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Antibody Production

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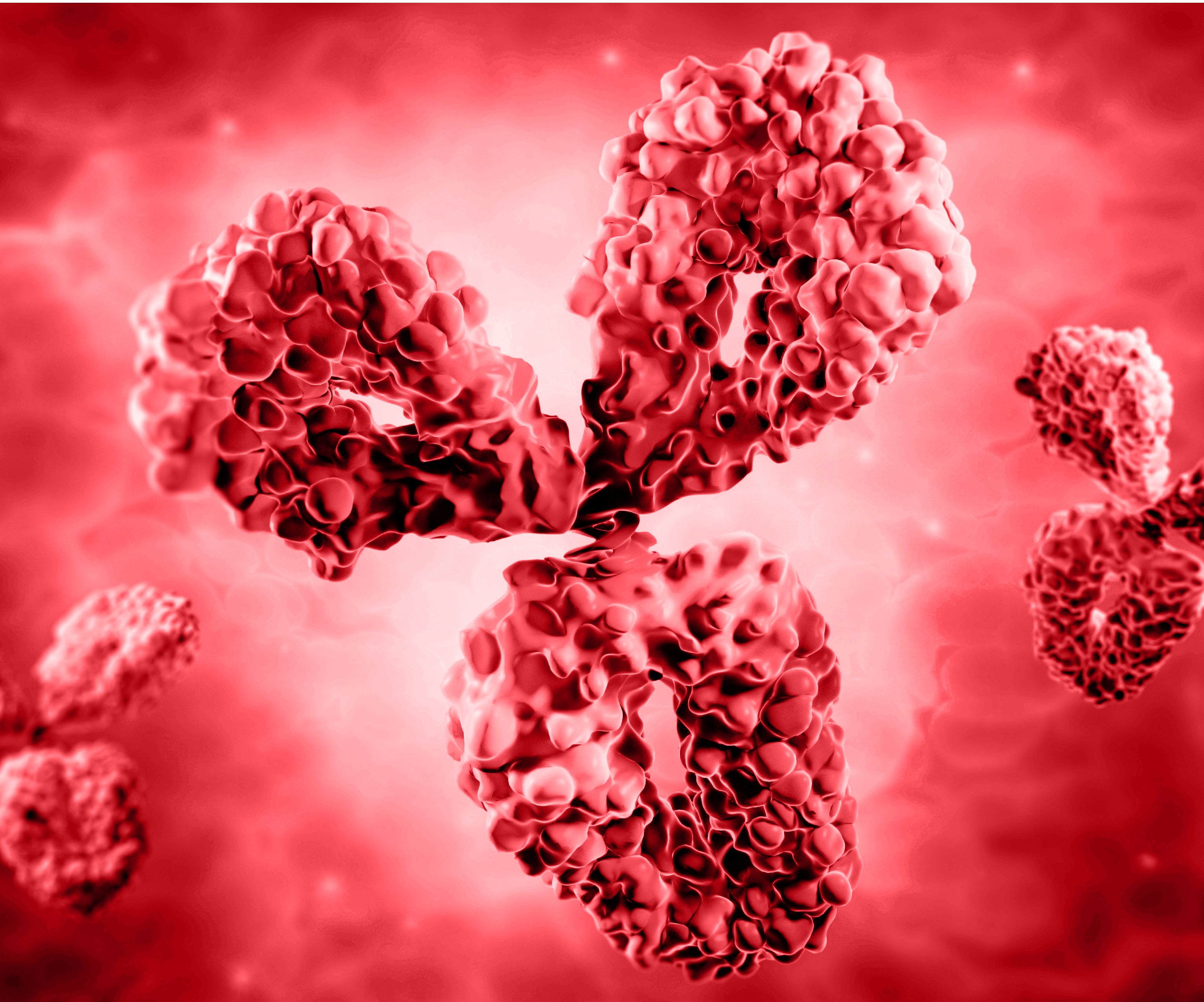
Are Your Antibodies Valid?



Overview of Antibody Production

Considerations for antibody production, from antigen design to validation

Gerald Edelman and Rodney Porter won the 1972 Nobel Prize for describing the chemical structure of antibodies. Bethyl was founded that same year. Only three years later, Köhler and Milstein published their work describing how to fuse myeloma cells to B cells, creating hybridomas. The creation of hybridomas was the foundational technology needed to produce monoclonal antibodies. Their work was subsequently awarded the Nobel Prize in 1984.

