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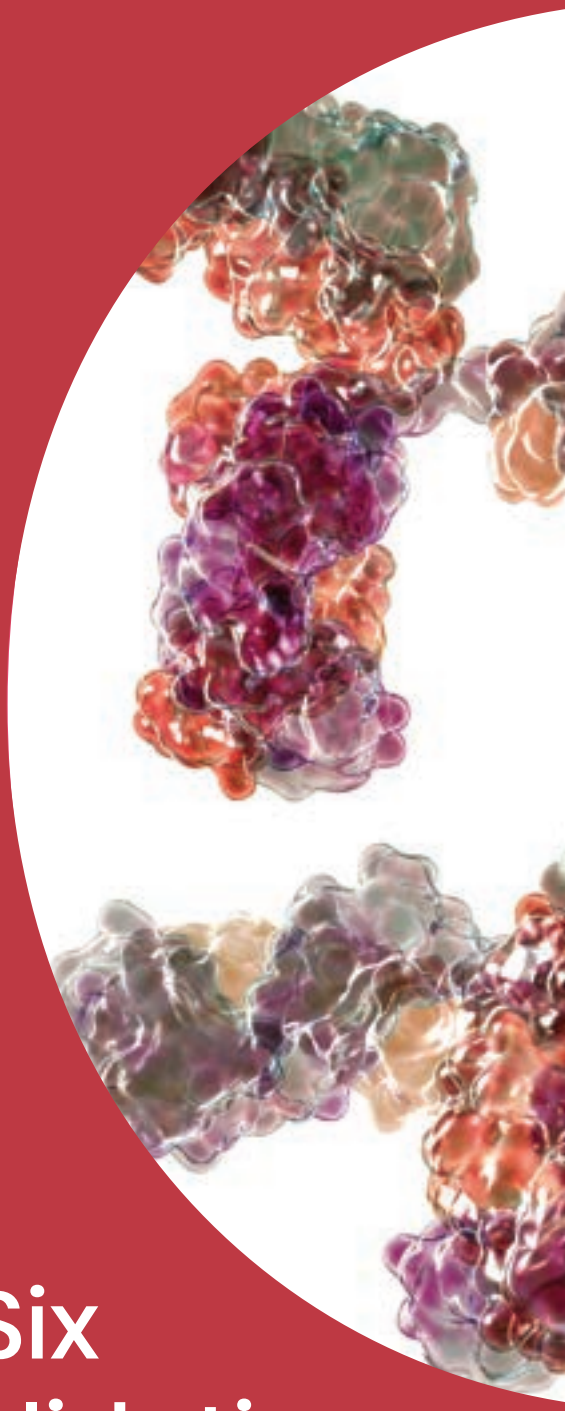


Ensuring Antibody Specificity and Reproducibility: The Six Pillars of Antibody Validation

The ideal antibody recognizes its target specifically, produces reproducible results, and retains these characteristics in its intended application. However, commercial antibodies are not always properly validated and unfortunately, many antibodies on the market cross-react with non-target proteins or give unreproducible results.

For example, an examination of 5,436 commercial antibodies from 51 providers found that only 1,410 monoclonal antibodies and 1,316 polyclonal antibodies passed validation using western blotting and immunohistochemistry (IHC) (Berglund et al., 2008). A survey of 49 antibodies against 19 G-protein-coupled receptors (GPCR) found that none were specific to one GPCR, likely due to the high degree of homology among GPCR subtypes in the same family (Michel et al., 2009). Poorly characterized antibodies can have a great financial and time impact across basic research, translational research, and clinical research.

In this whitepaper, we discuss six complementary methods for validating antibodies, the need to consider the application when using antibody validation data, and the responsibilities of antibody manufacturers and scientists in antibody validation.

What does antibody validation mean?

