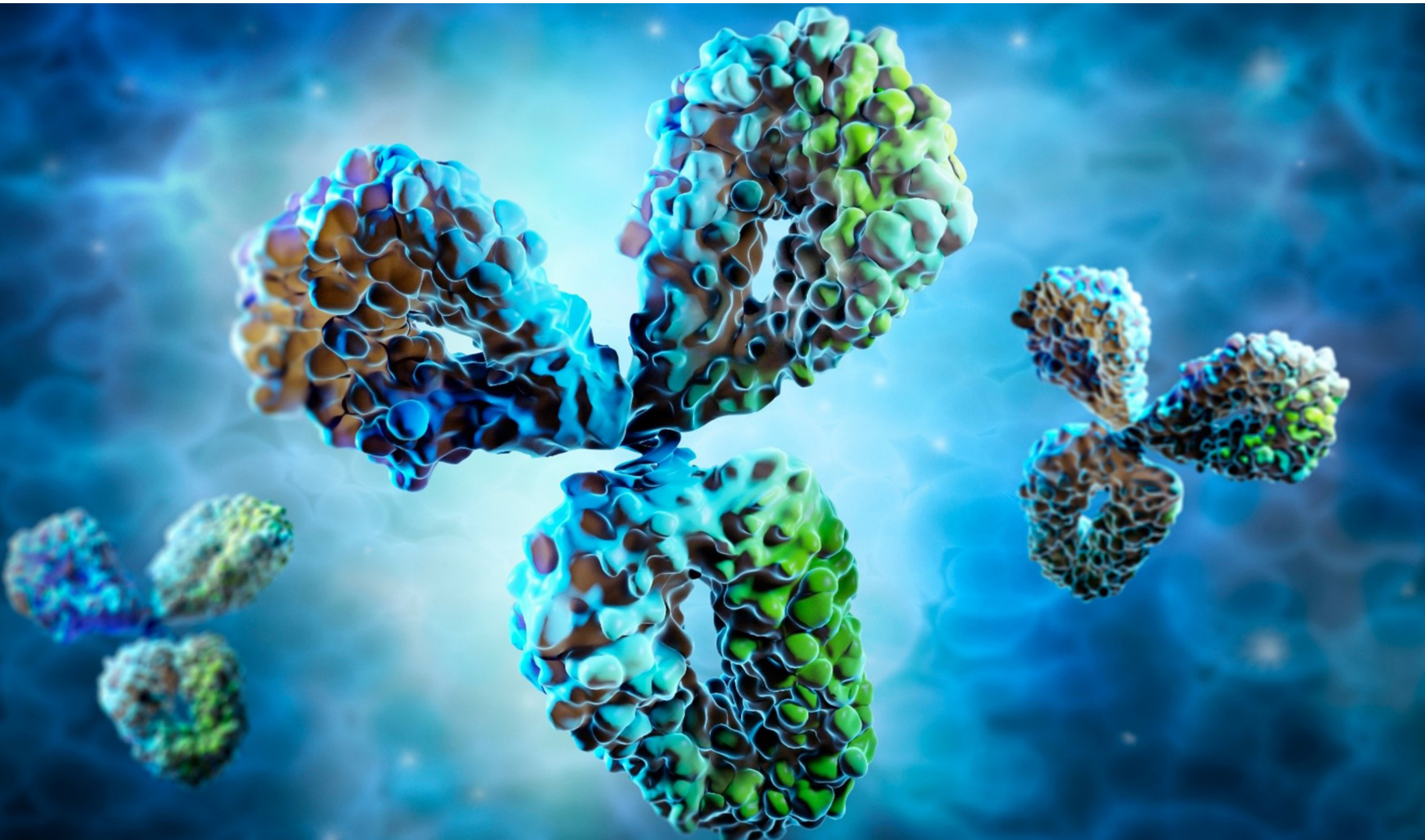
# Antibody Application and Selection



Creative Diagnostics

**Antibody Application and Selection Technical Guide**

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# 01 Antibody Application

Antibodies are important research tools in various biological laboratory techniques. Here we provide a brief overview of experimental techniques commonly used in the laboratory, with an emphasis on how they utilize antibody detection.

## Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a technology based on the interaction between solid-phase plates and antigen-antibody that can detect antigens or antibodies in biological samples. Like other immunoassays, ELISA relies on highly specific binding between antibodies and antigens. As an experimental technique, ELISA allows quantitative analysis and characterization of analytes and molecular interactions through the final color reaction. In ELISA, the antigen can be immobilized directly on the surface of a solid medium or, more commonly, by capture. The sample to be detected (which may contain antibodies or antigens to be tested) is added and incubated at the appropriate temperature. After removing the non-specific binding, antibodies conjugated to molecules such as enzymes or fluorophores are then added and incubated. Finally, the chromogenic substrate is added and the absorbance value is measured and analyzed on a plate reader. If antigen is present, the detection antibodies will remain bound to the plate and provide a signal. The intensity of this signal corresponds to the concentration of antigen or antibody in the sample.

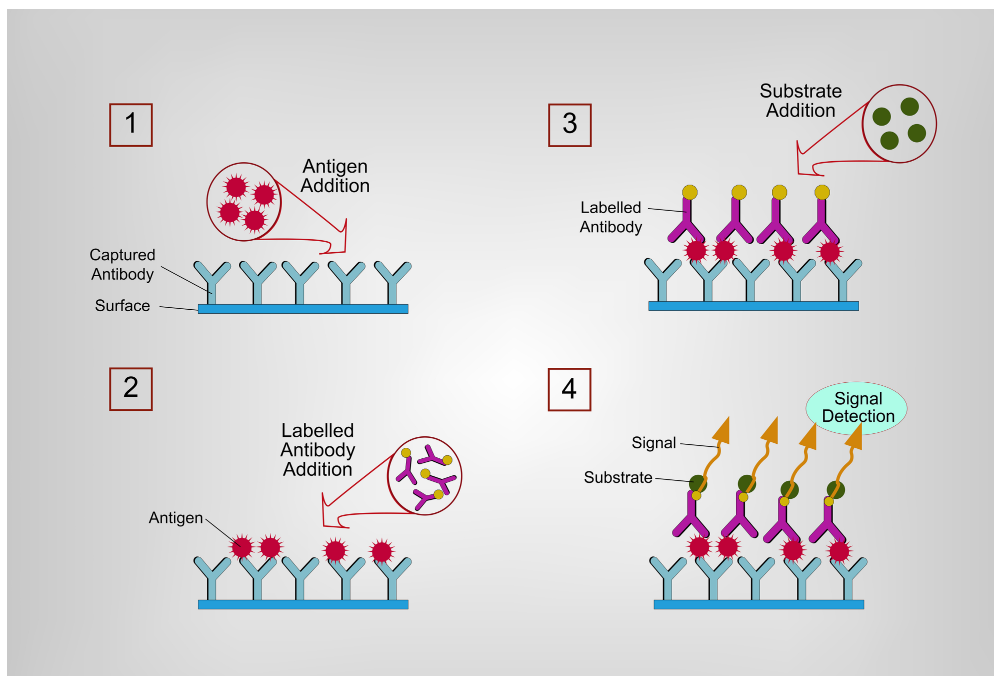


Figure 1. Sandwich ELISA.

ELISA experiments are typically performed in special multi-well plates (96 or 384 wells) where immobilization of the analyte allows separation of the antigen/antibody from the original sample components. These features allow ELISA to analyze multiple samples simultaneously.

There are four main types of ELISA:

Direct Methods, Indirect Methods, Sandwich Methods, and Competitive Methods. Each type of ELISA has its own advantages, disadvantages and applicability. The type of ELISA chosen for an experiment depends on many factors, including sensitivity, specificity, and the length of testing cycle required for the experiment. Please review the information below to help you choose the most appropriate type of ELISA.

## Enzyme-linked Immunospot (ELISPOT)

ELISPOT is a technology used to detect proteins secreted by cells, such as cytokines and growth factors. This method can be used to quantify and compare immune responses to different stimuli. Cells are cultured in 96-well plates with antibody-coated PVDF or nitrocellulose membranes. Primary and secondary antibodies are used to detect secreted proteins of interest. Cells secrete proteins of interest that appear as colored or fluorescent spots on the membrane. The membrane is then scanned and analyzed to determine the number or proportion of cells secreting the protein of interest.

