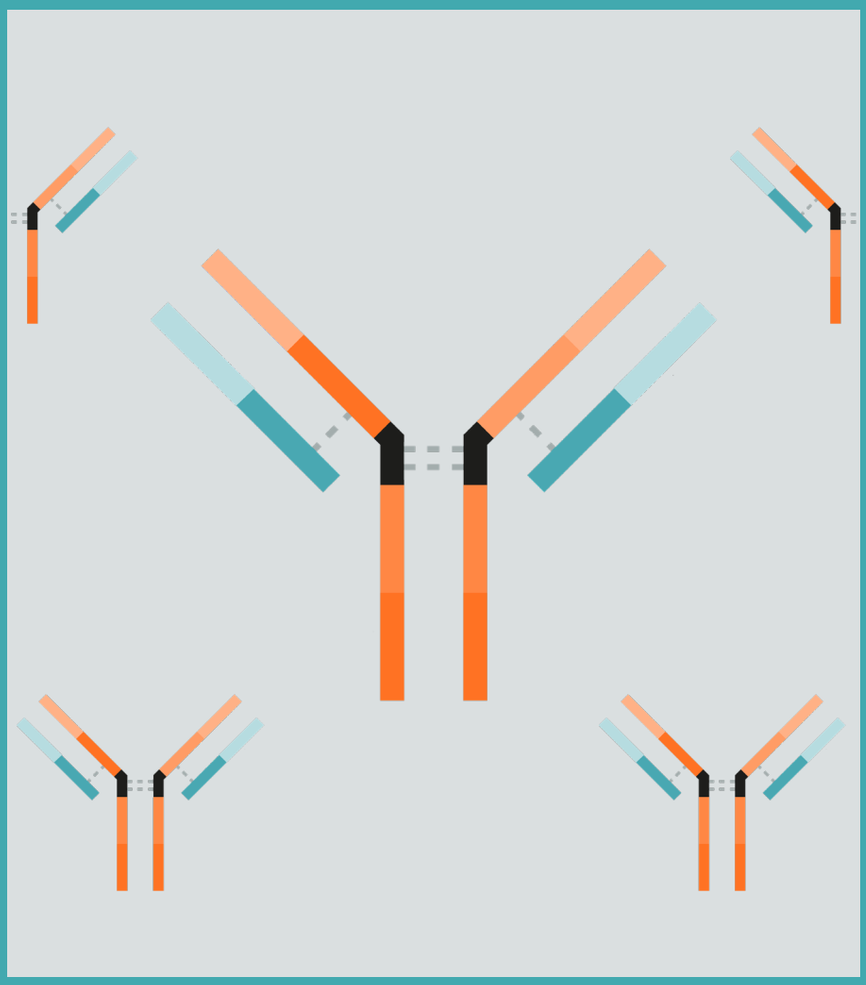
Antibody guide

A comprehensive guide to antibody basics: from antibody structure and production to antibody selection and handling.



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Chapter 1. Antibody basics

Antibody structure and isotypes

Antibody structure

Antibodies, also known as immunoglobulins (Ig), are large, Y-shaped glycoproteins produced by B-cells as a primary immune defense. Antibodies specifically bind unique molecules of a pathogen, called antigens. Antibodies exist as one or more copies of a Y-shaped unit composed of four polypeptide chains (Fig. 1).

Each Y unit contains two identical copies of a heavy chain (H) and two identical copies of a light chain (L); heavy and light chains differ in their sequence and length. The top of the Y shape contains the variable region (V), also known as the fragment antigen-binding (F(ab)) region. This region binds tightly to a specific part of an antigen called an epitope.

The antibody base consists of constant domains (C) and forms the fragment crystallizable region (Fc). This region is essential for the function of the antibody during an immune response.

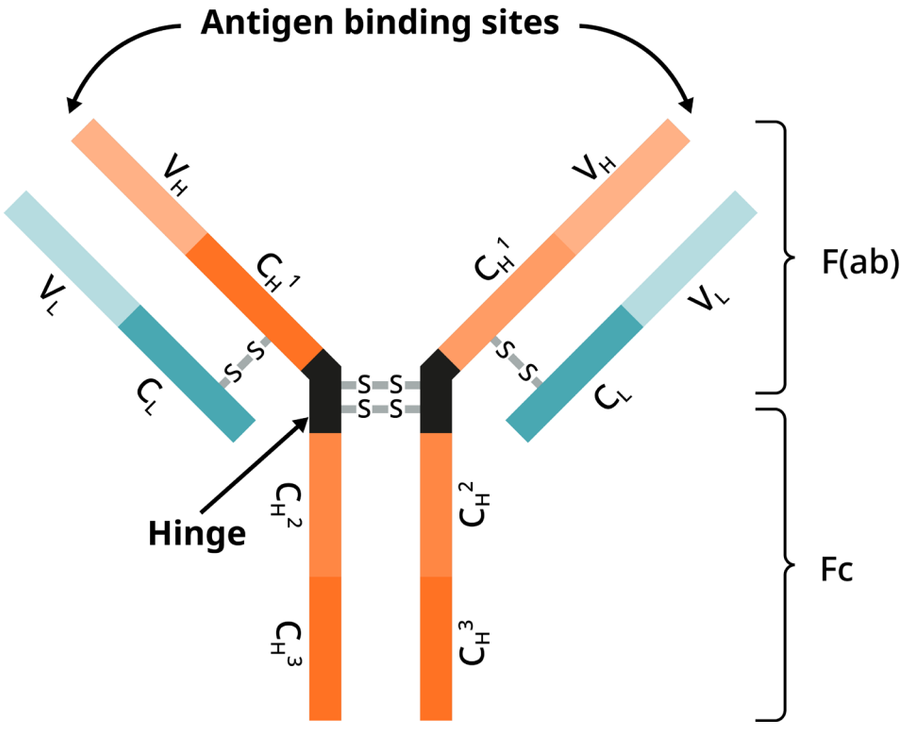


Figure 1. Antibody structure. The Y-shaped antibody is joined in the middle by a flexible hinge region. Antigen binding occurs at the variable domain (V), consisting of immunoglobulin heavy (H) and light chains (L). The base of the antibody includes constant domains (C). V_H – heavy chain variable domain, V_L- light chain variable domain, C_H – heavy chain constant domain, C_L – light chain constant domain.

F(ab) and Fc regions

The Y-shape of an antibody can be cleaved into three fragments by the proteolytic enzyme pepsin: two F(ab) regions and an Fc region. The F(ab) regions contain the variable domain that binds to cognate (specific) antigens. The Fc fragment provides a binding site for endogenous Fc receptors on the surface of lymphocytes and secondary antibodies. Also, dye and enzymes can be covalently linked to antibodies on the Fc portion of the antibody for experimental visualization.

Antibody fragments have distinct advantages in specific immunochemical techniques. Fragmenting IgG antibodies is sometimes useful because F(ab) fragments (1) will not precipitate the antigen, and (2) will not be bound by immune cells in live studies because of the lack of an Fc region. Often, because of their smaller size and lack of cross-linking (due to the Fc region's loss), F(ab) fragments are radiolabeled in functional studies. Fc fragments are often used as Fc receptor blocking agents in immunohistochemical staining.

Learn more about the advantages of F(ab) and F(ab')2 fragments.

Heavy chains

The type of heavy chain defines the overall class or isotype of an antibody. There are five types of mammalian Ig heavy chains denoted by Greek letters: α, δ, ε, γ and μ. These chains are found in IgA, IgD, IgE, IgG, and IgM antibodies, respectively. Heavy chains differ in size and composition; α and γ contain approximately 450 amino acids, while μ and ε have about 550 amino acids.

Each heavy chain has two regions: constant (C_H) and variable (V_H). The constant region is identical in all the same isotype antibodies but differs in antibodies of different isotypes. Heavy chains γ, α, and δ have a constant region composed of three tandem Ig domains – C_H^1, C_H^2, C_H^3 – and a hinge region for added flexibility. Heavy chains μ and ε have a constant region composed of four immunoglobulin domains. The heavy chain's variable region (V_H) differs depending on the B cell that produced it but is the same for all antibodies produced by a single B cell or B cell clone. Each heavy chain's variable region is approximately 110 amino acids long and composed of a single Ig domain.

Light chains

Mammals have only two types of light chains, lambda (λ) and kappa (κ), which have minor differences in the polypeptide sequence. A light chain has two successive domains: constant (C_L) and variable (V_L). The approximate length of a light chain is 211–217 amino acids. Each antibody contains two light chains that are always identical. Other types of light chains, such as the iota (ι) chain, are found in lower vertebrates like Chondrichthyes and Teleostei.

Antibody isotypes

In mammals, antibodies are divided into five isotypes: IgG, IgM, IgA, IgD, and IgE. Each isotype has a unique structure, as depicted in Figure 2. The isotypes vary based on the number of Y units and the type of heavy chain. They will also differ in their biological properties, functional locations, and the ability to deal with different antigens (Table 1).

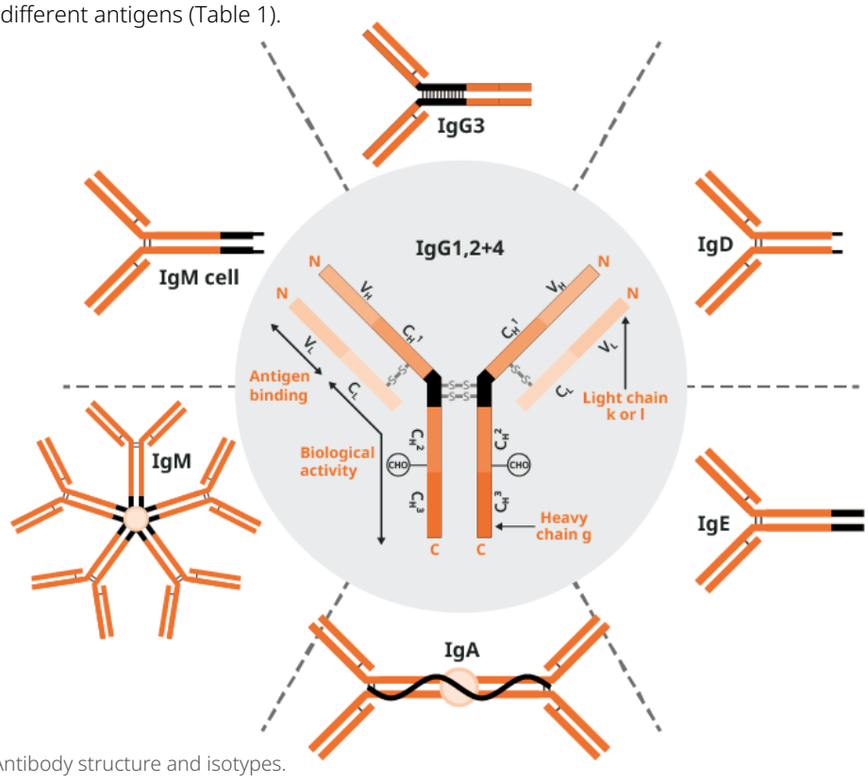


Figure 2. Antibody structure and isotypes.

Table 1. Structure and functions of different antibody isotypes.

Isotype	Heavy chain	Light chain	MW (kDa)	Structure	Function
IgA1 IgA2	α1 α2	λ or κ	150–600	Monomer - tetramer	Most abundantly produced antibody isotype in mice and humans. It is found in mucosal areas, such as the gut, respiratory, and urogenital tract, and prevents their colonization by pathogens. Resistant to digestion and is secreted in milk
IgD	δ	λ or κ	150	Monomer	Function unclear. Works with IgM in B cell development; mostly B cell-bound.
IgE	ε	λ or κ	190	Monomer	Binds to allergens, triggers histamine release from mast cells, and is involved in allergy. It also protects against parasitic worms.
IgG1 IgG2a IgG2b IgG3 IgG4	γ1, γ2, γ3, γ4	λ or κ	150	Monomer	The most abundant Ig in serum that accounts for ~75% of the total serum antibodies in humans. Provides the majority of antibody-based immunity against invading pathogens. Moderate complement fixer.
IgM	μ	λ or κ	900	Pentamer	First response antibody. Expressed on the surface of B cells and in a secreted form with very high avidity. Eliminates pathogens in the early stages of B cell-mediated immunity before there is sufficient IgG.

Antigen-antibody interactions: how and where antibodies bind

The F(ab) antibody region contains the antigen-binding site called paratope. The paratope binds to a specific part of an antigen called the epitope, which is a small part of the antigen – sometimes just a few amino acids long (Fig. 3).

The paratope and epitope are held together by complementary shapes and intermolecular interactions such as Van der Waals, hydrogen bonds, electrostatic and hydrophobic interactions; the strength of these forces determines the antibody's affinity.