For antibodies, validation means testing the antibody in various ways to answer the following questions:

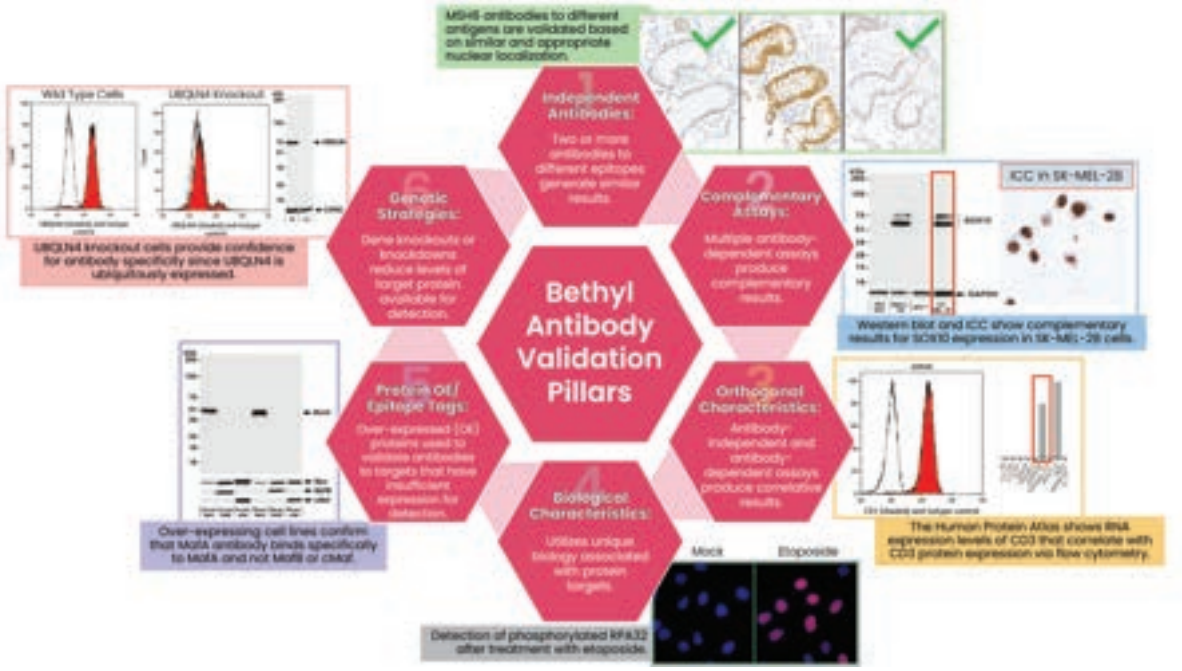
- Is the antibody specific for its target?
- Can it generate reproducible results?
- Can the antibody be specific and reproducible when it is used in its intended application?

It is important to note that an antibody does not necessarily produce completely clean results (ex: single band on a western blot) for the antibody to be useful and meet validation criteria.

Six pillars for validating antibodies for reproducibility and specificity

In 2016, the International Working Group for Antibody Validation published specific parameters for antibody validation in an application-specific manner (Uhlen et al., 2016). At Bethyl, we used these guidelines to enhance our previous antibody validation efforts and generated six complementary pillars for validating antibodies. We typically require antibodies meet at least two of the six following criteria:

- Independent antibodies
- Complementary assays
- Orthogonal characteristics
- Biological characteristics
- Protein overexpression / epitope tags
- Genetic strategies



Pillar 1: Independent antibodies

One way to validate antibodies is to use two or more independent antibodies that are raised against distinct epitopes of the same target protein. If these independently raised antibodies generate the same result, for example in iIHC assays, the antibody can be considered validated based on this pillar. Another way to meet the independent antibodies criteria is to use one antibody to generate a biological complex that can be detected by a second antibody (reciprocal immunoprecipitation, or reciprocal IP).

Reciprocal IP: At Bethyl, we use 3-4 different peptides from the same target protein to separately generate polyclonal antibodies. These antibodies are purified and used in separate immunoprecipitation assays to pull down the target protein. The immunoprecipitation products are then blotted against each of the other antibodies (Figure 1). Seeing the same banding pattern on the western blots suggests the antibodies are binding to the target protein.