## Western Blotting (WB)

WB is widely used in protein research for the isolation and identification of proteins. WB allows researchers to detect proteins, determine relative protein levels between different samples, and determine the molecular weight of a target protein to gain insight into its post-translational modifications.

WB involves three main steps: (1) separation of proteins by size differences on a gel, (2) transfer of proteins from the gel to a membrane, and (3) visualization of the protein of interest using primary and secondary antibodies.

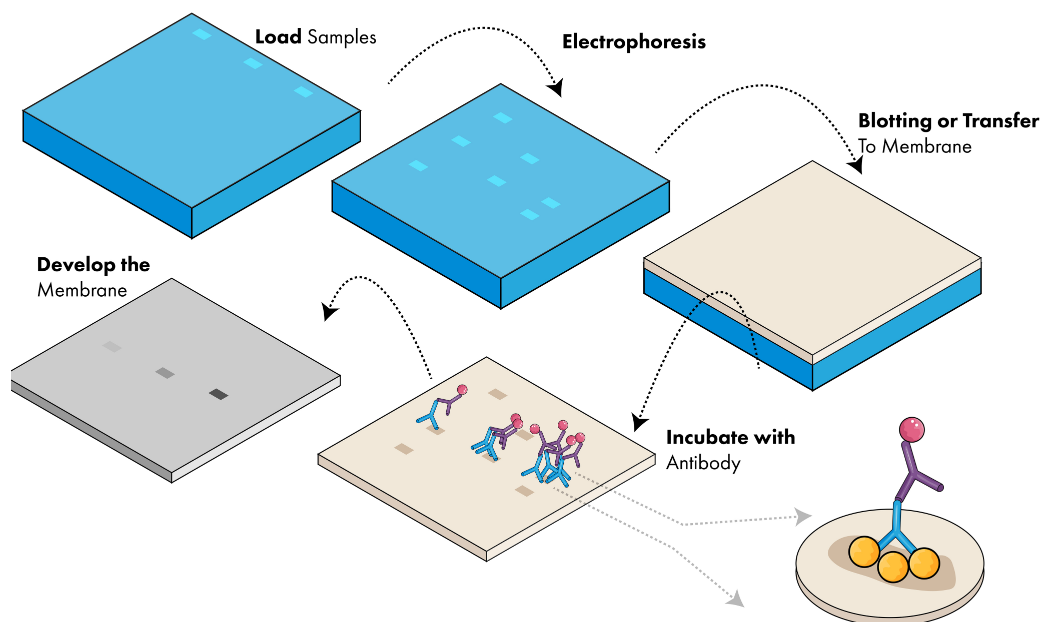


Figure 2. Schematic representation of WB workflow.

U In the first step, protein samples are loaded onto a gel and separated according to their size by gel electrophoresis. Subsequently, the protein band is transferred to the membrane through a transfer membrane device. Protein transfer to the membrane is important because the previous gel is not suitable for subsequent immunostaining, i.e., the antibodies do not adhere sufficiently to the proteins in the gel. Finally, the membrane can be immunostained with specific antibodies to further detect the target protein of interest and then visualized using secondary antibodies and detection reagents.

## Immunoprecipitation (IP) and Chromatin Immunoprecipitation (ChIP)

Immunoprecipitation (IP) is a versatile technique used to isolate and purify individual proteins and protein complexes. In this technique, antibodies are immobilized on a solid substrate, such as magnetic beads or agarose beads, to capture antigens from complex solutions.

Chromatin immunoprecipitation (ChIP) is used to determine whether a particular protein binds to a

particular sequence in DNA. ChIP allows researchers to determine the binding of proteins of interest to specific genes and genomic sequences, providing key clues to understanding their function and regulatory mechanisms.

