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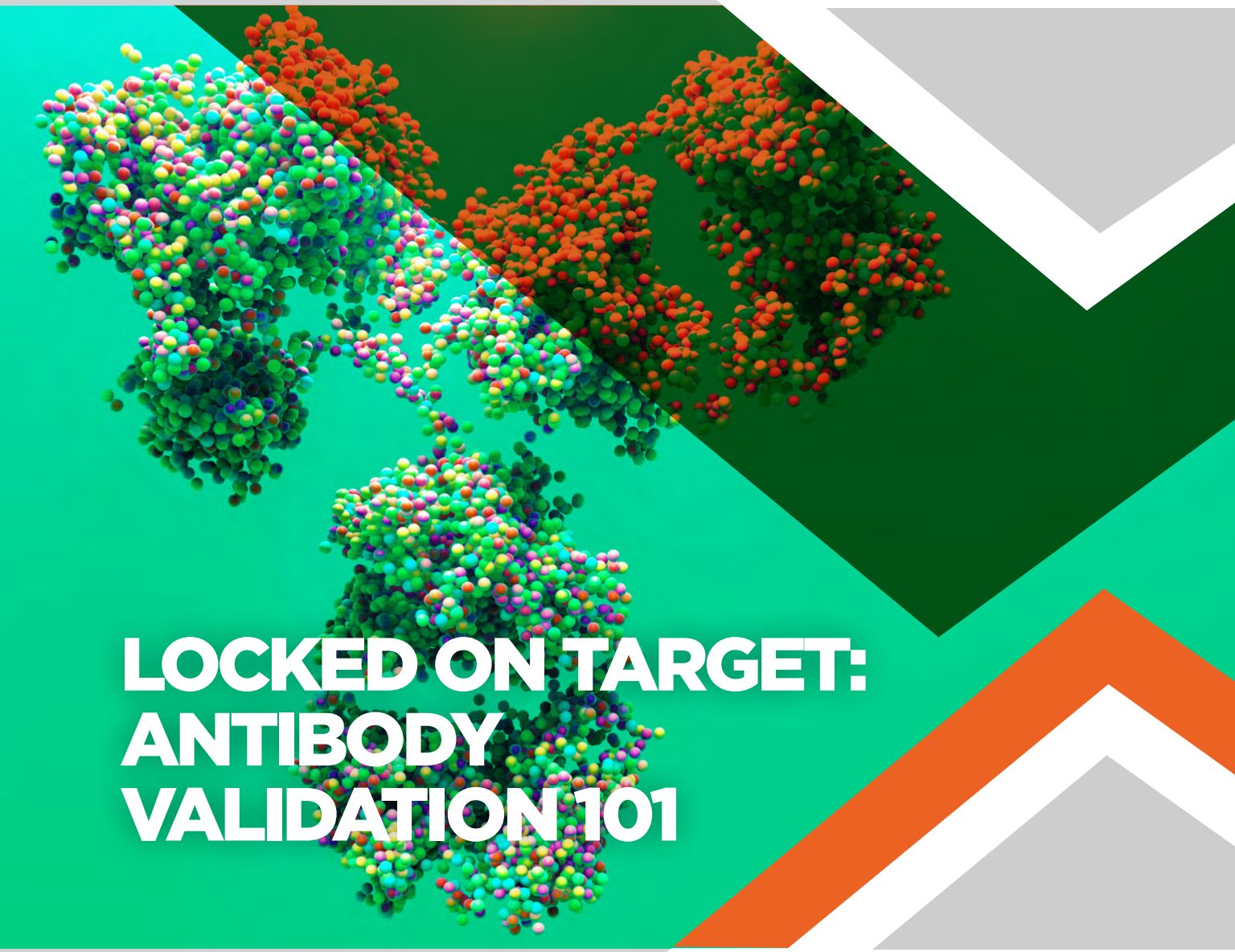