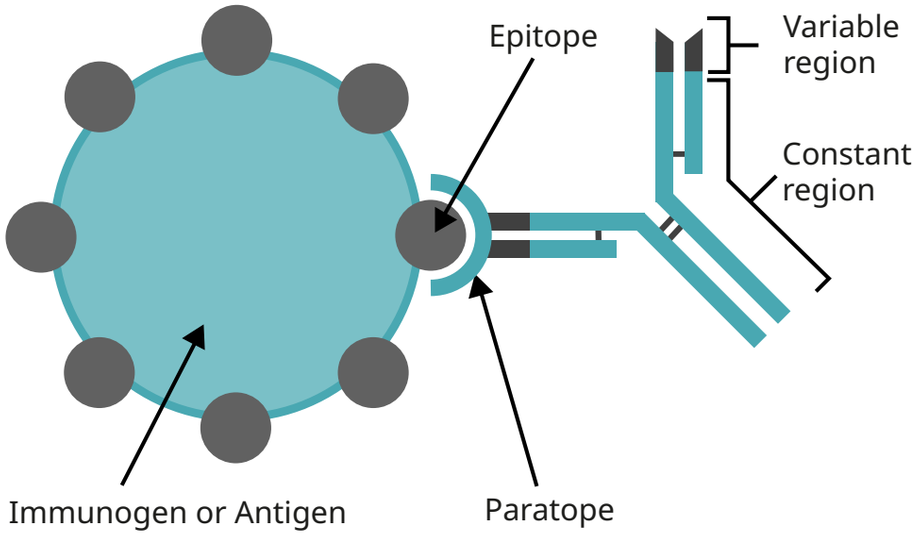


Figure 3. A schematic representation of antigen-antibody interactions.

Antigens: overview and considerations for your experiment

The basic principle of any immunoassay is that a specific antibody binds with its specific antigen, forming an exclusive antibody-antigen complex. This chapter defines what an antigen is and how to choose one to make an antibody.

Antigen: definition

An antigen is any foreign substance that can elicit an immune response in the body (eg, antibody production) and is bound by the specific antibodies produced against it by the immune system.

Antigens generally have high molecular weight and are commonly proteins or polysaccharides. Polypeptides, lipids, nuclear acids, and many other materials can also function as antigens.

Haptens

Haptens are smaller substances that can also generate immune responses if chemically coupled to a larger carrier protein. Common carrier proteins include bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), or other synthetic matrices.

Many molecules may function as haptens, including drugs, simple sugars, amino acids, small peptides, phospholipids, or triglycerides. Thus, given enough time, the immune system will identify just about any foreign substance and evoke specific antibody production. However, this specific immune response is highly variable and depends on the antigen's size, structure, and composition. Antigens eliciting strong immune responses are called strongly immunogenic.

Antigen-antibody interaction

An epitope is a part of an antigen that the specific antibody recognizes and binds to. For efficient interaction between the antigen and the antibody, the epitope must be readily available for binding. If the target molecule is denatured (eg, through fixation, reduction, pH changes, or during preparation for gel electrophoresis), the epitope may be altered, which may affect its ability to interact with an antibody.

For example, some antibodies might be ineffective in western blot but very good in immunohistochemistry (IHC) because a complex antigenic site might be maintained in the tissue in IHC. In contrast, in western blot, the sample preparation process alters the protein conformation sufficiently to destroy the antigenic site and eliminate antibody binding.

Thus, the epitope may be present in the antigen's native cellular environment or only exposed when denatured. In their natural form, epitopes may be cytoplasmic (soluble), membrane-associated, or secreted. The epitopes' number, location, and size depend on the antigen amount available during the antibody-making process.

Characteristics of a good antigen include:

- Areas of structural stability and chemical complexity within the molecule
- Significant stretches lacking extensive repeating units
- A minimal molecular weight of 8,000–10,000 Da, although haptens with molecular weights as low as 200 Da have been used in the presence of a carrier protein.
- The ability to be processed by the immune system
- Immunogenic regions accessible to the antibody-forming mechanism
- Structural elements sufficiently different from the host
- For peptide antigens, regions containing at least 30% of immunogenic amino acids: lysine, arginine, glutamic acid, aspartic acid, glutamine, and asparagine.
- For peptide antigens, significant hydrophilic or charged residues

The immune system and the antibody response

This chapter provides a general overview of the immune system and its role in generating specific antibodies.

Overview of the immune system

The function of the immune system is to protect animals from foreign agents and infectious organisms. It responds to pathogens in a specific way and can display a long-term memory of infectious agents' exposure. The immune system consists of two functional components:

1. The innate or non-specific immune system
2. The adaptive or specific immune system

The innate immune system

The innate immune system components provide the first line of defense against infection. Physical barriers to infection include skin, which prevents pathogen penetration, and bodily fluids, like mucus, which collect and clear pathogens.

Many cellular and biochemical components, including complement proteins, innate leukocytes, and phagocytic cells, identify and eliminate pathogens from the body.

The innate immune system's function and efficiency do not change with repeated exposure to foreign pathogens.

The adaptive immune system

The adaptive immune system is activated when the innate system fails to clear pathogens from the body. It consists of various cells and molecules, with lymphocytes and antibodies being the key elements.

Lymphocytes arise continuously from progenitor cells in the bone marrow. Lymphocytes synthesize cell surface receptors or secrete proteins that specifically bind to foreign molecules. These secreted proteins are known as antibodies. Any molecule that can bind to an antibody is called an antigen. The term antibody is used interchangeably with immunoglobulin.

Pathogens bound to antibodies are marked for clearance or destruction.

Most functions of the adaptive immune system can be described by grouping lymphocytes into three basic types:

1. B cells
2. Cytotoxic T cells (TC cells)
3. Helper T cells (Th cells)

The adaptive immune response can be either humoral or cell-mediated. B lymphocytes mediate the humoral response by releasing antibodies specific to the infectious agent. The cell-mediated response involves binding TC cells to foreign or infected cells, followed by the lysis of these cells.

Th cells are involved in both responses through the release of cytokine proteins. All three types of lymphocytes carry cell surface receptors that can bind antigens. All antigen receptors are glycoproteins, and only one kind of receptor is synthesized within any one cell. The specificity of the immune system is impacted by the fact that one cell recognizes only one antigen.

View our poster on human T cell development

Antibody response

The antibody-antigen interaction forms the basis of all immunoassays but is also the basis for the immune response.

The region of the antibody that reacts with the antigen is called the paratope. The region of an antigen that interacts with an antibody is defined as an epitope. Affinity measures the strength of the epitope's binding to an antibody and is often represented by the dissociation constant KD. Avidity measures the overall stability of the complex between antibodies and antigens.

Learn more about Kd and how it affects antibody performance

An antibody response is the culmination of a series of interactions between macrophages, T lymphocytes, and B lymphocytes. Infectious agent antigens are engulfed and partially degraded by antigen-presenting cells (APCs), such as macrophages, Langerhans cells, dendritic cells, lymph nodes, and monocytes.

The antigen's fragments will appear on the APC's surface attached to a cell surface glycoprotein known as MHC II (major histocompatibility complex). There are two types of MHC molecules: MHC class I, expressed on the surfaces of most cells, and class II, expressed exclusively on APCs' surfaces. The antigen-MHC II complex allows Th cells to bind to the APC, leading to a proliferation of Th cells and cytokine release. T cells then bind to the MHC complex on B cells, leading to B cells' proliferation and differentiation. B cells change into plasma cells, secreting large quantities of finely tuned antibodies specific to the foreign agent. Some B cells are transformed into memory cells, allowing for a faster antibody-mediated immune response upon future infection.

View our poster on antigen processing by MHCs

Chapter 2. An introduction to antibody production

Here we review the benefits and limitations of monoclonal, polyclonal, and recombinant monoclonal antibodies and how they are produced.

Polyclonal antibody production

Polyclonal antibodies represent a heterogeneous mix of antibodies, with each antibody recognizing different epitopes of a particular antigen.

Polyclonal antibody production typically starts with immunizing an animal with the target antigen to stimulate an immune response, involving the production of antigen-specific antibodies by the animal's B cells (Fig. 4). Immunizations of the same antigen are repeated at intervals of several weeks to increase the number and affinity of antigen-specific antibodies within the animal. The resulting immune-sera (a blood portion containing the antibodies) can be used in its crude form, or the antibodies can be isolated by affinity purification.

Polyclonal antibodies consist of a mixture of antibodies representing the natural immune response to an antigen. So, they can produce a strong signal against the target antigen in their relevant application and are not biased against a single epitope. However, the disadvantages to their use are that they are limited in supply, and batch-to-batch variation is higher than with monoclonal antibodies. Polyclonal antibodies can also exhibit cross-reactivity and lack of specificity because of a higher risk of binding to other proteins with similar sequences. These issues are often addressed by cross-adsorbing (ie, further purifying) the polyclonal antibody mixture to remove antibodies with unwanted binding characteristics, often against similar antigens in particular species.

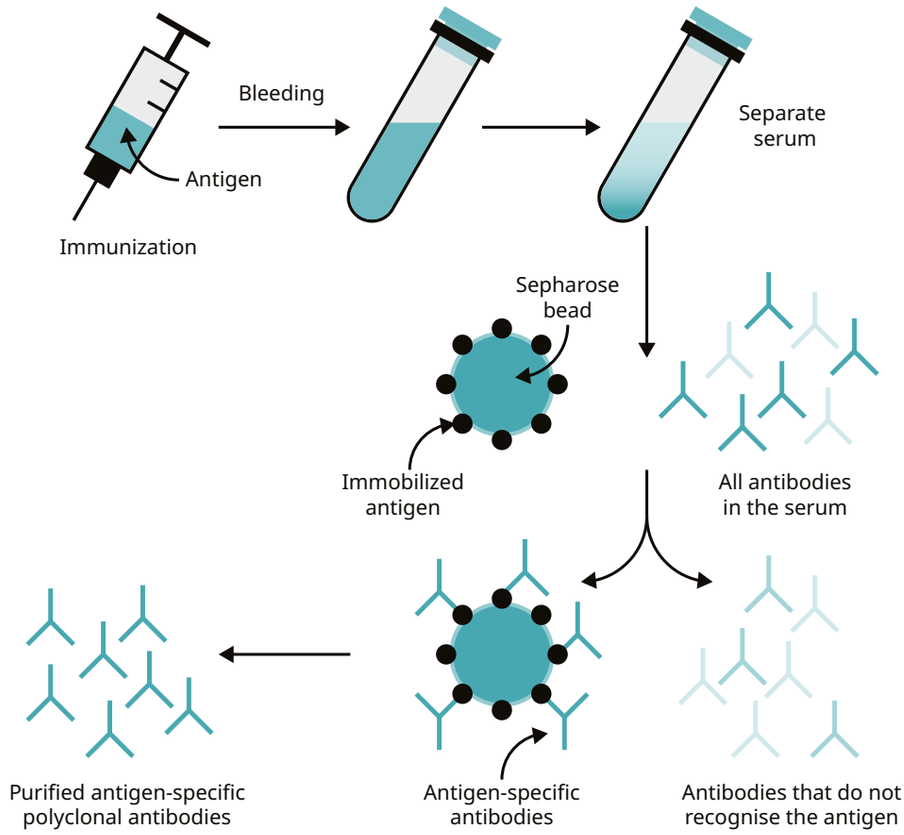


Figure 4. A typical process of polyclonal antibody production.

Monoclonal antibody production using hybridoma technology

In contrast to polyclonal antibodies, a monoclonal antibody is derived from a single B cell parent clone and will only recognize a single epitope per antigen (Fig. 5). In the hybridoma production method, B cells are immortalized by fusion with hybridoma cells, allowing for the long-term production of immunoglobulins (Ig).

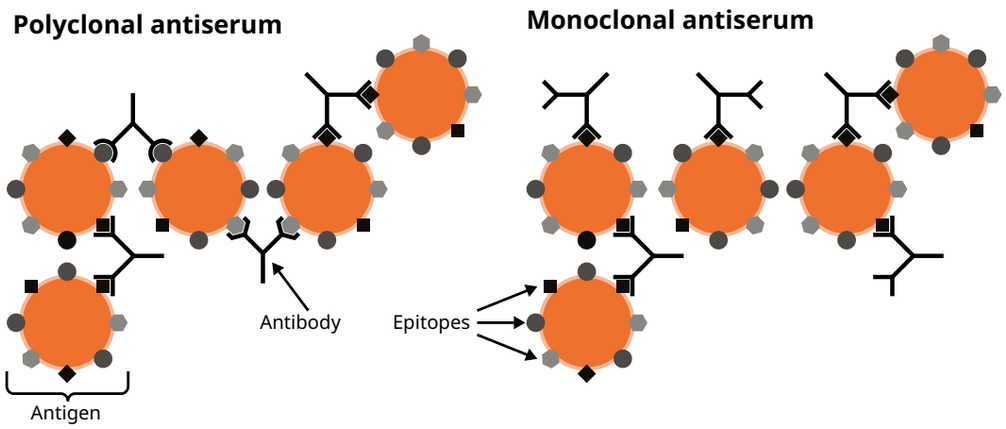


Figure 5. The difference in the specificity of polyclonal antibodies and monoclonal antibodies.

Monoclonal antibody production via the hybridoma method starts with the same immunization protocol used for polyclonal antibodies. After immunization, antibody-producing cells (B cells/plasma cells) are harvested from the spleen and fused with immortal tumor cells to become hybridomas screened for antibody production and performance (Fig. 6).

The antibody-producing hybridoma cells are cloned by isolation and cultivated using tissue culture techniques. Antibodies secreted by the cells into the culture media can be harvested and used either in their crude form or purified by affinity purification. Hybridomas may have unique clone names (eg, MJFF5 (68-7) or EPR19759) to identify the exact clone. Unlike polyclonal antibodies, monoclonal antibodies are homogenous with defined specificity to one epitope.