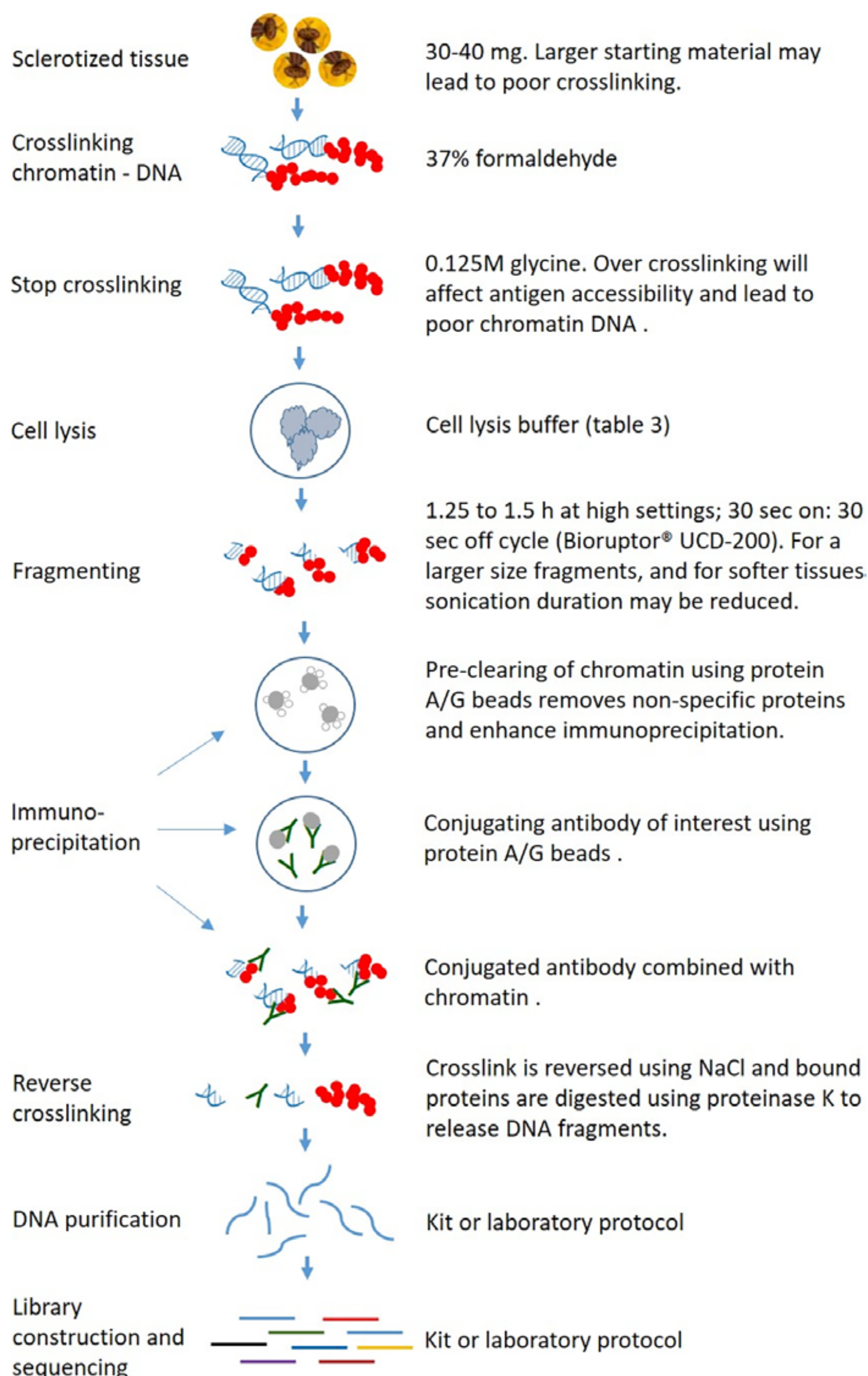


Figure 3. Schematic representation of ChIP workflow.( Nagalingam K, *et al.*; 2018)

In ChIP, specific antibodies are used for immunoprecipitation to capture proteins of interest (such as transcription factors) and their associated DNA. The DNA involved is then recovered and analyzed using methods such as PCR, microarrays or sequencing to determine the genomic sequence and where the protein binds to the DNA.

## Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is an experimental technique used to understand the distribution and location of antigens in tissue sections through the interaction of antibodies with antigens. Although IHC is less quantitative than Western blotting or ELISA, it has unique advantages in characterizing protein expression in intact tissues. IHC is often used to diagnose tissue abnormalities in diseases such as cancer and provides valuable insights and support that can be combined with data from other methods. IHC staining relies on antibodies that recognize the target antigen. Researchers can use chromogenic or fluorescence-based detection systems to visualize this antibody-antigen interaction. In chromogenic detection, the antibody binds to the enzyme and produces a colored precipitate upon contact with the chromogenic reagent. In fluorescence detection, antibodies are conjugated to a fluorescent label. There are many options for sample preparation and visualization methods that should be selected based on your sample type and desired sensitivity.

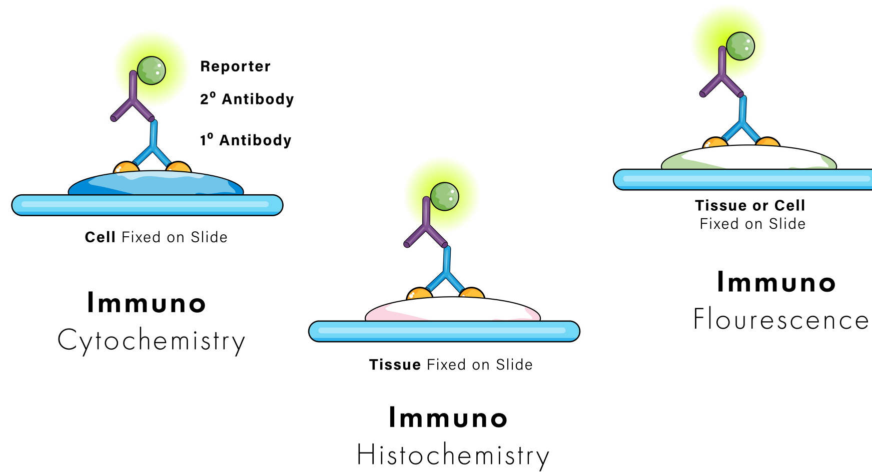


Figure 4. Immunohistochemistry (IHC) model diagram.

## Flow Cytometry

Flow cytometry is a widely used laser technique to study the properties of cells or particles. This technique measures the fluorescence emitted by labeled antibodies, which correlates with the binding of individual cells in a mixed cell population. Additionally, light scattering from different cells can be used to determine their size and properties.

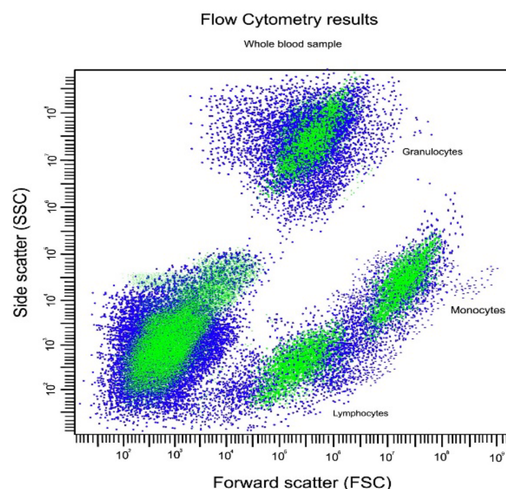


Figure 5. Flow Cytometry results diagram.

The flow cytometry method allows researchers to analyze the expression of cell surface and intracellular

molecules, characterize and differentiate different cell types in heterogeneous cell populations, evaluate the purification of isolated populations, and analyze cell size and volume. This technology can also be implemented using a flow cytometer, commonly known as FACS (Fluorescence Activated Cell Sorting).

# 02 Antibody Selection

## Primary Antibody Selection

A primary antibody is an antibody that can directly bind to the target protein and has a variable region capable of recognizing a specific epitope of the protein. When choosing a primary antibody, you should consider the following factors:

### Method of Cloning and Preparing Antibodies

The way in which antibodies are cloned depends on whether they come from different B cells (polyclonal antibodies) or from B cells of the same parental line (monoclonal antibodies).