LOCKED ON TARGET: ANTIBODY VALIDATION 101

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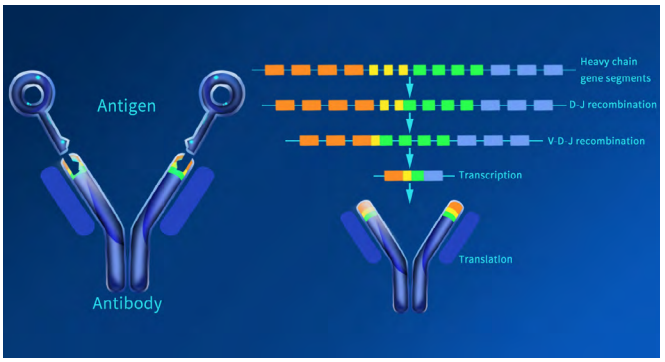

Antibody Specificity: The Secret Behind Antibody Versatility

The human immune system constantly protects against a dynamic range of pathogens, allergens, and other molecules. Antibodies, also known as immunoglobulins (Igs), play a critical role in this protection as they target specific molecules, or antigens, that may be harmful to the body. Antibodies bind antigens via a unique lock-and-key mechanism, which is essential to eliminating these threats.

Antibody Structure

Antibodies capture a wide range of antigens because of their unique structure (*see image*). Each individual antibody is composed of the following:

- Two heavy chains with variable and constant regions
- Two light chains with variable and constant regions

The two heavy chains are joined by a disulfide bond, creating a hinge that allows for the antibody's trademark Y shape. One heavy chain is bound to one light chain, and the antigen binding site, also known as the paratope, forms where the variable regions meet. Each paratope binds a specific antigen peptide sequence or conformation—an epitope—as a lock-and-key. The structure, shape, affinity, and specificity of the paratope depends on the sequence of the heavy and light chain variable regions.

V(D)J Recombination

Variable region properties are determined during B cell maturation by a process called V(D)J recombination. Here, the gene segments encoding the variable regions reorganize, forming unique binding sites in the resulting antibody[1]. For the heavy chain alone[1], humans carry an average of

- 51 variable (V) functional gene segments
- 27 diversity (D) functional gene segments
- 6 joining (J) functional gene segments

This means that more than 2.6×10^6 unique antigen-binding sites can be generated. The imprecise nature of the recombination process further enhances variable domain diversity by more than 10^8-fold[1]. Once an antigen is encountered by a B cell, a process called somatic hypermutation creates even greater variation in the antigen-binding region, which allows antibodies with high affinities for their respective antigens to be produced by affinity-matured B cells.

Antibodies are flexible and can bind multiple antigens at once, form dimers and polymers with other antibodies, and create antigen-antibody complexes. The flexibility of the hinge region also allows complexed antibodies to interact with cells, and this mediates and regulates a diverse range of immune responses.

Using Antibodies in the Laboratory

Following V(D)J recombination, each B cell produces only one specific antibody that structurally correlates to the stimulating antigen. This means that scientists can mass produce specific antibodies targeting proteins of interest by exposing B cells to specific antigens. This diversity has made antibodies very popular as labels for proteins, as it is theoretically possible to design and produce a specific antibody for every antigen in existence. Scientists now use antibodies extensively to identify, quantify, and characterize proteins in the laboratory.

Researchers can quantify highly specific antigen-antibody interactions by detecting signals emitted from antibodies directly bound to an antigen or by using secondary antibodies carrying fluorescent or colorimetric labels. This is the guiding principle behind many commonly used techniques such as Western blotting, enzyme-linked immunosorbent assays (ELISA), and immunohistochemistry[2].

The Need for Antibody Validation

Each antibody binds one specific epitope, but an epitope may be present in multiple proteins, especially if the proteins are similar in sequence. For example, an antibody generated against one isoform of a surface receptor may also cross-react with the other isoforms. Because of this, researchers must validate antibodies used for research to confirm that they bind the intended target—and only the intended target.

Because nonspecific antibodies can lead to incorrect conclusions, antibody validation is important for ensuring the accuracy of every antibody-based experimental protocol[2]. Antibody validation techniques examine binding specificity, reproducibility of experimental results, bond strength, reagent stability, and application suitability, among other key parameters[2,3]. Despite its absolute necessity, the amount and depth of validation information made available to researchers varies significantly from antibody to antibody and manufacturer to manufacturer[3].