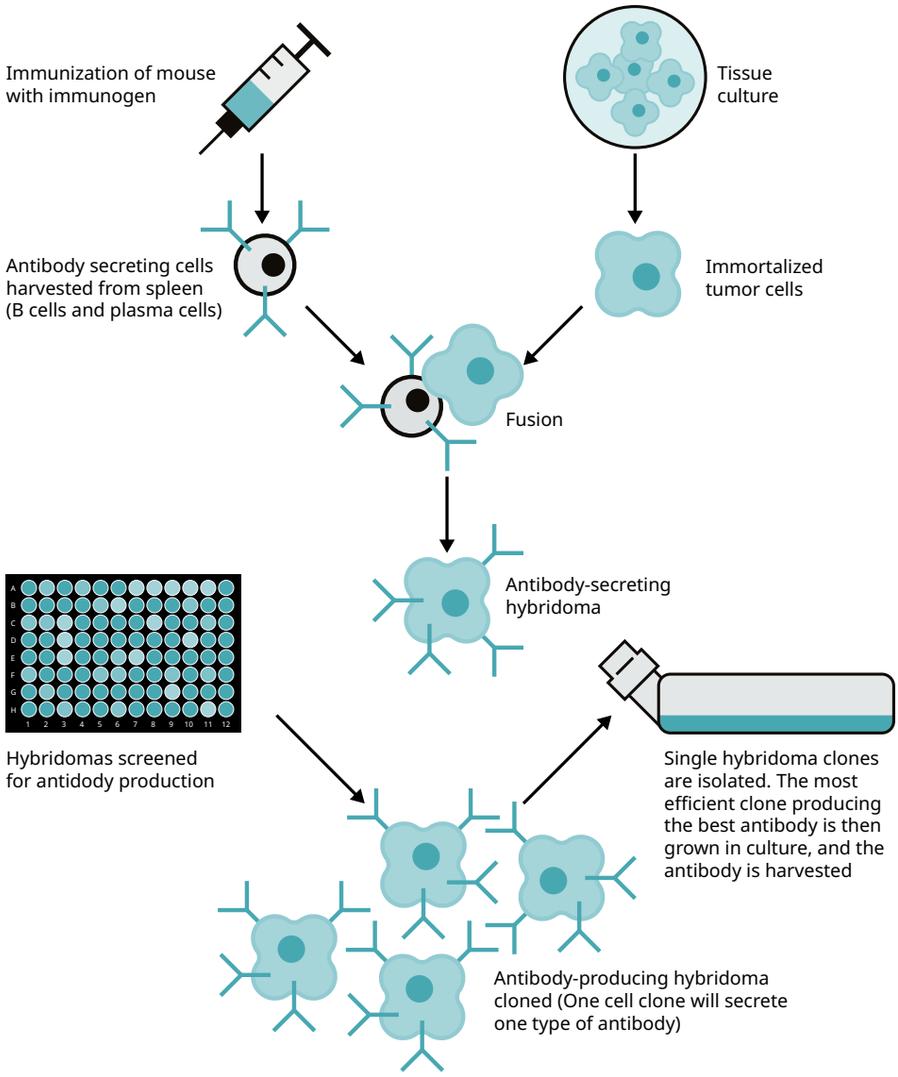


Figure 6. Monoclonal antibody production using hybridomas.

As they homogeneously detect a single epitope on an antigen, monoclonal antibodies are less likely to cross-react with other proteins and display less batch-to-batch variation than polyclonal antibodies. Monoclonal antibodies can be well characterized and defined to meet specificity and sensitivity criteria.

Monoclonal antibodies produced using hybridoma cell lines are prone to experiencing genetic drift over time. An antibody produced using the same cell line several years later may have slight variations from the antibody's original version. Therefore, to preserve the antibody for confirmed long-term supply and maintain product quality, hybridoma-derived antibodies are converted to the recombinant format.

Recombinant monoclonal antibody production

A recombinant antibody is an antibody generated *in vitro* using synthetic genes. The encoding sequences can be carefully controlled, allowing for optimized expression (higher antibody yields) and improved reproducibility over antibodies produced from hybridomas. Compared to traditional monoclonal and polyclonal antibodies, recombinant antibodies offer long-term, secured supply with a minimal batch-to-batch variation. Also, as the antibody-encoding sequence is known and defined, it can be further engineered and manipulated for its intended use. For example, the sequence can be modified to improve antibody binding characteristics, include tags, or incorporate an FC fragment from an alternative species.

Recombinant antibodies are produced by cloning the antibody-coding genes into a high-yield mammalian expression vector. The resulting vectors are then introduced into expression hosts to manufacture functional antibodies. Mammalian cell lines, such as HEK 293 or CHO-K1, are typically used as expression hosts to preserve the correct post-translational modifications (such as glycosylation).

Multiple methods are available to derive the antibody-coding genes, including hybridoma technology, phage display, B cell cloning, and Next-Generation Sequencing (NGS). Here we describe recombinant antibody production processes based on hybridoma and phage display technologies.

Recombinant monoclonal antibody production from existing hybridomas

Existing hybridoma-based monoclonal antibodies can be converted to recombinant monoclonal antibodies to ensure enhanced consistency and specificity. Recombinant conversion is achieved by obtaining the sequence of the antibody-producing genes from the hybridoma and expressing them in a mammalian cell line. We've outlined the process below.

1. Hybridomas are produced using the same procedure described above in Figure 6.
2. A particular hybridoma's mRNA is isolated, converted to cDNA, and then amplified using PCR to identify the antibody-encoding sequence to allow in vitro recombinant production.
3. The antibody-encoding sequence is sequenced and cloned into an expression vector.
4. The genes are then expressed in a mammalian cell line (eg, HEK 293).
5. The resulting recombinant antibody is validated to ensure the performance matches the hybridoma version.

Recombinant monoclonal antibody production by phage display

In vitro phage display technology provides the ability to discover recombinant monoclonal antibodies against targets more rapidly and without animal immunization (in the case of naïve libraries). It involves generating an antibody library, selecting antibodies binding to the target/antigen of interest, and affinity maturation (Groff et al., 2015).

The phage display process includes the following steps (Fig. 7):

1. Target proteins or peptides can be captured on an ELISA plate or coated onto magnetic beads. These antigen-coated surfaces are used to screen the phage display library (with bacteriophage expressing an antigen-specific antibody domain, such as scFv or Fab, sticking to this coated surface).
2. Plates are washed to remove non-specific binders.
3. The specific phage display binders are subsequently eluted and transduced into bacterial cells for amplification.
4. Additional rounds of panning/screening are processed to enrich the presence of antigen-specific bacteriophage. Typically 2-3 rounds are performed.
5. Target specificity is confirmed through binding assays, such as an ELISA. DNA from positive binders is then isolated and sequenced.
6. The antibody-encoding sequence is engineered into a mammalian or IgG expression vector for large-scale manufacture of the recombinant monoclonal antibody.
7. The antibody is subsequently validated in relevant assays, such as western blot, immunofluorescence, flow cytometry, and immunohistochemistry.

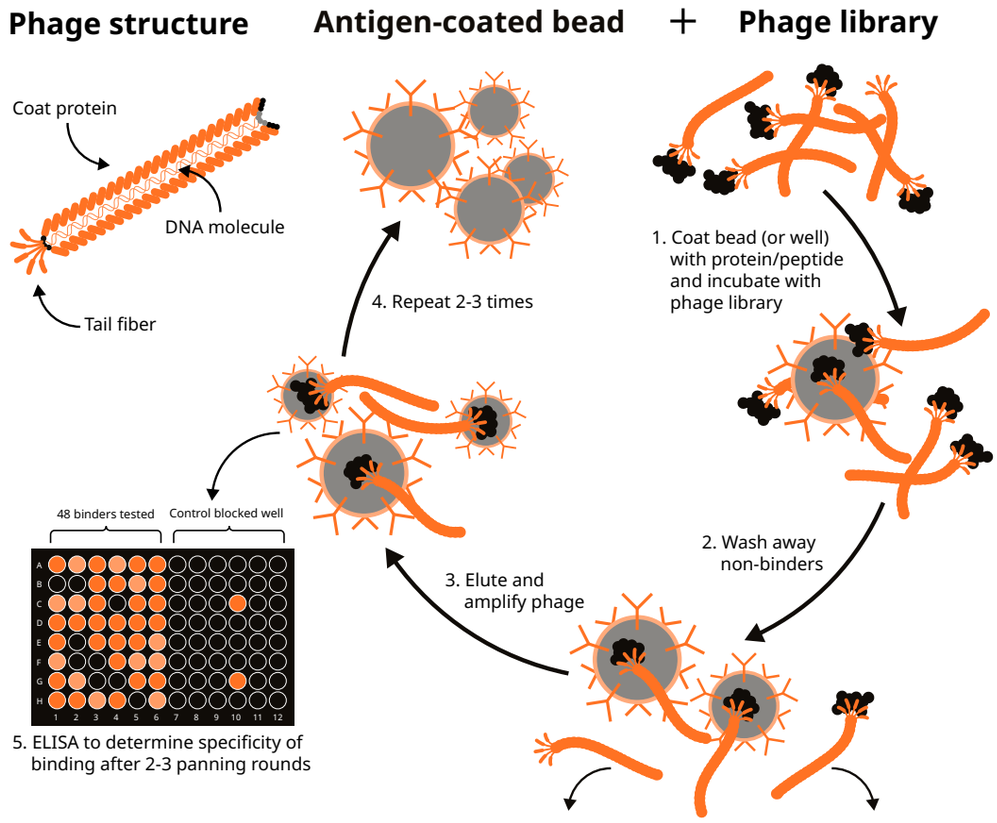


Figure 7. An example of the phage display process (Steps 1–5).

Chapter 3. Antibody methods and techniques

Antibodies are powerful research tools used in various lab techniques. Here we provide a brief overview of the most popular lab techniques, highlighting how they use antibodies.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a plate-based technique enabling the detection of antigens in biological samples. Like other immunoassays, ELISA relies on antibodies to detect a target antigen using highly specific antibody-antigen interactions. ELISA enables the quantification and characterization of analytes and molecular interactions.

In an ELISA, the antigen is immobilized to a solid surface either directly or more commonly via a capture antibody, itself immobilized to the surface (Fig. 8). The surface is washed, then incubated with detection antibodies conjugated to molecules such as enzymes or fluorophores.

In the antigen's presence, these detection antibodies will remain bound to the plate, providing a signal. The strength of this signal corresponds to antigen concentration within the sample.

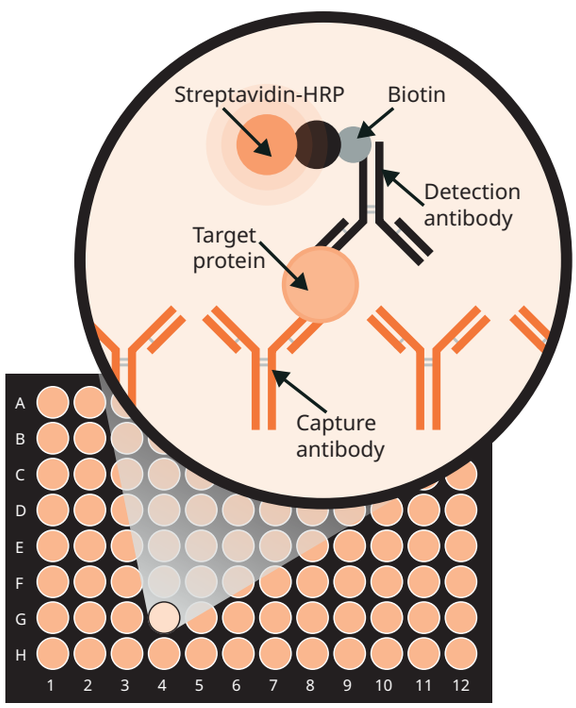


Figure 8. Sandwich ELISA setup. A capture antibody on a multi-well plate will immobilize the antigen of interest. This antigen will be recognized and bound by a detection antibody conjugated to biotin and streptavidin-HRP.

An ELISA is typically performed in a multi-well plate (96- or 384-wells), and the analytes' immobilization facilitates the separation of the antigen from the rest of the sample components. These characteristics make ELISA one of the easiest assays to perform on multiple samples simultaneously.

There are four main types of ELISA: direct, indirect, sandwich, and competitive – each with unique advantages, disadvantages, and suitability. The most appropriate ELISA format for each experiment will depend on many factors, including desired sensitivity, specificity, and assay time. See more information **here** to help you choose the right type of ELISA.

Enzyme-linked immunospot (ELISPOT)

Enzyme-linked immunospot (ELISPOT) is used to detect proteins secreted by cells, such as cytokines and growth factors. The technique enables quantification and comparison of immune responses to various stimuli.

Cells are grown in 96-well plates with antibody-coated PVDF or nitrocellulose membranes. The secreted proteins of interest are detected using primary and conjugated secondary antibodies. Cells secreting the protein of interest will appear as a spot of color or fluorescence. Membranes are scanned and analyzed to quantify the number or proportion of cells secreting the protein.

For the detailed procedure, please refer to our **ELISPOT protocol**.

Western blot (WB)

Western blot is widely used in research to separate and identify proteins. Western blot allows us to detect proteins, determine the relative protein levels between samples, and establish the target's molecular weight, providing insight into its post-translational processing.

Western blot involves three main steps: (1) separation of proteins by size, (2) transfer of proteins to a membrane, and (3) visualizing the target protein using primary and secondary antibodies (Fig. 9).