Polyclonal antibodies include a number of different antibodies capable of recognizing different epitopes of the target antigen. Polyclonal antibodies are important because they have the ability to bind to many different antigenic epitopes, allowing them to produce strong signals in related applications without bias toward one antigenic epitope. However, the use of polyclonal antibodies is limited due to limited quantities, different batches, high cross-reactivity, and lack of specificity.

Unlike polyclonal antibodies, monoclonal antibodies recognize only one epitope of each antigen. Monoclonal antibodies are highly specific to their target, and have low cross-reactivity and minimal batch-to-batch variation.

Recombinant antibodies are antibodies produced by *in vitro* gene synthesis. Compared to traditional monoclonal and polyclonal antibodies, recombinant antibodies provide a sustainable and reliable supply with minimal batch-to-batch variability. Because the coding sequence of the recombinant antibody is known, it can be further designed and modified to meet the needs of specific applications.

When selecting an appropriate clone, recombinant monoclonal antibodies should be considered based on your specific target and application needs to ensure experimental reproducibility and long-term supply. For applications where polyclonal antibodies are commonly used, recombinant polyclonal antibodies may provide the ideal solution.

### Antibody Suitability Needs to Be Validated for Specific Applications and Biological Species

When selecting a primary antibody, it is important to ensure that it has been validated for binding to the target in the appropriate species. The antibody product data sheet should list the experimental applications and biological species that have been successfully tested. In addition, the data sheet will clearly indicate whether the antibody has been tested in a specific application with successful results. If the relevant application and biological species information is not provided in the data sheet, the performance of the antibody in this regard cannot be determined.

### Confirmation of Antibody Specificity Through Gene Knockout Experiments

A high-quality antibody should have high specificity for the target, be able to reliably recognize the target protein even at low expression levels, and provide accurate experimental results. However, not all

antibodies can achieve such high specificity, and many antibodies will cross-react with non-target proteins in experiments, causing false positives. Knockout (KO) experiments in cells or animals are one of the effective methods to detect antibody specificity. This powerful technology enables the assessment of antibody specificity by examining cell lines, cell lysates, or tissues that do not express the target protein. High-quality specific antibodies will not produce a signal in the KO assay, but will show normal specific signals in the wild type. In this way, the KO experiment provides a true negative control. Therefore, when selecting a primary antibody, we recommend choosing antibodies that have been validated for multiple applications, especially those validated using KO technology. Alternatively, you can confirm the specificity of your chosen antibody yourself using KO cell lines, KO cell lysates, or appropriate tissues.

### Immunogen Details

Detection of antibody typically begins with immunization of the host animal, and these immunogens can be full-length proteins, peptides, or whole cells. Researchers can find relevant information in the data table. However, if the sequence of the immunogen is proprietary, this information will not be available.

The immunogen used determines the binding site of the antibody to the antigen. If the immune gene sequence is available, it should be checked to see if the immune gene matches or contains the target protein region to be detected.

### Sample Processing

Antibody is specific for an epitope in a specific shape of the antigen. Because sample processing can change the structure of epitopes, some antibodies can only detect samples that have undergone specific processing. For example, many antibodies only recognize proteins that have been reduced and denatured, because this reveals hidden epitopes. On the other hand, some antibodies only recognize the natural epitope of the protein. Therefore, we recommend that you consult the antibody data sheet for information on sample processing limitations.

### Host Species

When using indirect detection and secondary antibodies, it is best to prepare the primary antibody to a species different from the sample source. This will avoid cross-reaction of the secondary antibody with endogenous immunoglobulins in the sample. This can be an important issue for tissue samples, but is less obvious for cell line samples.

However, if researchers can only use primary antibodies from the same host species as the tissue sample, careful consideration needs to be given to reducing the possibility of background staining. Alternatively, chimeric antibodies composed of domains from different species can be used. For cell lysates that do not contain endogenous immunoglobulin (IgG) or for experiments using primary antibody conjugates for direct detection, it is not necessary to consider the host species of the antibody.