Today, antibodies are widely available from many different companies. Before you buy an antibody, though, it is important to understand who actually made your antibody. Dr John Carwile, President of Protein Technologies at Bethyl-Fortis explains, "The key reason for choosing a provider that is a manufacturer, really has to do with the complexity of the entire manufacturing process, because any slight deviation in any of the steps builds in errors, and even a slight percentage variation in any one of those steps can lead to dramatic differences in the finished product."

Life cycle of antibody production

Antigen identification or design

The first stage in antibody production is to identify the antigen. The antigen can be almost anything—a protein, a peptide, or a small molecule. This antigen needs to be either synthesized or purified for injection into the animal host deemed highly suitable, which most commonly includes rabbits, mice, or goats.

Antibody collection

Once the animal's immune system has had the opportunity to respond to the antigen, the serum of that host is recovered. If the scientist needs polyclonal antibodies, antibodies can be immediately purified to varying levels of purity.

If monoclonal antibodies are desired, the antibody producing B-cells are collected and manipulated to immortalize the genetic code for that antibody—specifically the variable region that is responsible for antigen binding. This can be achieved via traditional hybridoma techniques or—more commonly today—recombinant methodologies.

These antibodies can be collected from culture and purified for use. Creation of this cell line and capturing the genetic code ensures reliable preservation of this antibody.

Antibody validation

Once antibodies have been generated, collected, and purified for the application's needs, your manufacturer can characterize the antibodies. This is a vital step to ensure that the antibodies you receive will meet your needs. Recent interest in rigorous antibody validation is impacting the industry, but as Dr. Carwile explains, Bethyl-Fortis has been using "…the antibody validation pillars, using multiple validation techniques long before it was fashionable."

What to look for in a supplier?

Choose an antibody that is validated to be specific, selective, reproducible, and sensitive.

When shopping from a catalog or having a custom antibody made, consider asking your supplier about:

- Antigen design: are the immunizing peptides made in-house, ensuring reportability?
- Antibody validation: do they use techniques such as complementary assays or independent antibodies to validate the antibodies?
- Technical expertise: if your antibody supplier can understand and employ antibody validation tools, they also have the expertise to help when you need to optimize antibody techniques for lab- and experiment-specific conditions

Antibody purification

For both monoclonal and polyclonal antibodies, purification is the next step. There are numerous options for purification, depending on the needs of the scientist. With the vast number of options to consider for antibody purification, you need to have a manufacturer you trust to help guide you through the options and make sure they offer what you truly need. Purification on-site ensures better lot-to-lot reproducibility. Ideally your antibody vendor will also design and create the antigens. This ensures the creation of your antibodies progresses seamlessly from antigen design, through antibody purification.

Antibody purification techniques

Antibody purification options

- Antiserum: useful for some applications requiring polyclonal antibodies
- Immunoglobulin fractions: useful to isolate one particular class of antibody
- Antigen-specific antibodies: useful to isolate antibodies that bind to a particular antigen

Antibody fragmentation

Additional services offered by your antibody company may include antibody fragmentation. Not all end users need whole antibodies. There are many applications that only need fragments of an antibody, such as the Fab or F(ab')2 fragments. Either biochemical or molecular biology techniques can be used to generate antibody fragments.

Antibody conjugation

Many applications for antibodies require those antibodies to be linked to other molecular components. Your antibody provider can label the antibodies with fluorescent dyes, HRP, alkaline phosphatase, etc. They can also immobilize your

antibodies on chromatography media or attach them to beads.