In the first step, the proteins are loaded onto a gel and separated based on size by gel electrophoresis. Protein bands are then migrated to a membrane using an electrical current. Protein transfer to the membrane is essential because gels used for electrophoresis provide an inferior surface for subsequent immunostaining, ie, antibodies don't stick to the gel's proteins.

Finally, the membrane can be further immunostained with antibodies specific to the target of interest and visualized using secondary antibodies and detection reagents.

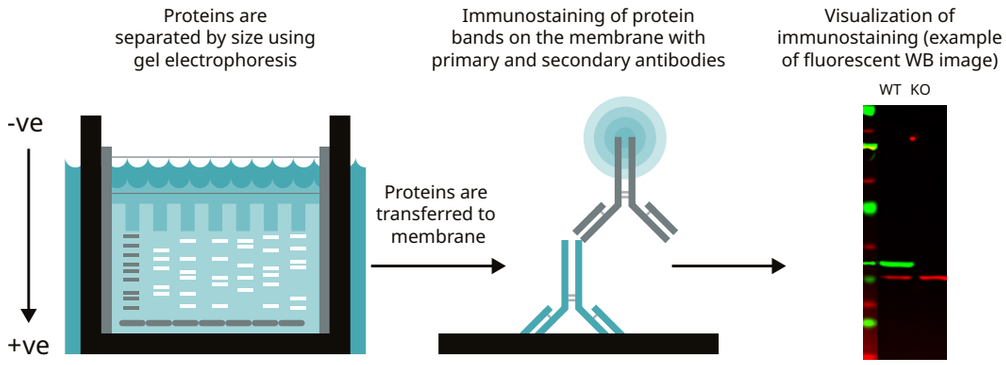


Figure 9. A simplified diagram of western blotting.

For a full procedure, please refer to our **western blot protocol.**

Immunoprecipitation (IP) and Chromatin immunoprecipitation (ChIP)

Immunoprecipitation (IP) is a versatile technique that isolates and purifies individual and complexed proteins. In this technique, antibodies are immobilized on solid-phase substrates (eg, magnetic/agarose beads), capturing antigens from complex solutions.

Chromatin immunoprecipitation (ChIP) is used to determine whether a given protein binds to a specific DNA sequence *in vivo*. ChIP allows researchers to identify specific genes and sequences where a protein of interest binds across the entire genome, providing critical clues to their regulatory functions and mechanisms.

The ChIP procedure (Fig. 10) utilizes an antibody to immunoprecipitate a protein of interest, such as a transcription factor, along with its associated DNA. The associated DNA is then recovered and analyzed by PCR, microarray or sequencing to determine the genomic sequence and location where the protein was bound.

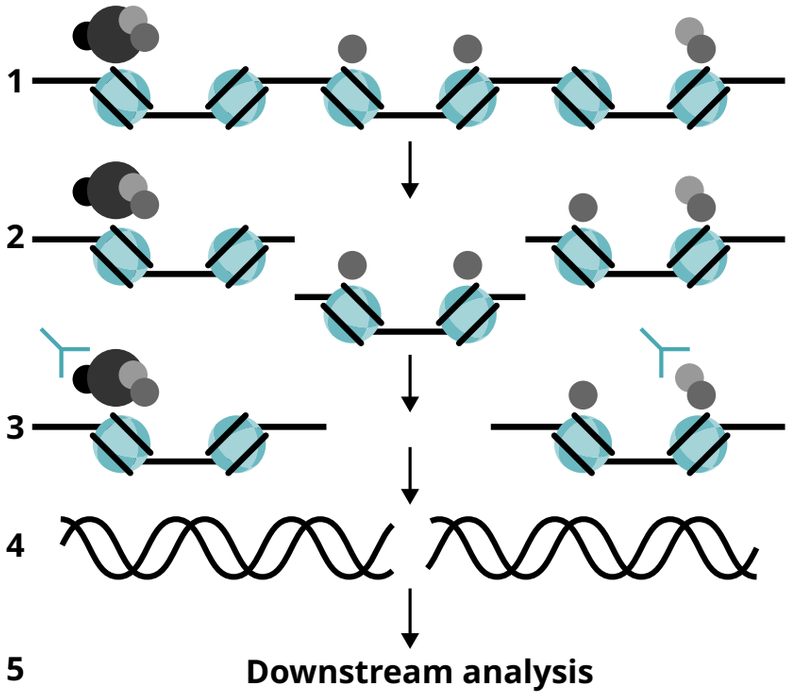


Figure 10. ChIP protocol workflow. A step-by-step approach to carry out a ChIP experiment, where 1 - cross-linking, 2 – chromatin fragmentation, 3 – immunoprecipitation, 4 – DNA recovery and purification, 5 – DNA analysis.

To learn more about the ChIP procedure, refer to our **ChIP guide.**

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a method to access the distribution and localization of antigens in tissue sections using antibody-antigen interactions (Fig. 11). Although less quantitative than western blot or ELISA, IHC offers the advantage of characterizing protein expression in the context of intact tissue.

IHC is often used to diagnose tissue abnormalities in diseases such as cancer. IHC provides valuable perspective and support that can contextualize data obtained from other methods.

IHC staining relies on antibodies that recognize the target antigen. You can use chromogenic or fluorescent-based detection systems to visualize this antibody-antigen interaction. In chromogenic detection, an antibody is conjugated to an enzyme that produces a colored precipitate when exposed to a chromogen. In fluorescent detection, an antibody is conjugated to a fluorophore. There are various techniques for sample preparation and visualization, and the method used should be tailored to your type of specimen and the degree of sensitivity required.

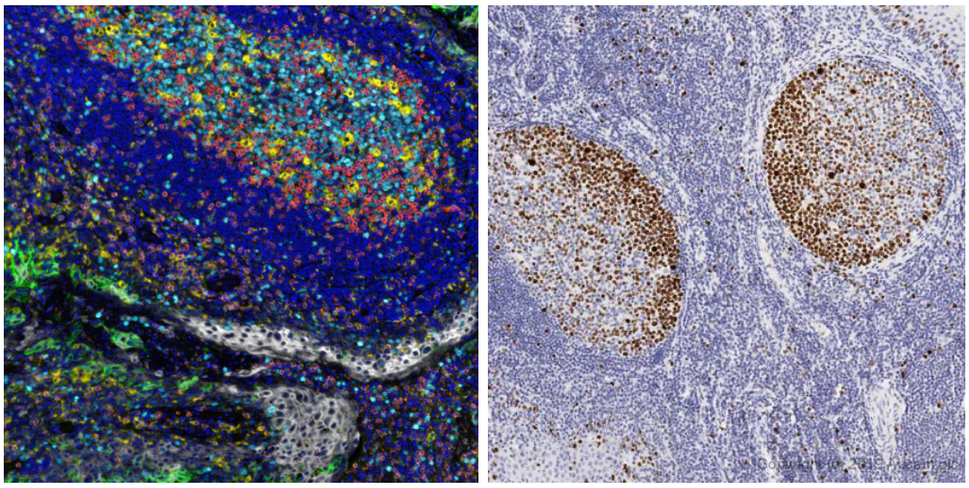


Figure 11. On the left, fluorescence multiplex IHC staining of normal human tonsil tissue (formalin-fixed paraffin-embedded section). Merged staining of anti-PD1 (ab237728; orange; Opal™520), anti-PDL1 (ab237726; green; Opal™540), anti-CD68 (ab192847; yellow; Opal™570), anti-CD3 (ab16669; red; Opal™620), anti-Ki67 (ab16667; light blue; Opal™650) and anti-PanCK (ab7753; grey; Opal™690). On the right, IHC staining with anti-Ki67 antibody (ab16667) in a section of formalin-fixed paraffin-embedded normal human tonsil.

Check here for more information on immunostaining in IHC.

Immunocytochemistry (ICC)

Immunocytochemistry (ICC) is used to study the subcellular distribution of proteins using labeled antibodies. In contrast to IHC, this technique focuses on samples of cells rather than blocks of tissues.

In ICC staining, antibodies raised against a protein of interest are applied to cell culture samples that have been fixed and permeabilized. There are two types of ICC: direct and indirect. Direct ICC uses conjugated primary antibodies, whereas indirect ICC involves an unconjugated primary antibody, which will then be detected by a conjugated secondary antibody (Fig. 12). For most ICC experiments, antibodies are labeled with fluorophores which is ideal for co-localization studies. Various imaging techniques, such as widefield, confocal or spinning disc microscopy, can be used to detect the signal.

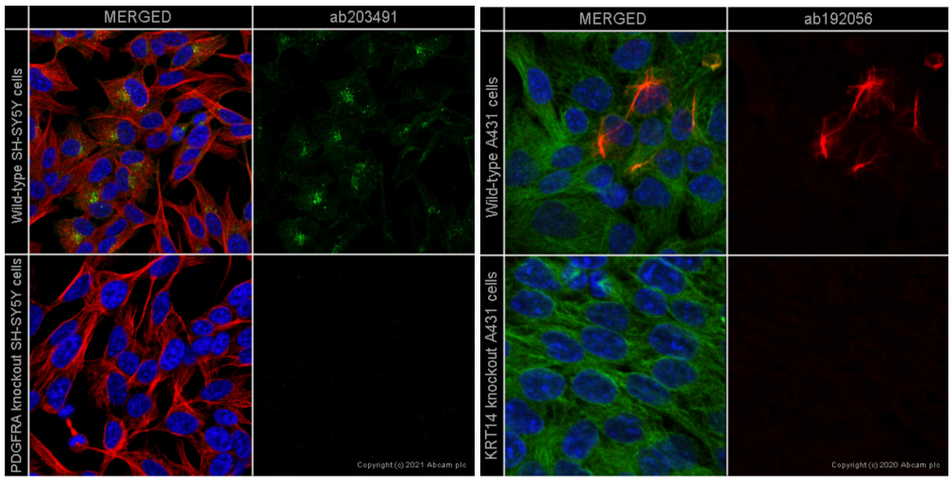


Figure 12. On the left, an indirect ICC staining in SH-SY5Y cells with an anti-PDGFRA antibody (detected with a secondary Alexa Fluor® 488 antibody - green) and an anti-tubulin antibody (detected with a secondary Alexa Fluor® 594 antibody - red) is shown. On the right, a direct ICC with a conjugated anti-KRT14 antibody (Alexa Fluor 647® - red) and a conjugated anti-tubulin antibody (Alexa Fluor 488® - green) is shown. Nuclei are stained with DAPI (blue). The top panels show the signal of interest in wild-type cells, and the bottom panels show the absence of the specific signal in knock-out cells. The image was taken with a confocal microscope.

You can find the detailed ICC protocol here.

Flow cytometry and FACS

Flow cytometry is a popular laser-based technology used to analyze the characteristics of cells or particles (Fig. 13). The technique measures fluorescence emitted by labeled antibodies bound to individual cells in a mixed population. Also, the scattering of light by different cells is used to determine their size and properties.

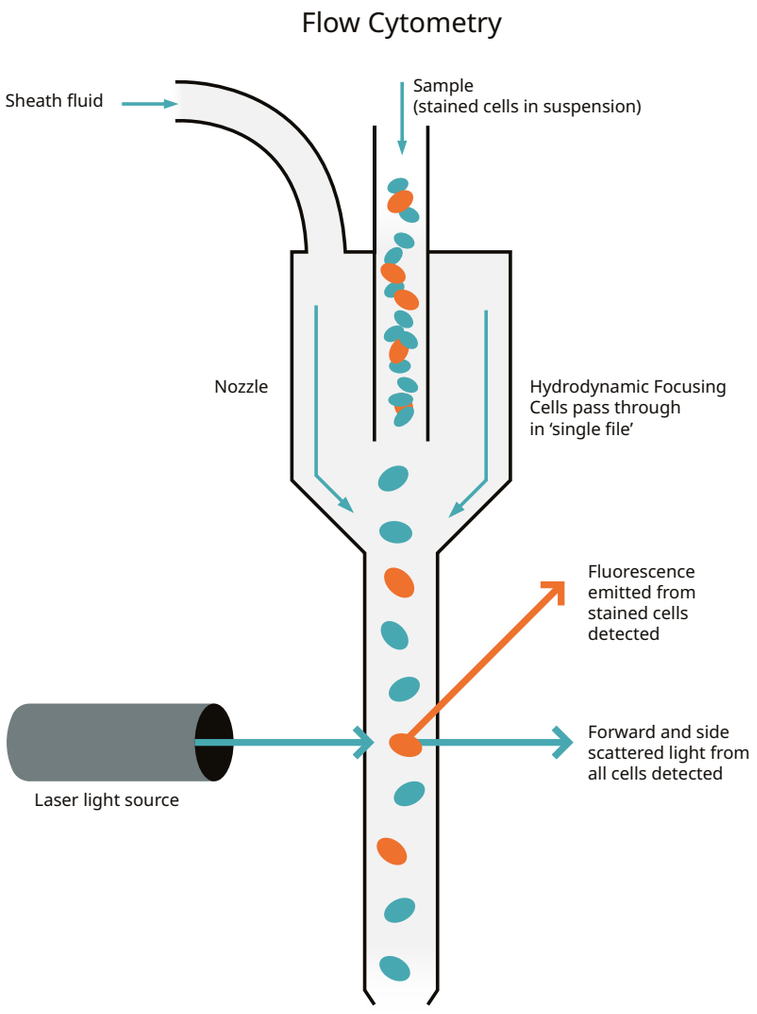


Figure 13. An overview of a flow cytometer.