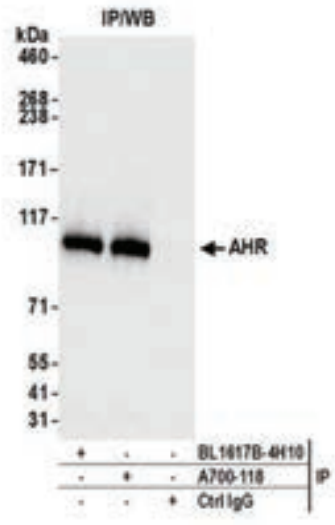


Figure 1: Western blot using one antibody against immunoprecipitation product from two distinct antibodies.

IHC: To validate an antibody using IHC, adjacent sections of the same tissue are stained by two independently generated antibodies. If these sections show the same staining pattern, the antibodies can be considered validated (Figure 2).

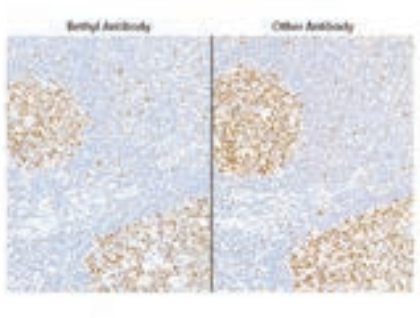


Figure 2: Adjacent sections of the same tissue stained with two independent antibodies.

Pillar 2: Complementary assays

To validate an antibody using this pillar, the antibody must produce the same result using different antibody-dependent techniques. There are a variety of methods to demonstrate this including IHC, immunocytochemistry (ICC), western blot, and flow cytometry to show specificity for certain cell lines, tissue types, or species; enzyme-linked immunosorbent assay (ELISA) to show phospho-specificity; chromatin immunoprecipitation (ChIP); or competitive ELISA.

In this example, SOX10 expression in the SK-MEL-28 cell line can be demonstrated using western blot and ICC (Figure 3). The antibody can be considered validated against this pillar as both methods produced the same result.

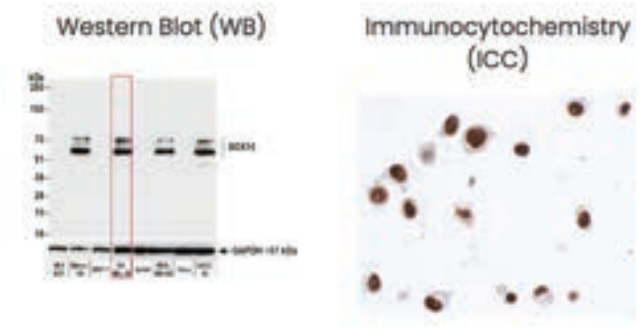


Figure 3: SOX10 expression detected by western blot and ICC in SK-MEL-28 cells. GAPDH was used as an internal loading control in the western blot.

Pillar 3: Orthogonal characteristics

Antibodies can also be validated using antibody-independent techniques, such as using data from publicly available datasets from mass spectrometry or mRNA expression studies (ex: ProteomicsDB.org or Genevisible.com), published research, or internal data. The goal is to produce comparable results between an antibody-dependent technique and an antibody-independent technique.

This example examines the performance of a CD19 antibody using western blot and compares the data from the western blot with mRNA expression data from the GeneVisible dataset (Figure 4). Since the presence of CD19 in Raji and Daudi cells is confirmed by both western blot and mRNA expression data, the antibody can be considered validated under this pillar.