Antigen design considerations and choosing the right vendor

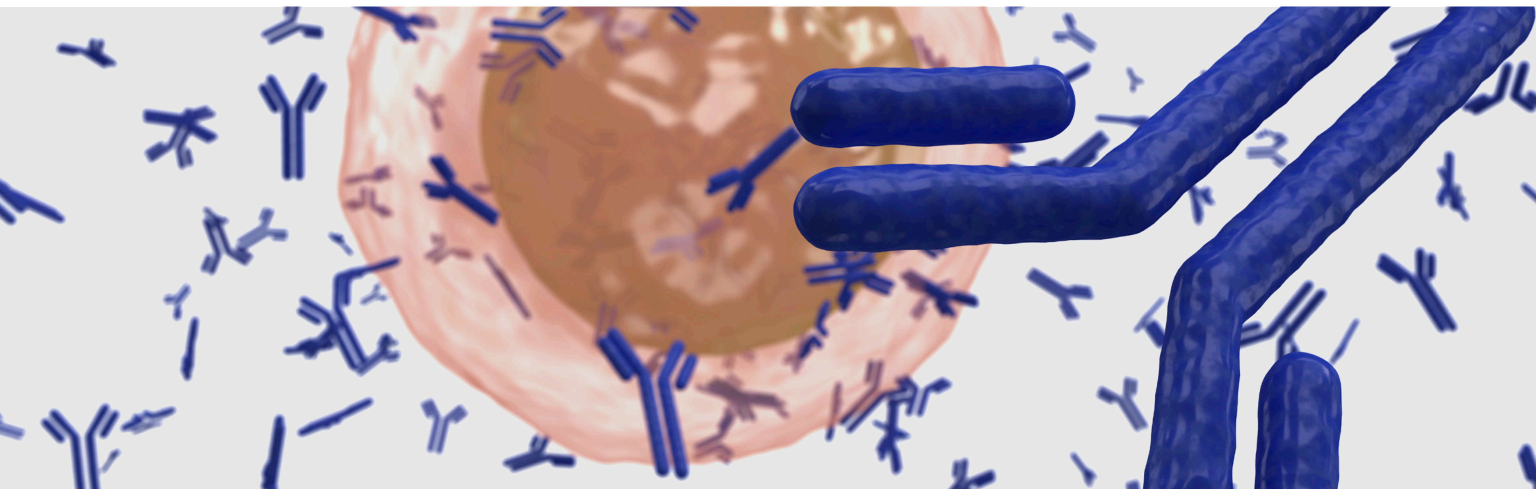
Aside from the obvious considerations of what antigen target and what animal's antibodies you will need, there are many ways to optimize your antigen design.

Using bioinformatics to guide your antigen selection can help identify antigenicity of your target. It can also help identify antigens that will either minimize human cross-reactivity, or utilize mouse or rat cross-reactivity.

When choosing your antibody manufacturer, ask them if they will manufacture the antigen as part of the process to creating the antibody. This helps improve reproducibility and helps with lot-to-lot reproducibility. It also provides full control and traceability over the supply chain. This will simplify your reporting. Finally, by reducing variability in the supply chain, it will aid in optimizing the use of your antibodies for your application and troubleshooting.

Just because a company can make an antibody doesn't mean it will work when you buy it. Bethyl-Fortis' vigorous antibody validation process protects you as an end-user. As Dr. Carwile says, Bethyl-Fortis won't sell an antibody until we are confident it will work, "We make everything that we sell, but we do not sell everything that we make."

Finding the right manufacturer simplifies your science, because you can rely on their products and focus on your own experiments. You can trust Bethyl to not only create and validate your antibodies, but their deep technical expertise is a resource to help you implement your antibody workflows and ensure they succeed.



Antibody-Based Applications

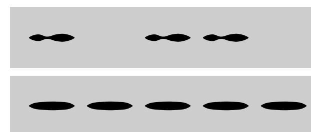
Common antibody techniques, how they work, and why you need them

Most life sciences researchers have used antibodies at some point in their careers. Although each antibody-based application relies upon quality antibodies, not all antibodies are appropriate for all techniques. Most antibodies are sold in catalogs where appropriate applications are listed. Although the applications are typically accompanied by usage recommendations, bench-side optimization is required. Read below to learn how antibodies work in these powerful techniques.

Antibody-based applications

The power of antibody-based applications for your research is immense. Characterization, isolation, interaction studies, and expression profiles are all possible with the right antibody. Make sure you understand when you purchase an antibody which of the following techniques it will work for. If your manufacturer hasn't validated the antibody in the specific assay, it may not work for you. Ask about how your antibody has been validated, so you understand your starting point for your own optimization.