Flow cytometry enables you to analyze the expression of cell surface and intracellular molecules, characterize and define different cell types in a heterogeneous cell population, assess the purity of isolated subpopulations, and analyze cell size and volume. It allows simultaneous multi-parameter analysis of single cells. See our **introduction to flow cytometry** for more information.

Fluorescence-activated cell sorting (FACS) is a derivative of flow cytometry that physically separates a population of cells into subpopulations based on fluorescent labeling.

Chapter 4. How to choose and use antibodies

Choosing a primary antibody

A primary antibody is an antibody that binds directly to a target protein, with a variable antibody region recognizing a protein's epitope. When choosing a primary antibody, consider the following:

Antibody clonality and manufacture method

Clonality is determined by whether the antibodies come from different B-cells (polyclonal antibodies) or identical B-cells derived from a parent clone (monoclonal antibodies). These antibodies have distinct advantages and limitations covered in Chapter 2.

To recap, polyclonal antibodies consist of a heterogeneous mixture of antibodies, with each antibody recognizing different epitopes of a particular antigen (Fig. 5, Chapter 2). By binding to several different epitopes, polyclonal antibodies can produce a strong signal against the target antigen in their relevant application and are not biased against a single epitope. However, they are limited in supply, subject to high batch-to-batch variability, and exhibit cross-reactivity and lack of specificity.

In contrast to polyclonal antibodies, monoclonal antibodies only recognize a single epitope per antigen (Fig. 5, Chapter 2). Monoclonal antibodies have high specificity for their target, low non-specific cross-reactivity, and minimal batch-to-batch variations.

The term 'recombinant' refers to antibodies produced in vitro using synthetic genes. Compared to traditional monoclonal and polyclonal antibodies, recombinant antibodies offer long-term, secured supply with a minimal batch-to-batch variation. Since the antibody-encoding sequence is known and defined, it can be further engineered and manipulated for its intended use.

We recommend using **recombinant monoclonal antibodies** when a suitable clone exists for your particular target and application to ensure experimental reproducibility and long-term antibody supply. For applications where a polyclonal antibody would traditionally be used (eg, when analyzing low-abundance targets or detecting multiple post-translational modifications at once), **recombinant multiclonal antibodies** can offer an ideal solution. Recombinant multiclonal antibodies are a defined mixture of carefully selected individual recombinant monoclonal antibodies designed to recognize different epitopes on the same antigen. So, they can provide excellent sensitivity combined with superior specificity and reproducibility, only available from a recombinant antibody.

Antibody validation in specific applications and species

When selecting a primary antibody, ensure it's validated to bind the target. Antibody datasheets should list the applications and species in which the antibody has been successfully tested.

The datasheet will also highlight if an antibody was tested in a particular application and failed. If the datasheet doesn't list an application and species, it is unknown how the antibody will perform in this specific application and species. In this case, check if there are any reviews from customers who tested the antibody in your application and species of interest. All published customer reviews for a given product are listed under the "Customer reviews & Q&A" tab of the datasheet on our website.

Our antibodies are continuously tested, and datasheets are updated with the latest information on validated applications and species.

Antibody specificity confirmed by knock-out validation

A good antibody exhibits target specificity, allowing it to identify the protein of interest even at low expression levels. However, many studies have shown that not all antibodies are specific in this way, with many displaying cross-reactivities with off-target proteins.

Knock-out (KO) validation is one of the most accepted and trusted validation processes for antibody specificity. This robust technique can confirm the antibody's specificity by testing it in a KO cell line, cell lysate, or tissue that does not express the target protein. A specific antibody should produce no signal in the KO cell line but give a specific signal in the wild-type cell line. In this way, KO validation serves as a true negative control.

Figure 14 below shows an example of KO validation for KI-67 antibody in immunocytochemistry (ICC), with Ki67 knock-out HAP1 cells (bottom) showing no expression of Ki67 (green).

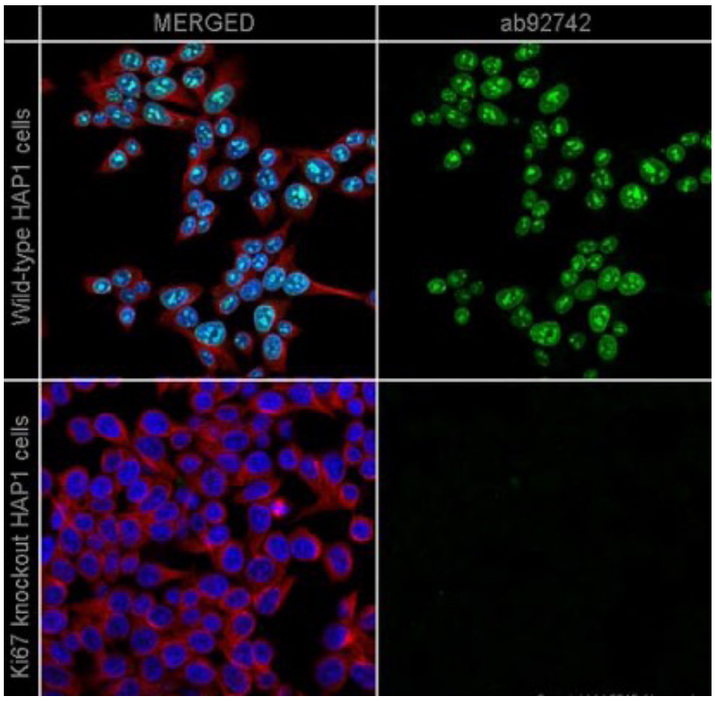


Figure 14. ICC/immunofluorescence image of knock-out testing for Ki67 antibody in wild-type (top) and Ki67 knock-out HAP1 cells (bottom). Green is anti-Ki67 [EPR3610] (ab92742) with goat-anti-rabbit IgG (Alexa Fluor® 488) (ab150081), red is anti-alpha-tubulin [DM1A] (Alexa Fluor® 594) (ab195889), and blue is nuclear DNA labeled with DAPI.

We recommend you choose antibodies that have been validated in multiple applications, ideally using KO technologies. Alternatively, you can validate an antibody yourself, using the appropriate KO cell line, KO cell lysate, or tissue.

Immunogen details

As described in Chapter 2, antibody discovery often starts by immunizing host animals with an immunogen. These immunogens can be full-length proteins, peptides, or whole cells. Usually, you can find information about the immunogen on the datasheet. However, the immunogen sequence won't be available if it's proprietary information.

The immunogen used will define which region of the protein antibody binds. If the immunogen sequence is publicly available, check that the immunogen is identical to or contained within the region of the protein you are trying to detect. For example, if you are trying to detect a cell surface protein on live cells by FACS, choose an antibody raised against the protein's extracellular domain.

Sample processing

An antibody is specific to an epitope in a particular conformation. Since sample processing will change epitope conformation (eg, fixation will lead to protein cross-linking by formaldehyde-induced methylene bridges), some antibodies only work on samples processed in a certain way. Many antibodies will only recognize proteins that have been reduced and denatured because this reveals epitopes that would otherwise be obscured. On the other hand, some antibodies will only recognize epitopes on proteins in their native state.

For immunohistochemistry, some antibodies are only appropriate for unfixed frozen tissue. Others that have been formalin-fixed and paraffin-embedded need an antigen retrieval step to expose the epitope. We recommend you check if the antibody datasheet lists any restrictions on sample processing.

Host species

If you intend to perform indirect detection with secondary antibodies, you should ideally choose a primary antibody raised in a different species to your sample. This allows you to avoid cross-reactivity of the secondary (anti-immunoglobulin) antibody with endogenous immunoglobulins in the sample. For instance, if you study a mouse protein, choose a primary antibody raised in a species other than a mouse – eg, rabbit. Since cross-reactivity emerges from the presence of host antibodies in the sample, it's a pitfall for tissue samples but not cell lines.

Suppose you have to use a primary antibody with the same host species as your tissue sample. In that case, you'll need to carefully consider how to **modify your protocol** to reduce background staining. Alternatively, to avoid cross-reactivity, you can use chimeric antibodies made up of domains from different species.

You don't need to worry about the primary antibody's host species with applications like western blot that use a cell lysate without any endogenous immunoglobulin (IgG) or direct detection experiments that use primary conjugated antibodies.

A note on non-model organisms

If you work in a non-model organism (ie, species not commonly used in research), you may need to use an antibody that hasn't been tested in your species. In many cases, the protein sequences are often conserved enough across several species and can be recognized with an antibody not validated in this species.

If there's no alternative to using a non-validated antibody, we recommend checking the antibody's immunogen sequence alignment with your protein of interest.

- You can find antibody immunogen sequences using the **UniProt/SwissProt protein database** link on online datasheets.
- Take this immunogen sequence and compare it with the protein you're interested in using an online tool like **CLUSTALW**.
- An alignment score of over 85% indicates that the antibody may bind to your protein. However, this doesn't guarantee the antibody will perform well; you'll need to run several controls to ensure it works as intended.

Carriers and preservatives

Typically, antibodies are stored in a phosphate-buffered saline (PBS) solution with carrier proteins like bovine serum albumin (BSA) and preservatives like glycerol and sodium azide. While these are essential components for maintaining antibody stability and preventing contamination, they can hinder the effective conjugation of labels (eg, fluorochromes, enzymes, and metals), affect live-cell systems, and even possibly interfere with highly specialized hardware setups.

During a typical conjugation reaction, BSA will compete with the primary antibody to attach to the label of interest, significantly reducing the conjugation efficiency. The presence of sodium azide in the antibody solution can be toxic to cells, limiting the antibody's use in cell culture and negatively affecting conjugation. Therefore, if you intend to conjugate your primary antibody or use it to stain live cells, we recommend choosing antibody **formulations without carriers or preservatives**.

A checklist for choosing primary antibodies

Here's a helpful checklist for choosing your primary antibodies.

- Check antibody clonality, application suitability, host species, and species reactivity – you can find these on the datasheet.
- Keep in mind that our **Abpromise™ guarantee** will cover only applications and species listed on the antibody datasheet.
- Take a look at the images and data provided – do the data look robust?
- Read recent **publications citing the antibody and customer reviews** on the datasheet; these should give you a good idea of how the antibody performs.

Choosing a secondary antibody

Secondary antibodies bind primary antibodies to allow detection, sorting, and purification of target antigens. They allow you to detect your protein of interest due to their specificity for the primary antibody species and isotype. Whether you need to use a secondary antibody depends on your antibody detection method.

Direct vs indirect detection methods

The method of detecting the antigen of interest can be either direct or indirect (Fig. 15):

- Direct: antigen is detected by a primary antibody directly conjugated to a label (ie, conjugated primary antibody), so no secondary antibody is required.
- Indirect: antigen is detected by a conjugated secondary antibody that has been raised against the primary antibody's host species and binds to the primary antibody. Indirect methods provide higher signal intensity because several labeled secondary antibodies can bind indirectly to each antigen (Fig. 15). Indirect detection may also include amplification steps to increase signal intensity.

The choice of direct or indirect detection will often depend on the expression level of the target antigen. Direct detection is suitable for analyzing highly expressed antigens. On the contrary, indirect detection is preferable for studying poorly expressed antigens, which benefit from signal amplification provided by the secondary antibody.

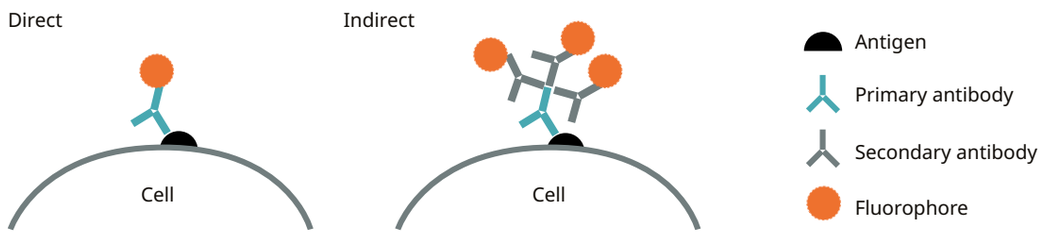


Figure 15. A schematic representation of direct and indirect detection of antigens using antibodies labeled with a fluorophore.

Both methods have several benefits and limitations to consider before choosing the most appropriate method for your experiments (Table 2).

Table 2. Comparison of direct vs indirect detection methods.

	Direct	**Indirect**
Time	Usually shorter as it only requires one labeling step.	Using conjugated secondary antibodies results in additional steps and a longer time.
Complexity	Fewer steps in the protocol make this a more straightforward method.	You need to select an appropriate secondary antibody or combinations of antibodies in multiplex experiments, which adds complexity.
Sensitivity	Signal may seem weaker compared to indirect methods because of the absence of secondary antibodies, which typically provide signal amplification.	Several secondary antibodies may bind to the primary antibody resulting in an amplified signal.
Background	Non-specific binding is reduced.	Samples with endogenous immunoglobulins may exhibit a high background.

Choosing a directly conjugated primary antibody

Using directly conjugated primary antibodies (eg, conjugated to enzymatic or fluorescent labels) allows you to speed up and simplify the protocol, omitting the need for a secondary antibody staining step. Also, conjugated primary antibodies will enable you to minimize species cross-reactivity and eliminate any non-specific binding that may occur with secondary antibodies. Fluorescent conjugated primary antibodies are ideal tools for multicolor experiments as they give you the flexibility to assemble the multiplex panel you need.