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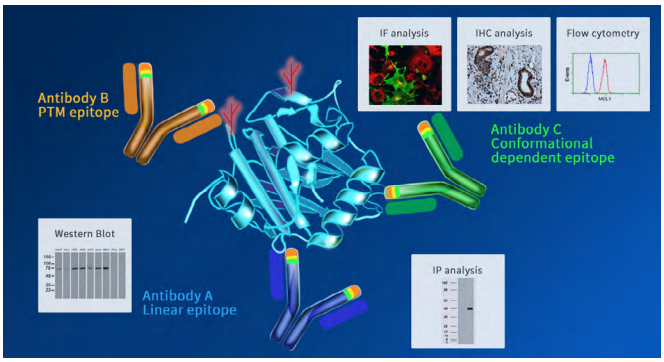
The Importance of Antibody Validation for Laboratory Assays



Numerous laboratory assays rely on antibody-antigen interactions for signal detection and data acquisition. Antibody binding specificity greatly impacts the accuracy of the data from each experiment, so validating the antibody specificity is important. The specific nature of an antibody-antigen interaction varies depending on the assay being performed. Some antibodies are better suited for particular assay types, so it is necessary to validate an antibody for each assay where it will be used.

Western Blotting

Western blotting is arguably the most prevalent antibody-based laboratory assay in use today. For this technique, researchers perform these steps:

- Separate proteins by molecular weight using gel electrophoresis
- Probe them with antibodies specific to a protein of interest
- Determine the relative protein abundance

Nonspecific antibody binding can result in fuzzy or smeared Western blots that are difficult to interpret. It can also result in the appearance of multiple bands, leaving the task of identifying the correct band up to the researcher's interpretation. Scientists may also assume that a band in the right location represents the protein of interest. However, it is entirely possible for an antibody to bind nonspecifically to other proteins similar or identical in size, leading to either a false positive result or an overestimation of relative abundance.

Using an antibody with validated specificity gives researchers confidence that any signal they see represents the protein of interest. This may reveal previously unknown information. For example, they may detect different forms of the target protein, including dimers and polymers, truncations, mutations, or even proteins with post-translational modifications.

Immunohistochemistry (IHC) and Immunofluorochemistry (IF)

By applying antibodies directly to tissue samples, researchers can use colorimetric (IHC) or fluorescent (IF) signals to correlate expression patterns with specific locations, structures, or cells. IHC data is also widely used for clinical diagnostic assays. Here, a false positive signal caused by a nonspecific antibody can result in incorrect diagnoses and serious therapeutic planning issues. When validating an antibody for IHC/IF, both antibody specificity and spatial distribution accuracy needs to be confirmed. To do this, researchers may

- Use multiple antibodies targeting the same protein to see if the signals overlap
- Compare antibody signal patterns to a distribution profile acquired using a technique that does not use antibodies, such as in-situ hybridization, genetic-level tagging, or RNA sequencing

Antibody binding specificity for IHC/IF applications can vary depending on how a sample was prepared and stored prior to assaying. Factors such as fixation, storage, and the application of antigen retrieval techniques can all affect antibody performance. Using the same antibody on a formalin-fixed paraffin-embedded (FFPE) sample and an OCT-embedded frozen sample may lead to data discrepancies and inconsistencies[1-3]. Therefore, antibody validation should be performed for each different set of experimental conditions experienced by the test samples.

Flow Cytometry (FC)

Flow cytometric analysis is becoming more accessible and more popular, especially for the characterization of novel cellular subsets. In FC, researchers detect fluorescent signals from antibodies bound to cells as they pass through a column. Nonspecific binding results in false positives, skewing cell type ratio data or possibly indicating cell types or subsets that are not contained in the sample. Antibodies can be validated for flow cytometry using samples with known cellular identities and quantities[1]. Cells known to express the marker being probed can serve as positive controls, while cells known to lack the marker can serve as negative controls.

Because FC is commonly used to identify new cellular phenotypes using marker expression profiles, any antibody nonspecificity can lead to incorrect identification of a cell type, masking of small and rare cellular subsets, or an incorrect determination of cell counts and proportions within a heterogeneous sample.