Western blots



Western blots were named to align with the naming conventions for other related techniques—Southern blots (to visualize DNA—named after its inventor), and northern blots (for RNA). Western blots are used to visualize proteins that have been separated from a mixture based on their size via electrophoresis. The most common uses for this technique are to quantify the relative amounts of protein under unique conditions and to determine the presence or absence of a protein.

Western blot samples can be from any tissue, cell, or extract. Sample preparation will vary dramatically based on the source. Some methods require mild approaches, while others may need more stringent approaches. These lysates are commonly mixed with denaturants that help to linearize the proteins. For quantitative techniques, initial total protein quantification is performed to control for the amount of protein being loaded.

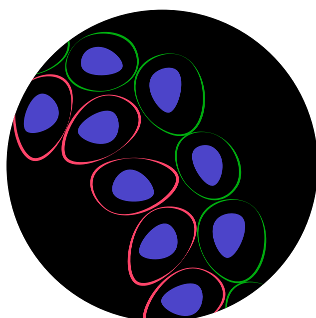
The next step uses acrylamide gel electrophoresis to separate the proteins based on size and then electrotransfer those protein bands to a membrane. After blocking the exposed membrane and washing, the primary antibody is applied and allowed to adhere. Every step in a western blot is followed by numerous washes to remove any unbound antibody. The primary antibody binds directly to the target antigen. A secondary antibody is used to visualize where the primary antibody bound the antigen. The secondary is a generic antibody raised to bind all antibodies from the target species. For example, if the primary antibody is a rabbit antibody, the secondary antibody may have been made in a goat to recognize all rabbit antibodies (called goat anti-rabbit).

The secondary antibody is typically modified by conjugation with a reporter subunit. This subunit will react in some way that makes it easy to see. It could be something like β-galactosidase to catalyze the cleavage of X-gal to yield a blue, insoluble precipitate at the location of binding. More modern western blots use chemiluminescent reporters, like horseradish peroxidase (HRP), which are far more sensitive and quantitative.

When using western blots for quantitative assays, the western blot membrane can be stripped and re-blotted to examine an independent, ubiquitous protein as a control. Ratios of luminescence of the control protein to the test protein are used to quantify western blots.

Immunohistochemistry and immunocytochemistry

Immunohistochemistry (IHC) and immunocytochemistry (ICC) are powerful techniques used to directly visualize the location of the target antigen in either tissue sections (IHC) or cultured cells (ICC).

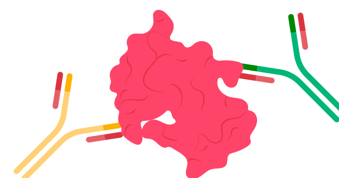


For IHC, tissues and cells need to be prepared on slides that can be treated for visualization. In general tissues are fixed, dehydrated, embedded in a matrix, and then sectioned. Fixation of samples preserves the morphology of cells within the tissues, and embedding further supports the structures. Whole tissue samples are typically treated to fix the samples with formaldehyde and embed them in a paraffin-based matrix. However, frozen samples can also be prepared if the heating and chemical processing risks damaging the protein target. In either case, a researcher would take the fixed tissues and slice ultra-thin sections onto slides using a microtome or cryostat. Cell or tissue thickness is an important consideration when preparing samples.

The only difference between ICC and IHC is that the tissues don't need to be prepared for sectioning. One way to prepare samples is to seed slides directly with the cells in a culture dish. Care must be taken to limit the thickness of the samples. An alternative approach for ICC is to create a cell pellet and section it, similar to IHC.

The sample slides can then be treated to visualize the antigens for photography under a microscope using a primary antibody and a secondary antibody for visualization.

Immunoprecipitation

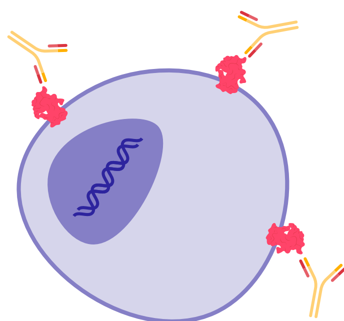


Immunoprecipitation (IP) is a powerful technique that allows for the enrichment of target antigens/proteins, but it can also be used to co-precipitate other biochemical components that interact with the target.