When choosing primary antibody conjugates, pay attention to antibody specificity. Ideally, go for recombinant monoclonal antibodies, which provide high specificity and batch-to-batch consistency.

Compared to secondary antibodies, primary conjugates don't provide signal amplification, so your protein of interest should be abundant in the sample. Abcam offers a wide range of primary recombinant antibodies directly conjugated to **fluorescent labels** or **enzymes**. If your antibody of choice is not available in a suitable conjugated format, you can use Abcam's **antibody conjugation kits**.

To learn more about how to conjugate antibodies, refer to our **Antibody conjugation guide**.

Choosing a suitable secondary antibody

If you are using indirect detection, you will need to select an appropriate secondary antibody.

Secondary antibodies are generated by immunizing an animal with antibodies that act as immunogens. The secondary antibodies produced will bind to the antibody type with which the animal has been immunized.

Secondary antibodies have descriptive names that reveal the type of primary antibody they'll bind to (see Fig. 16, 17, and 18). These names include the prefix 'anti-' to denote their reactivity. For example, if an animal has been immunized with rabbit IgG, the secondary antibodies produced will bind to rabbit IgG and are referred to as anti-rabbit IgG.

When choosing a secondary antibody, you need to consider whether it will bind selectively to your primary antibody and enable you to detect the antigen, which is determined by several key factors outlined below.

Host species

The host species used to raise the secondary antibody must be different from that of the primary antibody. For example, if the primary antibody is raised in rabbit, your secondary antibody will need to be raised in an alternative species; a donkey anti-rabbit secondary antibody would be suitable.

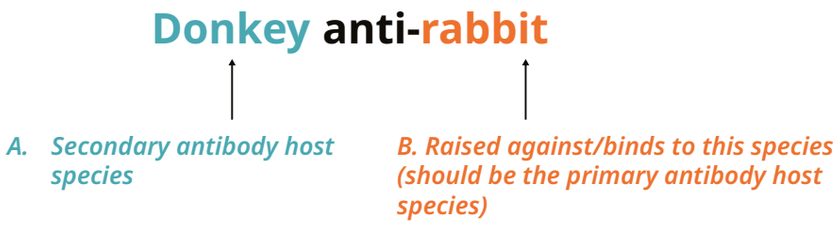


Figure 16. Using the name of a secondary antibody to understand with which species it reacts. The secondary antibody is raised in donkey (A) and binds to rabbit antibodies (B).

Binding isotypes and specificity

The secondary antibody must bind to the isotype of the primary antibody.

Primary antibodies are typically IgG isotypes. Therefore, the secondary antibody will need to be raised against IgG. Usually, anti-IgG secondaries bind to the heavy & light chains (H&L) but can also be made to bind to other regions of the primary antibody.

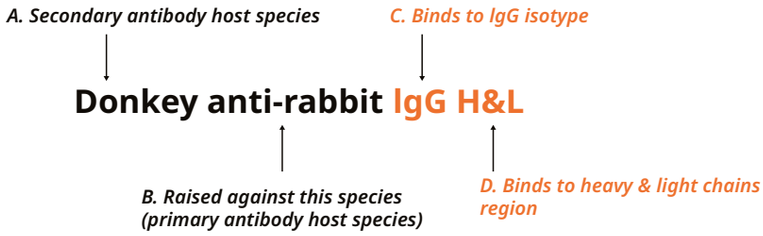


Figure 17. Using the name of a secondary antibody to understand the species, isotype, and region of the primary antibody it binds. This secondary antibody binds specifically to the H&L region of rabbit IgGs (B, C, D).

Conjugate selection for different applications

Labels, such as fluorescent dyes, proteins, enzymes, and biotin, are conjugated to secondary antibodies to visualize the target protein's presence.

- Fluorescent labels emit light in the visual range when excited by light of a wavelength. There are several available, all with their own excitation and emission characteristics.
- Enzymatic labels, such as horseradish peroxidase (HRP) and alkaline phosphatase (AP), form a colored precipitate when combined with the appropriate substrate.
- Biotinylated antibodies are useful for signal amplification when followed by an avidin-biotin-enzyme or fluorochrome complex (commonly abbreviated as ABC reagent), which is avidin or streptavidin conjugated to an enzyme or fluorochrome.

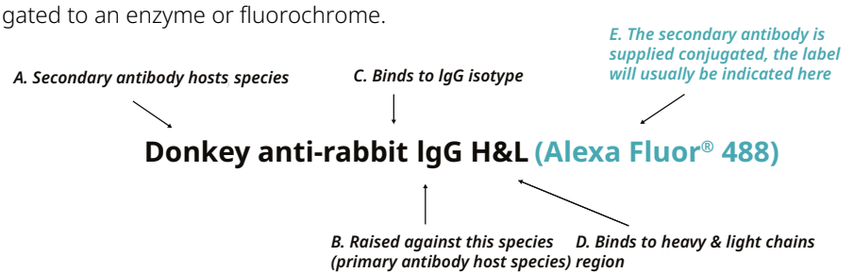


Figure 18. The secondary antibody label is usually indicated at the end of the antibody name. This secondary antibody is conjugated to Alexa Fluor® 488, a green fluorophore.

The conjugate choice depends on the application. Enzyme-linked secondary antibodies tend to be the most popular for ELISA or western blot applications. In contrast, there is a preference for secondary antibodies conjugated to fluorescent proteins or dyes (such as Alexa Fluor®) for flow cytometry and ICC.

Below we outline some suggested secondary antibodies for the main applications you're likely to use (Table 3).

Table 3. Choosing a secondary antibody labeled with an enzyme or fluorochrome for different applications.

Secondary antibodies	Enzyme	Fluorochrome
IHC	HRP, HRP polymer, biotin (avidin/ streptavidin-conjugated to enzyme or fluorochrome)	Alexa Fluor®, Cy® dyes, FITC, PE
ICC	-	Alexa Fluor®, Cy® dyes, FITC, PE
Western blot	HRP, AP	IRDye®, Alexa 680, Alexa 790
ELISA or ELISPOT	HRP, biotin (avidin/ streptavidin conjugated to enzyme or fluorochrome)	-
Flow cytometry or FACS	-	Alexa Fluor®, Cy® dyes, FITC, PE

IHC = immunohistochemistry, ICC = immunocytochemistry, FACS = fluorescence-activated cell sorting, HRP = horseradish peroxidase, AP = alkaline phosphatase

Avoiding cross-reactivity with pre-adsorption and fragments

When selecting a secondary antibody, you need to ensure that it won't cross-react with non-target proteins in the sample. You can minimize species cross-reactivity by using pre-adsorbed secondary antibodies and F(ab) antibody fragments.

Pre-adsorbed secondary antibodies

Pre-adsorbed secondary antibodies are ideal for eliminating species cross-reactivity in multicolor experiments that simultaneously use several primary antibodies and corresponding secondary antibodies. Pre-adsorption (also called cross-adsorption) is an extra purification step introduced to increase antibody specificity. The pre-adsorption process reduces the risk of cross-reactivity between the secondary antibody and endogenous immunoglobulins present on cell and tissue samples.

F(ab) and F(ab')2 antibody fragments

Using **F(ab) and (Fab')2 fragment antibody fragments**, rather than whole antibodies, can eliminate non-specific binding between Fc portions of antibodies and Fc receptors on cells (such as macrophages, dendritic cells, neutrophils, NK cells, and B cells). F(ab) and F(ab')2 fragments also penetrate tissues more efficiently due to their smaller size. As fragment antibodies do not have Fc portions, they do not interfere with anti-Fc mediated antibody detection.

Choosing antibodies for dual staining

Double immunostaining of cell cultures or tissue requires two primary antibodies raised in different species and two secondary antibodies exclusively recognizing one species. To avoid cross-reactivity, you can choose secondary anti-IgG antibodies, which have been pre-adsorbed against immunoglobulins from other species. Alternatively, using directly conjugated primary antibodies will remove the need for secondary antibodies.

Quick tips on choosing secondary antibodies

- Select the brightest fluorophore to label a protein with the lowest expression levels
- All secondary antibodies should come from the same host species when you use multiple labels
- Pre-adsorbed secondary antibodies are helpful for multicolor analysis to ensure low cross-species reactivity
- Fragment antibodies are smaller and penetrate tissues more efficiently (useful for IHC)
- Biotin conjugates can detect low-abundance proteins

Antibody specificity validation

Here we discuss all essential factors you need to consider to successfully validate antibody specificity in your experimental setup, such as choosing and preparing the appropriate positive and negative controls and validating antibodies in specific applications.

Why validate antibody specificity?

Antibody validation revolves around proving three key aspects:

- Specificity and functionality - showing an antibody can differentiate between various antigens in the intended application.
- Affinity - showing the strength of binding between antibody and epitope.
- Reproducibility - showing that your validation data can be reproduced in any lab.

Here we will focus on how you can validate antibody specificity in your experimental setup to ensure accurate and consistent results. Although a good manufacturer usually tests an antibody in several applications and species, it's impossible to account for numerous protocols and reagents with which researchers may use the antibody. Therefore, your antibody validation steps are essential because they are specific to your setup.

Key points to consider when validating antibody specificity

1. Choice and preparation of positive and negative controls

Identifying and using appropriate positive and negative controls is essential for successful antibody validation.

- **A positive control** is a relevant cell line or tissue sample strongly expressing the target protein of interest that can be used to confirm the selective binding of your antibody.
- **A negative control** is a cell line or tissue sample that does not express the target protein and, therefore, can provide the data on the non-selective binding properties of your antibody. When a true negative control is not available, a sample expressing low levels of the target proteins can work as an acceptable alternative.

It's often challenging to determine cell or tissue types that do or do not express the target protein. You can find information about target protein expression in different tissues or cell lines in peer-reviewed papers and online protein databases, including:

- **https://www.proteomicsdb.org/proteomicsdb/#overview**
- **www.genecards.org**
- **portals.broadinstitute.org/ccle**
- **www.ncbi.nlm.nih.gov/gene**
- **www.proteinatlas.org**
- **https://www.uniprot.org/**

However, there are a few limitations to defining protein expression profiles using online databases:

- Expression data may not be complete in some cases
- It can be challenging to find negative cell lines or tissues, particularly for essential housekeeping genes.
- RNA levels are often very unreliable in determining the quantity of proteins.

Furthermore, antibodies may or may not recognize the protein in its native or denatured state. It is therefore essential to prepare the test samples accordingly. For example, an antibody that recognizes the protein only in its native form should not be used on samples using denaturing conditions, such as western blot.

2. Protocols

Ensure you use an optimized protocol to give the antibody the best chance of passing the validation process. For instance, incubation times can vary dramatically from a minimum of one hour to overnight at 4°C, so you'll need to determine the optimal incubation period for each antibody. If the incubation period is too short, you may encounter sensitivity issues, while prolonged incubation time may lead to background staining. You will also need to optimize other factors, such as working dilutions, blocking conditions, and the use of native vs denatured conditions.

3. The choice of buffers

The majority of antibody assays will use two buffer types: PBS or TBS. You will need to determine the optimal buffer for your experiment, considering parameters that can influence buffer performance, such as pH.

Models for designing positive and negative controls

Cell lines or tissues that endogenously express or lack the target protein can serve as positive or negative controls, respectively. You can use several various cell lines with different protein expression levels to provide a range of controls. Alternatively, appropriate positive and negative controls can be designed using multiple methods, including knock-out models, siRNA knockdown and cell treatment (Table 4).

Table 4. Models for designing appropriate positive and negative controls.

Validation	Benefits	Limitations
Knock-out (KO) models Cell lines, tissues, or lysates, where the protein-encoding gene of interest is eliminated with genetic tools (eg, CRISPR)	• KO models function as a true negative control • Guaranteed no expression of the target gene • You can use KO cell lines or tissues in all assays: western blot, IHC, ICC, flow cytometry • You can save time by using commercially available KO cell lines or lysates for your gene of interest (subject to availability), rather than generating your own KO	• KO cell lines against 'essential' genes are not always viable • The lack of signal in a KO sample shows that the antibody detects the protein of interest in the wild-type sample. It does not guarantee that the Ab will not bind unspecifically to an unrelated protein in a different sample background.

siRNA knockdown Protein-encoding gene expression is lowered using post-transcriptional gene regulation tools, such as small interfering RNA (siRNA).	• Confirms specificity through target protein being downregulated • Knockdown cells lines may be used in all assays: western blot, ICC, flow cytometry	• Knockdown is transient • Knockdown is rarely 100% effective, so good controls are needed, such as real-time PCR and well-established siRNA for a control gene. • Non-specific reduction in expression might be observed where the siRNA binds and silences "off-targets" due to binding to similar transcripts
Cell treatment Protein expression level is manipulated within cells	• Can increase or reduce expression levels of specific proteins or affect post-translational modifications, such as phosphorylation, (eg, via starvation) • These could serve as positive or negative controls	• Additional controls are required to ensure the cell treatment worked • It can be challenging to design the experiment

Validating antibody specificity in various applications

You can use several different methods to validate antibodies. Below we outline some popular applications, their benefits and limitations.