Enzyme-linked Immunosorbent Assays (ELISAs)

ELISAs are highly sensitive, making them popular for quantifying small protein concentrations. Slight variations in signal magnitude—potentially caused by antibody nonspecificity—can result in significant data discrepancies. Critically, these variations can render an entire experiment inaccurate if they affect the controls and calibration curves. When validating antibodies for ELISA, it's important to

- Run replicates for each well and for each run
- Keep an eye on intra- and inter-run signal variation in controls, calibration curves, and sample wells

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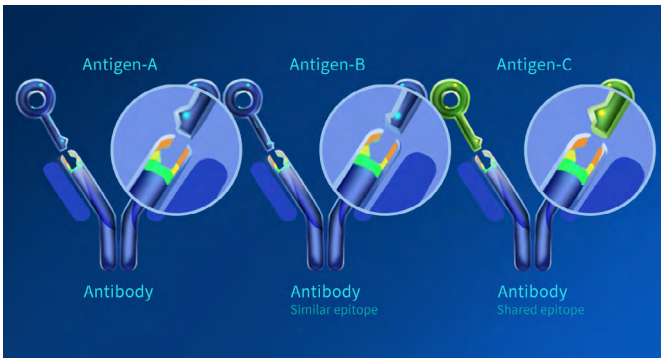

How Do I Validate an Antibody?

Antibody validation experiments determine whether an antibody binds to its intended antigen in a specific and reproducible manner. Because nonspecific binding can lead to false positives and a lack of binding contributes to false negatives, antibody validation is essential for obtaining accurate results from antibody-based assays.

One Lock, Multiple Keys?

Each antibody binds one specific epitope, but that epitope might be found in multiple proteins. This scenario is most common in closely related proteins or structural variants of the same protein.

Western blotting offers a simple and accessible method for validating antibody specificity[1]. Separating proteins by molecular weight allows researchers to distinguish between specific binding to the protein of interest and nonspecific binding to other proteins of differing sizes. However, Western blotting is limited in terms of throughput; it is time-consuming and can only validate a small number of samples per run. The presence of multiple labeled Western blot bands does not always indicate nonspecific binding, but may result from alternative variants of the protein of interest with a common epitope, such as protein degeneration products, splice variants, or post-translational modifications[1]. A good negative control, such as a cell lysate that does not express the antigen of interest, is necessary to distinguish between specific binding and false positives, regardless of whether the band size matches expectations.

Another method for validating antibody binding is mass spectrometry to detect immunoprecipitated antibody-antigen complexes (IP-MS)[2]. While IP-MS is highly sensitive and can be automated[2], it is a specialized technique and requires considerable equipment investments. Additionally, not all antibodies are suitable for immunoprecipitation.

Finally, for extremely high-throughput antibody validation, protein or peptide microarrays can be used to examine potential off-target antibody interactions with tens of thousands of different proteins or peptides at once. Protein and peptide arrays enable researchers to investigate epitope conformations, protein isoforms, and epigenetic and post-translational modifications[3]. Currently, the main drawback for microarrays is increased complexity in both execution and analysis. However, the ever-increasing availability of commercial kits is helping to bridge that gap.

The Importance of Proper Controls

Proper controls are essential to proper validation of binding capacity and specificity. Signal from positive controls confirms antibody binding to the protein of interest, while the absence of signal from negative controls confirms a lack of nonspecific binding. For negative controls, it is insufficient to simply include a sample that has not been incubated with the antibody[1]. Instead, scientists should use a sample that is known not to express the protein of interest.

Both positive and negative controls for antibody-based assays are often obtained from native cells or tissues that are known to either express or not express the protein of interest. The availability and accessibility of these sources is limited by logistical roadblocks and knowledge gaps. Genetic engineering advances such as CRISPR are helping researchers develop more appropriate controls for their antibody validation needs.

Transfected cell lines overexpressing the target protein serve as excellent positive controls. Because the researcher can control the sequence of the protein being produced, he or she can produce a positive control that directly mimics the antigen used to raise the antibody being validated. For negative controls, cells where the gene encoding the protein of interest has been removed or deactivated, or where RNA interference has downregulated the protein of interest, are optimal[4,5].

One Size Does Not Fit All: Cross-Application and Cross-Protocol Validation

Although the fundamental principle behind antibody-based assays is the same, the same antibody may not demonstrate the same binding efficacy across different techniques. Not only are the epitopes present in different formats (most Western blots use linear epitopes while immunohistochemistry and Western blots that do not incorporate a denaturing step use conformational isotopes), but differences in protocol can impact epitope accessibility and antibody performance[6]. It is necessary to further validate antibodies for each potential application, and even perhaps for specific sets of experimental parameters, with an eye on both specificity and reproducibility.