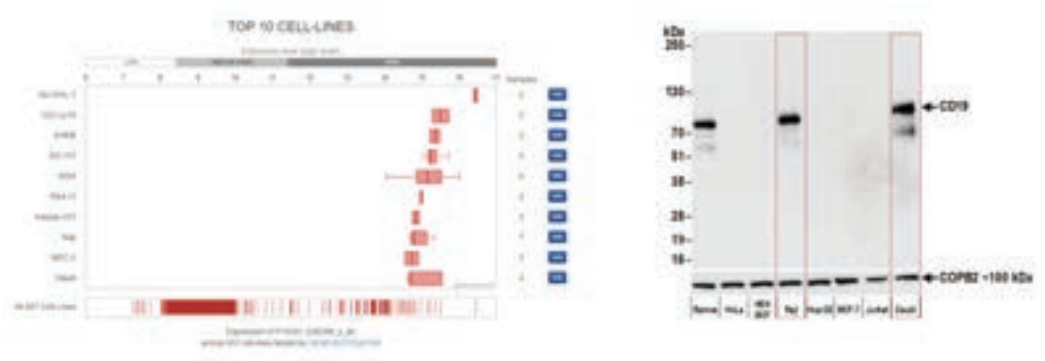


Figure 4: (Left) Expression of CD19 in 10 different cell lines. (Right) Expression of CD19 detected by western blot. COPB2 was used as an internal loading control.

Pillar 4: Biological characteristics

Proteins are often differentially expressed under various biological conditions such as during hypoxia, drug treatments, or phosphopantetheinyl transferases treatment. In other cases, post-translational modifications to proteins only occur under specific conditions. When the outcome of these treatments are known, they can be used to validate antibody specificity.

For example, hydroxyurea treatment is known to induce the phosphorylation of CDK1. This can be demonstrated using western blot where the antibody of interest detects an increase in phosphorylation of CDK15 only when cells are treated with hydroxyurea (Figure 5).

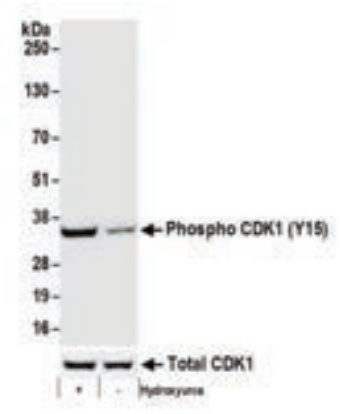


Figure 5: Western blot against CDK1 detects differences in phospho-CDK1 levels under hydroxyurea treatment.

Pillar 5: Recombinant expression strategies

Overexpression (OE) in specific OE cell lines can be used to demonstrate antibody binding and specificity. OE cell lines are used when the target protein is not natively expressed in another cell line or when the protein is expressed at a level below the threshold for detection. This pillar can be used to ensure that the antibody does not cross-react with other proteins or confirm the relative molecular weight of the target in other cell lines.

For example, overexpression of MafA, MafB, and cMaf in mouse and human cell lines can be used to show that the antibody of interest is specific to its target. A western blot of cell lysates from these cell lines demonstrates that the anti-MafA recombinant monoclonal antibody is specific to MafA and does not cross-react with MafB or cMaf. Additionally, the western blot shows that the MafB antibody is specific to MafB and the cMaf antibody is specific to cMaf. This recombinant expression strategy was chosen as the validation method for these antibodies because MafA, MafB, and cMaf are closely homologous proteins.

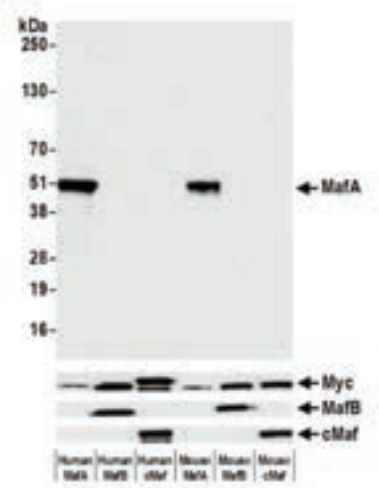


Figure 6: Western blot using MafA, MafB, and cMaf antibodies demonstrate specificity to their target proteins. Myc was used as an internal loading control.

Pillar 6: Genetic strategies

Gene knockout or knockdown can also be used to validate an antibody. A gene knockout or knockdown should completely or partially reduce the band intensity in a western blot, respectively (Figure 7). In an ICC experiment, the knockout or knockdown should reduce the signal.