Validation	Benefits	Limitations
Mass spectometry/ Immunoprecipitation - Mass spectometry (IP-MS) Protein complexes are first immunoprecipitated from a cell lysate and then analyzed with mass spectrometry	• Amenable to a high-throughput format • Potential to estimate abundance of target protein bound to the antibody of interest using normalization techniques • Can recognize all protein isoforms to which the antibody binds • Can identify post-translational modifications, interacting partners and complexes, • Confirms specificity based upon digested protein fragments	• Too many washes of your IP could remove weak or moderate binders • Not all antibodies are suitable for IP • It can be challenging to distinguish partner proteins pulled down in a complex from off-target binding • Interpreting the data can be tricky as the highest enrichment score does not always mean that this is the target the antibody preferentially binds to, for example: 1. Off-target binding can be difficult to demonstrate. An isotype control Ab is required, but there may still be discrepancies between IPs.
Western blot Protein is detected in a sample via initial size separation and then blotting onto a membrane to be visualized by an antibody	• Useful for determining antibody specificity against target protein based on molecular weight • Ideal for detecting reduced/ denatured proteins • Qualitative assay	• Time-consuming assay • Not easily automatable compared to some of the other applications

Immunocytochemistry (ICC) Proteins in cells are detected via specific antibodies and reporter molecules	• Validates whether an antibody recognizes the correct protein based on cellular localization • Specificity is confirmed in cells that either do or do not express the target protein • Qualitative assay	• Unable to determine if an antibody recognizes other proteins non-specifically with identical cellular localization
Immunohistochemistry (IHC) Proteins in tissues are detected via specific antibodies and reporter molecules	• Validates whether an antibody recognizes the correct protein based on localization within the tissue • Specificity is confirmed in tissue samples that either do or do not express the target protein • Qualitative assay	• Unable to determine if an antibody recognizes other proteins non-specifically with identical tissue localization
Protein/peptide array Antibody binding events are detected by first spotting arrays with the peptides/proteins and then adding the antibody (similar to ELISA)	• Allows screening for antibody binding with numerous different proteins/peptides • High-throughput screening process	• Protein array only: unable to screen for post-translationally modified proteins if the arrays are made of E.coli (bacteria)-synthesized proteins • Arrays made by peptides and denatured proteins only present linear epitopes for interrogation

Note that the two following methods can't be considered exhaustive tests of antibody specificity; therefore, we do not recommend using them alone:

- **Blocking with an immunizing peptide** can confirm that an antibody binds its immunogen. However, the immunizing peptide will block both specific and non-specific antibodies, so it can't serve as a comprehensive method to confirm antibody specificity.
- **Omitting a primary antibody** can evaluate the tissue or secondary detection reagents but not the primary antibody specificity.

Antibody storage and handling

With proper storage and handling, most antibodies should retain activity for months, if not years. In this section, we provide general guidelines on antibody storage and handling. Please always refer to the manufacturer's datasheet for specific storage recommendations.

General storage guidelines

Upon receiving the antibody, you will need to centrifuge it at 10,000 x g for 20 seconds to pull down the solution trapped in the vial threads and then aliquot it into low-protein-binding microcentrifuge tubes. Aliquotting minimizes damage due to repeated freeze/thaw cycles that can denature an antibody, causing it to form aggregates that reduce its binding capacity. Aliquotting also helps minimize contamination introduced by pipetting from a single vial multiple times.

Aliquots should be frozen and thawed once, with any remainder kept at 4°C. It's usually recommended to store antibodies at -20°C as there's no significant advantage to storing them at -80°C.

The size of the aliquots will depend on how much you typically use in an experiment. Aliquots should be no smaller than 10 µL. The smaller the aliquot, the more the stock concentration is affected by

evaporation and adsorption of the antibody onto the surface of the storage vial.

In most cases, storage at 4°C upon receiving the antibody is acceptable for one to two weeks. It is essential to follow the recommendations on the datasheet.

Preventing freeze/thaw damage

Make sure not to use a frost-free freezer: it's unlikely your lab would, but the cycling between freezing and thawing should be avoided. For the same reason, antibody vials should be placed in the freezer area with minimal temperature fluctuations, for instance, towards the back rather than on a door shelf.

Some researchers add the cryoprotectant glycerol to a final concentration of 50% to prevent freeze/thaw damage because glycerol lowers the freezing point to below -20°C. While this may be acceptable for many antibodies, you should check the datasheet to see if the manufacturer tested antibody stability in this storage condition.

Storing solutions with glycerol at -80°C is not advised since this is below the freezing point of glycerol. Also, glycerol or any other cryoprotectant can be contaminated with bacteria, so you must obtain a sterile preparation.

Storing conjugated antibodies

Conjugated antibodies often require additional storage and handling precautions since they're more complicated than non-conjugated antibodies. For example, conjugated antibodies – whether conjugated to fluorochromes, enzymes, or biotin – should be stored in dark vials or wrapped in foil because exposure to light will compromise conjugates' activity. Fluorescent conjugates, in particular, are susceptible to photo-bleaching and should be protected from light during all phases of an experiment.

Table 5 below provides the detailed guidelines for proper conjugated antibody storage and handling.

Table 5. Handling, aliquoting, and storage guidelines for conjugated antibodies.

	Fluorescent labels, eg, Alexa Fluor®, Dylight®, FITC, PE	**HRP**
Handling	Aliquot upon delivery Avoid the freeze/thaw cycle Store in the dark or UV protected containers	Aliquot upon delivery Avoid the freeze/thaw cycle Store in the dark or UV protected containers
Aliquoting	Aliquot away from a direct light source When you receive the antibody, centrifuge at 10,000 x g for 20 seconds Aliquot after gently mixing with a pipette. Repeat 3-4 times. Do not mix by inversion, as this may cause the solution to foam	Aliquot away from a direct light source When you receive the antibody, centrifuge at 10,000 x g for 20 seconds **Do not add sodium azide to HRP-conjugated antibodies since this preservative inhibits HRP activity**
Long-term storage	Follow the manufacturer's datasheet recommendations Store at -20°C if it contains a cryoprotectant (eg, glycerol)* **Storing in amber vials or tubes covered with foil**	Follow the manufacturer's datasheet recommendations Store at -20°C if it contains a cryoprotectant (eg, glycerol)* **Storing in amber vials or tubes covered with foil**
Short-term storage	Store at +4°C short term (1-2 weeks)	Store at +4°C short term (1-2 weeks)

*Freezing and thawing enzyme-conjugated antibodies will reduce enzymatic activity and affect the antibody binding capacity. Therefore, enzyme-conjugated antibodies should not be frozen at all and should instead be kept at 4°C unless an antibody contains a cryoprotectant, and its stability has been validated for long-term storage at -20°C.

Avoiding contamination with sodium azide

To prevent microbial contamination, you can add sodium azide to an antibody solution to a final concentration of 0.02% (w/v). If an antibody already contains this preservative, this will be indicated on the datasheet in the storage buffer section.

When not to use sodium azide

Sodium azide should be avoided when staining or treating live cells with antibodies or conducting in vivo studies. This antimicrobial agent is toxic to most other organisms as it blocks the cytochrome electron transport system.

Sodium azide will interfere with any conjugation involving an amine group and should be removed before proceeding with the conjugation. After conjugation, you can store antibodies in sodium azide, except for HRP-conjugated antibodies, since sodium azide inhibits HRP. An acceptable alternative to sodium azide is 0.01% thimerosal (merthiolate), which does not have a primary amine. Also, sodium azide can be removed from antibody solutions by dialysis, ultrafiltration or gel filtration.

Protein concentration and stability

Proteins are generally less susceptible to degradation when stored at higher concentrations, ideally ≥1 mg/mL. Therefore, you should avoid diluting antibodies to working concentration and keeping them at 4°C for more than a day.

If the antibody concentration is low, stabilizer proteins such as BSA can be added to the antibody solution. The stabilizer protein also serves to minimize the antibody loss due to binding to the vessel wall. However, you shouldn't add stabilizing protein to the antibodies you intend to conjugate because it will compete with the antibody and reduce the conjugation efficiency.

Antibody formats and purification

If an antibody is received in an unpurified format, you may need to purify it before using it in your experimental setup. Antibody purification methods range from very crude to highly specific, and the necessary level of the purification depends on your intended application for the antibody. Here we briefly overview the most common unpurified antibody formats and antibody purification methods.

Antiserum

Polyclonal antibodies are often available in relatively unpurified forms, such as "serum" or "antiserum". Antiserum refers to the blood serum from an immunized host containing antibodies of interest (as well as other serum proteins and antibodies).

In addition to antibodies recognizing the target antigen, antiserum contains antibodies to various non-target antigens that sometimes react non-specifically in immunological assays. For this reason, raw antiserum is often purified to eliminate serum proteins and enrich the immunoglobulin fraction that specifically reacts with the target antigen.

Tissue culture supernatant

Monoclonal antibodies can be produced using hybridoma cell cultures (cytokine-secreting cells) and harvested as hybridoma tissue culture supernatants. Please refer to Chapter 2 for more information about monoclonal antibody production.

Ascites fluid

Ascites fluid is a historical *in vivo* antibody production method, which is now only used in exceptional cases, ie, when an antibody can't be produced by *in vitro* technologies.

In this method, monoclonal antibodies are produced by growing hybridoma cells within the peritoneal cavity of a mouse (or a rat). The hybridoma cells are injected into a host's abdomen, where they multiply and generate fluid (ascites), which can be harvested. This ascites fluid contains a high antibody concentration, usually providing higher antibody yields than hybridoma cell culture. However, the ascites fluid also includes many non-specific immunoglobulins from the host.

Antibody purification

Antibody purification is achieved by selective enrichment or specific extraction of antibodies from serum (for polyclonal antibodies), ascites fluid, or cell culture supernatant of a hybridoma cell line (for monoclonal antibodies). Below we describe typical purification methods for polyclonal antiserum or monoclonal ascites fluid/tissue culture supernatant.

Protein A/G purification

Proteins A and Protein G (expressed by *Staphylococcus aureus* or *Streptococcus bacteria*, respectively) are antibody binding proteins often used in antibody purification. Protein A/G purification is based on protein A or G's high affinity to the immunoglobulin Fc domain. Protein A/G purification eliminates the bulk of the serum proteins from the raw antiserum. However, it does not eliminate the non-specific immunoglobulin fraction. As a result, the protein A/G purified antiserum may still possess some undesirable cross-reactivity.

Find out more about antibody binding properties of Protein A, Protein G, and other proteins.

Affinity purification

Affinity purification isolates a specific protein or group of proteins with similar characteristics using affinity tags. The technique separates proteins based on a reversible interaction between the protein and a specific ligand coupled to a chromatographic matrix (Fig. 19).

Antigen affinity purification takes advantage of the specific immunoglobulin fraction's affinity for the immunizing antigen against which it was generated. This purification method eliminates the bulk of the non-specific immunoglobulin fraction while enriching the immunoglobulin fraction that specifically reacts with the target antigen. The resulting affinity-purified immunoglobulin will primarily contain the immunoglobulin of the desired specificity.

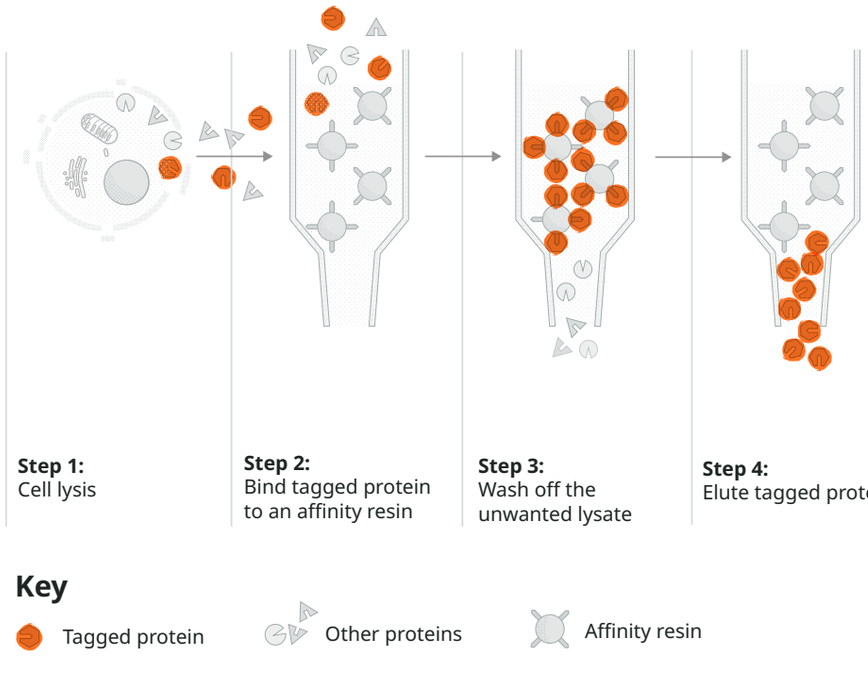


Figure 19. This diagram shows the steps for affinity chromatography. First, lyse your sample to release the proteins. When this lysate is added to the column, the fusion tag will bind to the affinity resin. Unwanted proteins are washed through the column, and then your protein of interest can be eluted.

You can find commercially available **antibody purification kits based on protein A/G or affinity purification methods.**

Pre-adsorption

Polyclonal antibodies are sometimes **pre-adsorbed**, meaning they have been adsorbed with other proteins, or serum from various species, to eliminate any antibodies that may cross-react. The solution containing secondary antibodies is passed through a column matrix containing immobilized serum proteins from potentially cross-reactive species (Fig. 20). Non-specific secondary antibodies are retained in the column, while highly specific secondaries flow through. The resulting purified antibody should exhibit significantly reduced cross-reactivity.