Tissue or cell sample lysates can be either native or denatured depending on the needs for the application. These samples are then exposed to an immobilized antibody, either by passing over a column matrix or by incubation with beads covalently bonded to the antibody. Once bound, the samples can be washed to purify the protein, or to

remove interacting proteins under whatever wash conditions are desired. Beads are precipitated either magnetically or via centrifugation, and columns are washed gravimetrically. Once washed to the specification of the experiment, the samples are eluted from the beads by altering the bonds to the antibody with detergents, urea, or by using a low-glycine buffer, depending on the application for the purified protein.

Flow cytometry



Flow cytometry analyzes single cells or particles as they flow past a laser. Light scattering of the laser is measured in the cytometer to determine the characteristics of the sample. There are many applications for flow cytometry, from the simplest of counting cells to the detection of biomarkers or engineered proteins. This technique can be very powerful when combined with antibodies with varying color reporters.

One of the biggest strengths of using flow cytometry for antibody-based applications is for multiplexing. A flow cytometer can distinguish, sort, and count the cells that are emitting different colors depending on the conjugated antibodies bound to target antigens in the sample population. Intensity can also be measured in this application to provide quantification information. This means multiple antibodies can be used to characterize a population simultaneously. However, you will need to find an antibody supplier that can provide antibodies that can be used for such multiplexing applications.

Antibody validation for your specific application

The power of antibody-dependent techniques has facilitated dramatic leaps forward in all aspects of protein studies. But any researcher who has worked with these techniques can attest to how finicky they can be. From buffer concentrations and components to lysis techniques, there are a vast number of places that antibody studies need to be optimized. However, when using purchased antibodies, the last thing you need is batch-to-batch variability. You also need to know if your antibody will even work for the application you need—many antibodies will not work in all applications.

Finally, understand how your antibodies are validated. For off-the-shelf products, see “Are Your Antibodies Valid?” for more information on antibody validation. For your custom project, discuss your needs with your manufacturer to understand the level of validation required. Bethyl-Fortis has used industry-leading validation for antibody quality for over 20 years, but their experts can also guide you to the right product, tested to work for your specific application. Finally, Bethyl-Fortis experts are available to help you optimize their antibodies for your lab and applications.

Recombinant Antibody Engineering

Genetic flexibility using recombinant antibody technologies

The creation of monoclonal antibodies using hybridoma cells lines altered how protein sciences were studied, allowing single antibodies to be isolated to antigen targets. These cell lines are created by isolating the B-cells from an immunized animal and fusing them to myeloma cells. Although this method offered an important tool for protein studies, there are limitations to monoclonal antibody generation using hybridomas. Hybridomas, like any cell line, can experience genetic drift over time, losing the specificity of the antibody being created.

Recombinant antibody technology

The solution to protecting your antibody supply is to use recombinant antibodies instead of those produced via hybridomas. Recombinant antibodies are created *in vitro* using an expression vector. This method of cloning the antibody-producing gene protects the integrity of the gene, allowing perpetual, identical antibody production.

Recombinant technology also offers the power of genetic modifications. Antibodies to be used in therapy can be humanized, by replacing structural parts of the antibody with human versions. This reduces the reactivity of the immune system to the treatment. Cloning a variable region onto a different backbone can also be done with other species for researchers working with mice, rat, rabbits, or other animal models. Bispecific antibodies, where a single molecule recognizes two different antigens, can also be generated using recombinant technologies.

The final, and most important feature of recombinant antibodies for bulk antibody production, is the inherent scalability of using a recombinant antibody vector in cell cultures. Whichever recombinant antibody product you need, it can be created in any format.

Recombinant antibody generation process

B-cell isolation

Antibody producing B-cells can be isolated from a variety of sources, for example peripheral blood, spleen tissue, lymph tissue, or whole blood. Cells can either be sorted using a cell sorting system, or isolated using a microbead or column-based extraction.

There are two approaches to microbead or column B-cell purification. Negative selection isolates B-cells that are unaltered, or "native." This is the most common technique, because the cells remain functionally unchanged. The alternative is positive selection, where B-cells are labelled and extracted from the sample using that label.