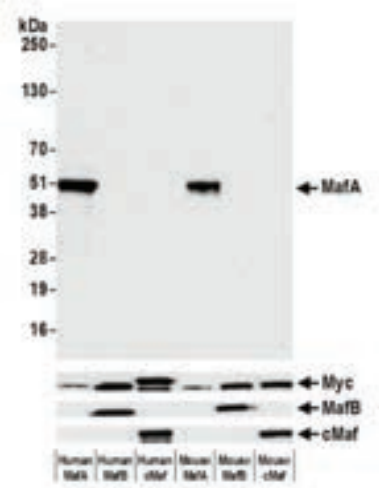


Figure 7: Western blot against UBQLN4 in wild-type cells vs. knockout. COPB2 was used as an internal control.

Overcoming challenges in antibody validation

Challenge 1: Apparent molecular weight does not equal predicted molecular weight

In some cases, the apparent molecular weight of a protein on a western blot is not the same as the predicted molecular weight of the protein. Validating antibodies in this context can be addressed using the independent antibodies and complementary assays pillars. Reciprocal IP can be done using an antibody against a different epitope of the target protein. If the western blot shows banding at the same molecular weight using both antibodies, the antibody of interest can be considered validated even when the band is not at the predicted molecular weight.

Challenge 2: Ubiquitous expression and localization

Some proteins are ubiquitously expressed or don't have a specific localization within the cell. This makes it difficult to find a negative control. However, these challenges can be addressed with the pillars described above.

For example, if it is known that the target protein is expressed in one cell line but not others using an antibody-independent technique, mass spectrometry or mRNA data can be used to verify western blot results across multiple cell lines. Additionally, the reciprocal IP strategy described in Pillar 1 can also be used.

Challenge 3: Cell-specific isoforms

In some cell types, a protein can exist as several isoforms and an antibody can cross-react with multiple isoforms. This results in multiple bands and it can be unclear if the antibody is specific to the target. This challenge can be overcome by reciprocal IP.

For example, the antibody of interest targeting GATA3 reveals one band on a western blot in Jurkat cells. However, the same antibody gives two bands in MCF-7 cells. To determine whether the banding pattern is specific or a sign of cross-reactivity, the two cell lines can be used in reciprocal IP to test two independent antibodies. When two independent antibodies give the same banding pattern in the same cell type, the antibody can be considered specific and not cross-reacting.

Validating antibodies for intended analytical use

It is important to consider the difference between antibody validation and application validation. While antibody validation demonstrates reproducibility and specificity in binding the appropriate target, antibodies can also be validated for specific applications such as western blot, ELISA, flow cytometry, IP, ICC, IHC, immunofluorescence, and multiplex immunofluorescence.

Application validation data is often included in antibody datasheets and on the manufacturer's website and can tell you about the applications the antibody is validated for, what species, the concentration and dilutions used, etc.

Whose responsibility is antibody validation?

Antibody manufacturers and scientists are both responsible for antibody validation.

For the manufacturer, it is important to provide as much data to the customer as possible including detailed experimental results, the applications tested, and negative data. Manufacturers must also take a collaborative approach when it comes to customer inquiries and offer technical support to customers using purchased antibodies.

In selecting an antibody, scientists also need to understand how the antibody was validated by the manufacturer. They should check manufacturer data, understand how the validation was done, and check the literature to see how the antibody has been used before. Finally, they should validate the antibody using their own protocols in the lab before proceeding with an experiment.

Guidance for selecting antibodies

To help select the best antibody, customers should look for the following when choosing an antibody:

- The antibody was validated using biologic expression of the target (pillar 4), rather than solely recombinant protein or overexpressing cell line techniques.
- The antibody was validated using several applications. If the manufacturer can show that the antibody can be specific and reproducible under several applications, the customer can have more confidence about the antibody performing in multiple applications than if it was validated using one application. It is not a guarantee that the antibody will work in an untested application, but it is possible.

- The manufacturer shows several images demonstrating validation in different applications.

Although careful consideration before purchasing antibodies greatly increases the chances of a successful experiment, it would not be unusual for an antibody to not perform as expected in the first attempts of an experiment. Customers may still need to further optimize the conditions in their specific experiment and should reach out to the manufacturer for additional technical support when needed.

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