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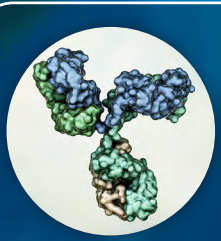
VALIDATION CHECKLIST

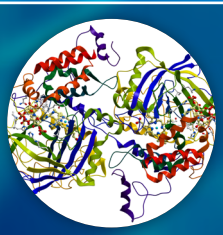
Many factors determine whether an antibody is properly validated.

HAVE YOU CONSIDERED THEM ALL?



IS THE ANTIBODY VALIDATED AGAINST YOUR PROTEIN OF INTEREST?

Test and confirm that your antibody binds only its intended target.



DO YOU KNOW YOUR EPITOPE?

Epitopes can be blocked, changed, or found on multiple proteins.

IS YOUR ANTIBODY VALIDATED FOR YOUR APPLICATION?

Different applications require different conditions and measure different things.

WHAT POSITIVE CONTROLS WERE USED?

Positive controls should contain a well-characterized sample of the target protein.

WHAT NEGATIVE CONTROLS WERE USED?

Knockdown and knockout cells are ideal negative controls because they do not express the target protein.

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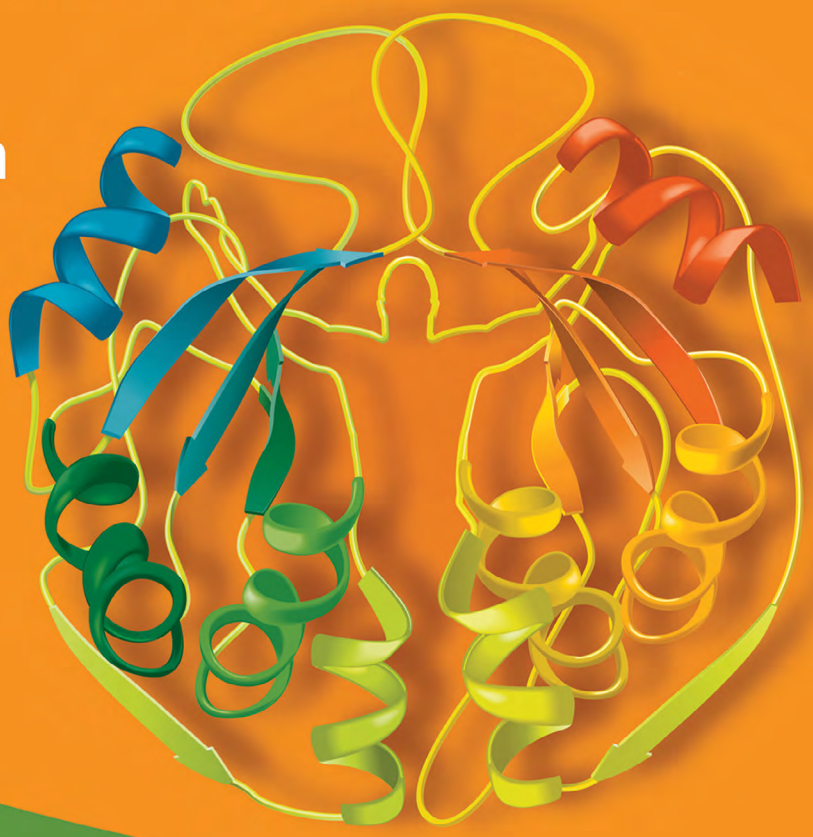

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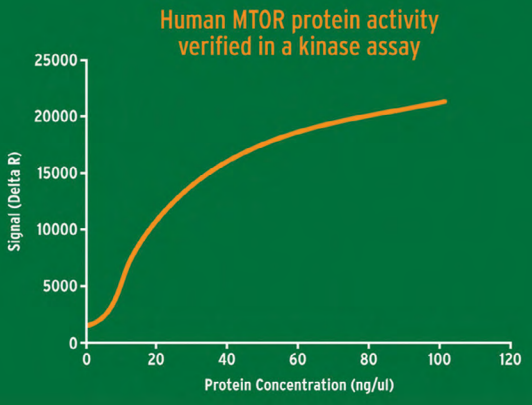

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