Assessing antibodies secreted by the B-cell

B-cells are the antibody production factories of the immune system. There are many different types of B-cells. How B-cells are differentiated during development determines where they are located in the mature immune system. Each type of B-cell also produces different types of antibodies, such as IgA, IgG, or IgM, each with unique functions in the immune system.

For the scientist or clinician, their antibody manufacturer must ensure the antibodies live up to expectations. When shopping for antibodies, ensure you understand how they have been assessed, or what your options are for custom antibodies.

Several types of antibody validation techniques* exist, such as:

- Independent antibodies: compare the results of an antibody-dependent assay using two or more differing antibodies and the get the same result
- Complementary analysis: compare two or more antibody-dependent techniques using the same antibody to confirm the results
- Protein overexpression or epitope tags: compare the results using the antibody from cells either overexpressing the target or with epitope-tag results to validate the antibody
- Orthogonal characteristics: compare antibody-dependent to antibody-independent results, such as using mRNA expression to predict the protein expression seen via antibody-dependent analysis

Cloning the variable region into expression vectors

Recombination of the genetic code for the antigen-binding regions of an antibody circumvents the drawbacks of genetic drift known to occur when using monoclonal antibodies. This sequence can be preserved and reused, not only for the most basic mAb, but it can also be subcloned into expression vectors for a variety of uses.

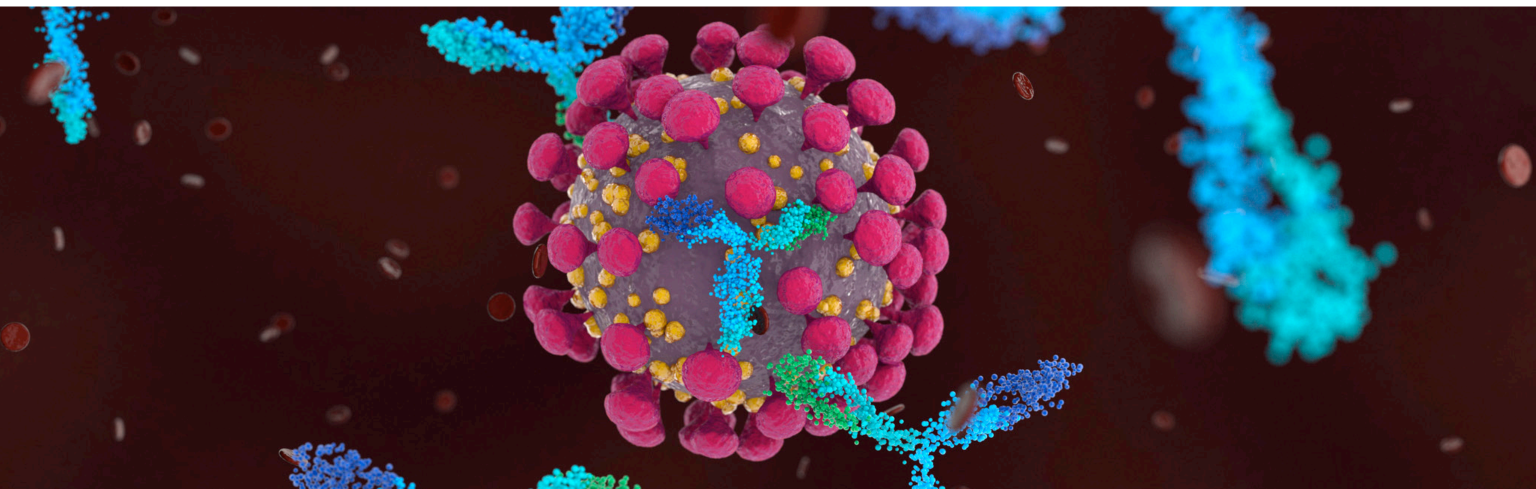
The variable region coding sequence can also be used to generate antibody fragments, or humanize the antibody to help improve its biological half life.

Custom antibodies

Catalogs of antibodies exist for a multitude of applications, but where do you turn if you need an antibody for your specific research project that isn't in a catalog? Custom antibodies can be created for any application, in any quantity. Bethyl-Fortis has decades of experience and can create recombinant monoclonal antibodies to an antigen, in any formulation. Bethyl-Fortis also offers custom polyclonal antibody services.