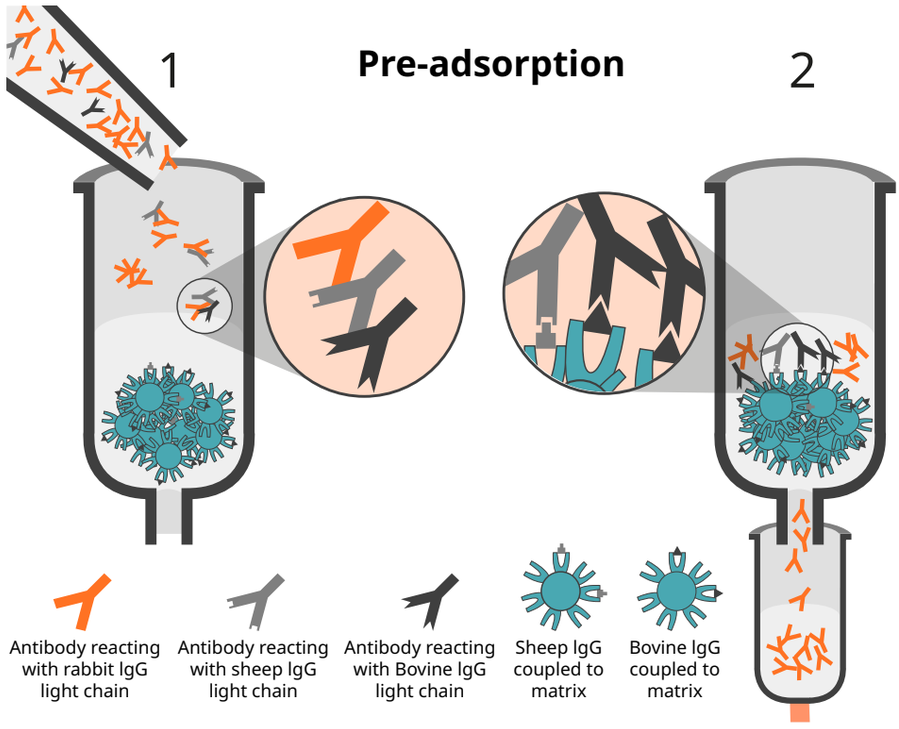


Figure 20. In this example, a solution of secondary antibodies recognizing rabbit IgG light chains is passed through a matrix containing immobilized serum proteins from potentially cross-reactive species such as sheep and bovine IgGs. Only antibodies highly specific to rabbit IgG light chains will flow through the column, while secondary antibodies cross-reacting with sheep or bovine IgG light chains will remain bound to the proteins in the column.

Antibody dilution and titer

When using an antibody for the first time, you may need to optimize its dilution for your specific application and experimental setup. Here we describe how to define the optimal antibody concentration by titration and provide suggested dilutions for antibodies without recommended dilution on the datasheet.

Why do you need to define antibody dilution for your experimental conditions

The rate of binding between antibody and antigen – affinity constant – can be affected by temperature, pH, and buffer constituents. Varying the relative concentrations of an antibody and an antigen solution can also control the extent of antibody-antigen complex formation. As it is not usually possible to change the antigen concentration, the optimal working concentration of each antibody must be determined with dilutions for each application and set of experimental conditions.

Usually, antibodies have recommended dilutions for various applications included in the datasheet.

However, they may require some optimization in your specific experimental setup.

Optimizing the antibody dilution: titration experiments

The optimal antibody concentration, which gives the best staining with minimum background, must be determined experimentally for each assay and is usually determined using a series of dilutions in a titration experiment. For example, if a product datasheet suggests using a 1:200 dilution, it is recommended to make dilutions of 1:50, 1:100, 1:200, 1:400 and 1:500.

A titration experiment is done by first selecting a fixed incubation time and then a series of experimental dilutions of the antibody. Each dilution should be tested on the same sample type to keep the same experimental conditions.

Many antibodies will have similar batch-to-batch consistency; therefore, only one titration experiment is required in most cases. However, especially for polyclonal antibodies, when there is a change in the staining results between batches of the same antibody, we recommend performing another titration experiment.

Suggested dilutions for antibodies with no recommended dilution on the datasheet

Unpurified antibody preparations differ significantly in antibody concentration. If the specific antibody concentration of a given unpurified antibody preparation is unknown, we recommend using a concentration/purification kit and refer to the table below as a guideline.

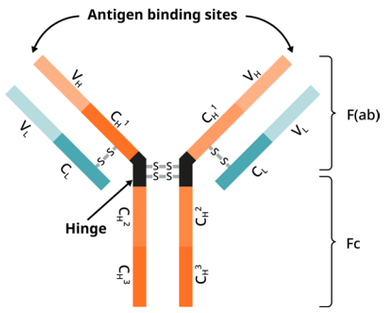
Table 6 provides various dilutions for each application for different antibody formats.

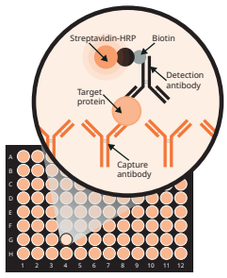
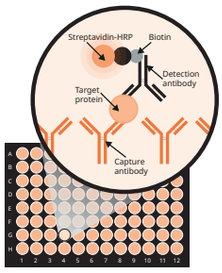
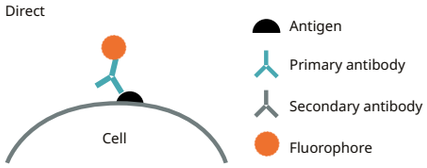
Table 6. Suggested antibody dilutions for different applications.

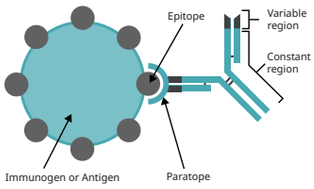
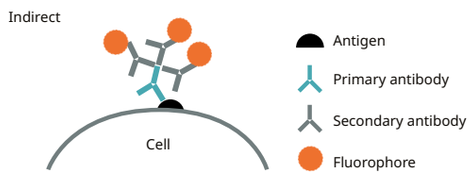
	Tissue culture supernatant	Ascites	Whole antiserum	Purified antibody
WB/dot blot	1/100	1/1000	1/500	1 µg/mL
IHC/ICC	Neat –1/10	1/100	1/50–1/100	5 µg/mL
EIA/ELISA	1/1000	1/10000	1/500	0.1 µg/mL
FACS/Flow cytometry	1/100	1/1000	1/500	1 µg/mL
IP	-	1/100	1/50–1/100	1–10 µg/mL
Approximate IgG concentration estimate	1–3 mg/mL	5–10 mg/mL	1–10 mg/mL	-

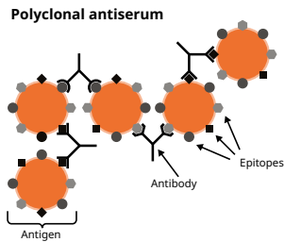
EIA=enzyme immunoassays, FACS=fluorescence-activated cell sorting, ICC=immunocytochemistry, IHC=immunohistochemistry, IP=immunoprecipitation, WB=western blot.

Chapter 5. Antibody glossary

Term	Definition
Adjuvant	Compounds/chemicals that are sometimes added to immunizing peptides during antibody production to help stimulate the immune response.
Affinity	A measure of the binding strength between antibody and antigen at a single binding site. Affinity refers to the strength with which the epitope binds to an individual paratope (antigen-binding site) on the antibody.
Affinity purification	Purification of an antibody based upon the strength of that antibody's binding to the antigen with which it was made.
Affinity constant	A numerical value indicating the strength of binding between antibody and antigen. It is also known as association or equilibrium constant.
Antibody	An immunoglobulin protein capable of specific binding with an antigen that has induced an immune response. Antibodies are produced by B cells as a primary immune defense in response to the presence of antigen. 
Antigen	Any substance foreign to the body that elicits a specific immune response.
Antigen retrieval	Methods that enable an antibody to access the target protein within the tissue or cells. Typically used after fixation (particularly the use of paraformaldehyde) or embedding techniques that may cause chemical changes (eg, cross-linking of amino acids within the epitope) that mask epitopes and restrict antigen-antibody binding.
Antiserum	Blood serum from an immunized host containing antibodies of interest (as well as other serum proteins and antibodies).
Ascites fluid	Fluid taken from the abdomen of a living host animal, which will contain unpurified monoclonal antibodies produced by hybridoma cells grown within the host.
Avidity	A measure of the total binding strength of the antibody-antigen complex.
BSA	Bovine serum albumin. Often used as a carrier protein in antibody production.

Capture antibody	An antibody coating onto an ELISA plate that binds an antigen from an applied sample/solution. 
Carrier protein	A large, highly antigenic molecule conjugated with a small antigen, called hapten. Carrier proteins induce a more effective and specific immune response in an immunized animal when creating antibodies for commercial use (eg, BSA or KLH).
Chromogen	A chemical substrate used to detect enzyme-tagged antibodies, for example, DAB. The chemical substrate will change color in the presence of the enzyme.
Conjugated antibody	Conjugated antibodies are chemically bound to labels, such as fluorochromes or chromogens, to enable visual detection of the antibody. Chemical substrates can be directly conjugated to a primary antibody or bound to a secondary antibody.
Cross-reactivity	The binding of an antibody to similar epitopes on other antigens or proteins.
Denatured	This term refers to the conformational change in an antigen away from its native state. Denaturing your sample may help expose an epitope or destroy it.
Detection antibody	The primary antibody used in sandwich ELISA, which detects the immobilized antigen. This antibody can be directly conjugated or visualized following the application of a conjugated secondary. 
Direct staining	An antigen is detected by a primary antibody directly conjugated to a label (ie, conjugated primary antibody), so no secondary antibody is required. 

Epitope	The specific site on an antigen molecule where an antibody binds via its variable region. Note that an epitope is not the same as an immunogen, which includes the epitope but is often much larger. 
Fluorochrome (fluorophore)	A chemical compound that emits fluorescent light within a measurable color spectrum following excitation in response to a specific wavelength of laser light or chemical interaction.
Hapten	A small molecule that only elicits a specific antibody response when directly coupled to a larger carrier protein.
Hybridoma	A cell line created following the fusion of antibody-producing B cells from the spleen with an immortalized tumor cell line. A purified hybridoma culture/cell line will secrete its own specific monoclonal antibody. Read more about the hybridoma method of antibody production in Chapter 2.
Host species	The animal species in which an antibody has been raised.
Immunogen	A peptide sequence, chemical, or other substance capable of inducing an immune response.
Immunogenicity	The ability of an antigen to induce antibody production.
Immunoglobulin (Ig)	General term for a family of proteins that function as antibodies, which includes several subclasses.
Indirect staining	An antigen is detected by a conjugated secondary antibody that has been raised against the primary antibody's host species and binds to the primary antibody. 
Isotype control	An antibody of the same immunoglobulin subclass and from the same species as the primary antibody. This antibody is not raised against anything specific and is used to confirm the primary antibody binding is specific and not a result of non-specific Fc receptor binding or other protein interactions.
KLH	Keyhole limpet hemocyanin. Often used as a carrier protein in antibody production.
Monoclonal antibody	A homogenous population of antibodies that recognize a single epitope per antigen. Monoclonal antibodies are often produced using the hybridoma method.
Negative control sample	Any tissue, cell line, lysate, or purified protein known not to express/contain the antigen of interest.

Normal serum	Blood serum from non-immunized animals, which is often used as a control.
Paratope	An antigen-binding site, ie, the antibody part that recognizes and binds to an antigen.
Optimal working dilution	Antibody concentration (or dilution) maximizing the positive signal while minimizing background and non-specific staining. Optimal working dilution must be optimized for different antibodies using a titration experiment.
Peptide	A short chain of amino acids.
Polyclonal antibody	A heterogeneous mixture of antibodies, with each antibody recognizing different epitopes of a particular antigen. 
Positive control sample	Any tissue, cell line, lysate, or purified protein known to express/contain the antigen of interest.
Pre-adsorbed	An antibody is adsorbed with other proteins or serum from various species to eliminate any antibodies that may cross-react with a target of interest.
Pre-immune serum	Serum extracted before immunization, which is often used as a control.
Primary antibody	The antibody that directly binds the antigen of interest. For direct staining, it will already be conjugated to a label. For indirect staining, a conjugated secondary antibody will be required for primary antibody detection.
Protein A/G purification	Column purification where the antibody's Fc domain binds the high-affinity S. aureus protein A or G.
RabMAb®	Abcam's patented technology for the generation of high-quality rabbit monoclonal antibodies.
Recombinant antibody	An antibody generated in vitro using synthetic genes.
Secondary antibody	A conjugated antibody that binds to the primary antibody and allows its visualization.
Specificity	This term refers to the antibody's ability to bind only the desired protein of interest. Various techniques can assess antibody specificity, including knock-out validation models. To learn more about these techniques, please refer to the Antibody specificity validation chapter.
Titration	An experiment designed to find the optimal antibody concentration for the desired application. The antibody is tested at a range of dilutions, and the results are assessed to identify the optimum concentration. Check out the Antibody dilution and titer chapter to learn more about titration.

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