Leveraging nearly 50 years of antibody creation, their experts will work with you to choose the antigen target and design an approach that is specific to your needs. They will then synthesize the antigen(s), and produce, isolate, and validate the antibodies for your individual needs. Each project starts after detailed discussions—you will know what the price, scope of work, and expected milestones will be. You choose the formulation and scale. Bethyl-Fortis delivers.

*See "Are Your Antibodies Valid?" for more information about antibody validation



Are Your Antibodies Valid?

Internationally recognized validation standards ensure antibodies are specific, selective, sensitive, and reproducible

If you have suffered through western blot troubleshooting, you understand how vital your antibody supply is. The most common problems in antibody-dependent experiments are when the antibody binds off-target proteins or when the conditions in the cell or lysate under study differ from those the antibody was originally validated in. Tremendous amounts of work are needed when using a new antibody to determine the conditions for its use for each individual experiment. The last thing you want to do is waste those hours and days with an antibody that isn't reproducibly specific, selective, or sensitive enough. When choosing an antibody, ask the manufacturer what validation pillars they use to assess the quality of their antibodies before you get them.

Pillars for antibody validation

In 2016, an international group of scientists, who were frustrated with the lack of consistency in antibody production, met and formed the ad hoc International Working Group for Antibody Validation (IWGAV). In their 2016[1] and 2018[2] publications, they established a list of validation approaches. At least one of these techniques should be used to validate antibodies before experimental optimization and use.

Independent antibody studies

One common method to validate antibodies is to use more than one. A good manufacturer will utilize two or more independent antibodies to the same protein, but different epitopes. They can compare the results of both of these antibodies to ensure the data are similar.

Orthogonal validation

Comparing antibody-independent data with the results from antibody-dependent studies done during manufacture is another common validation approach. This approach confirms that the expression patterns seen with antibodies match up with what is expected from other techniques, such as proteomics or transcriptomics.

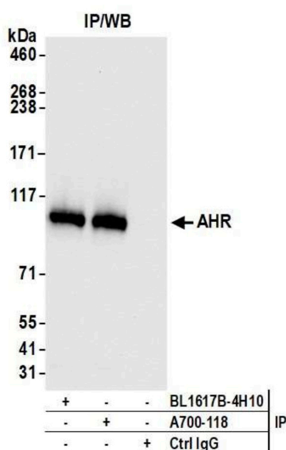
Other approaches

IWGAV suggested three other approaches to antibody validation: genetic knockdowns to

demonstrate the antibody recognizes the target protein in the presence of its intact gene compared to the knockdown; recombinant expression to show the absence of signal in a wild-type line compared to the presence of signal when using an expression vector; or immunocapture followed by mass spectrometry to confirm the antibody recognized the right protein.

Trust your source

Ask your current antibody manufacturer how they validate their antibodies. Do they do the minimum or go the extra mile? Years before these pillars were published, Bethyl has validated their antibodies using a combination of orthogonal methods and independent antibodies. This culminated in the creation of the reciprocal immunoprecipitation method (see the figure). For proteins with difficult expression levels or patterns, they use protein overexpression or knockdown validations as well. Bethyl also uses unique biological characteristics of the target protein, such as differential expression or other alterations to validate antibody to target recognition.



Independent antibody and complementary assays for validation. The target protein was immunoprecipitated with two different antibodies (see table below the image) and then immunoblotted with the antibody being validated (A700-118).

Validation methods are not universal for every type of antibody application. Optimization done with one method may not be applicable to another method. Bethyl uses extensive validation techniques such as complementary assays to compare results from more than one immunoassay, such as western blotting to immunocytochemistry. These processes validate the antibody, but they also help scientists by giving them a better starting point for their own optimization.

As a final point of trust, Bethyl checks batches of new antibodies against previous batches to ensure batch-to-batch reproducibility. Count on Bethyl's experience and quality for reproducible, specific, sensitive, and selective antibody supply and manufacture.

1. Uhlen, M., et al. A proposal for validation of antibodies. *Nat. Methods* 13, 823-827 (2016).

2. Edfors, F., et al. Enhanced validation of antibodies for research applications. *Nat. Commun* 9, 4130 (2018).

To learn more, visit :
www.bethyl.com/validation