### Buffers and Preservatives

Typically, antibody buffers are based on phosphate-buffered saline (PBS), supplemented with carrier proteins (such as bovine serum albumin-BSA) and preservatives (such as glycerol and sodium azide). Although these components are essential for maintaining antibody stability and preventing contamination, they can affect the effective binding of markers (such as fluorescent dyes, enzymes and metal) with antibody. In a typical conjugation reaction, BSA competes with the primary antibody for binding to the target marker, thereby significantly reducing conjugation efficiency. Sodium azide can be toxic to cells, limiting the use of this antibody in cell culture. Therefore, if researchers need to conjugate a primary antibody or use it to stain live cells, we recommend choosing an antibody preparation that does

not contain BSA or preservatives.

## Secondary Antibody Selection

Secondary antibodies are labeled antibodies used to bind to the primary antibody. The detection, classification and purification of target antigens can be achieved through specific binding. Because they are specific to the primary antibody species and isotype, they can detect and amplify the target protein signal. The need for secondary antibodies depends on the antibody detection method used.

Direct and Indirect Detection Methods

## Target antigen detection methods can be direct or indirect:

- Direct

The labeled primary antibody binds directly to the detection substance to complete the detection.

- Indirect

Antigen detection is performed by conjugation of a secondary antibody prepared against the host species with which the primary antibody interacts. Indirect methods can amplify signal intensity because a primary antibody can bind to multiple labeled secondary antibodies, i.e., multiple labeled secondary antibodies can bind indirectly to each antigen.

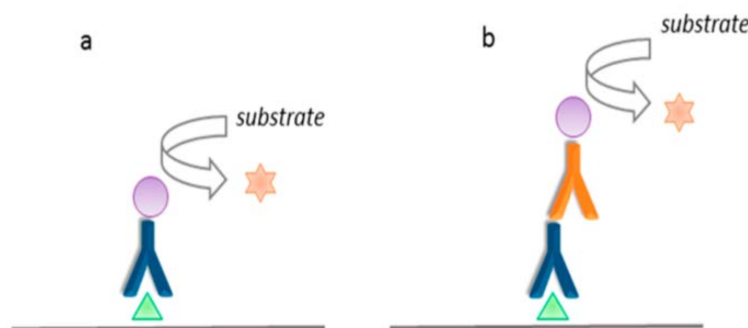


Figure 6. Schematic representation showing the (a) direct, (b) indirect.(Tony O Hara, *et al*.; 2021)

The choice of direct or indirect detection method often depends on the level of expression of the target antigen. Direct detection is suitable for the analysis of highly expressed antigens. In contrast, indirect detection is more suitable for studying antigens expressed at lower levels, as it benefits from the signal enhancement provided by the secondary antibody. The advantages and limitations of both methods should be considered, depending on the experimental detection needs.

## Selecting the Appropriate Primary Antibody Conjugate

When selecting a primary antibody conjugate, the specificity of the antibody should be considered. Recombinant monoclonal antibodies are preferred because they are highly specific and consistent from batch to batch. Unlike secondary antibodies, primary antibody conjugates do not enhance the signal. Therefore, you must ensure that the target protein is abundant in your sample.

## Selecting the Appropriate Secondary Antibody

When using indirect detection, you must select the appropriate secondary antibody. Secondary antibodies are prepared using antibodies that immunize the target animal. The secondary antibody produced corresponds to the type of antibody used in the vaccinated animal.

Secondary antibodies have descriptive names that reflect the type of primary antibody to which they will bind. These names have the prefix “anti” to indicate their reactivity. For example, if an animal is immunized with rabbit IgG, the secondary antibody produced will bind to the rabbit IgG and is called anti-rabbit IgG.

When choosing a secondary antibody, you need to consider several key factors to ensure that it will selectively bind to the primary antibody, allowing you to detect the antigen.

### Selection of Host Species

The host species used to produce secondary antibodies must be different from the host species used to produce primary antibodies.

### Isotype Binding

The secondary antibody must be able to bind to the isotype of the primary antibody. Primary antibodies usually have an IgG isotype. Therefore, it is necessary to generate secondary antibodies corresponding to the IgG isotype. Typically, secondary anti-IgG antibodies bind to the heavy and light chains (H&L), but can also bind to other regions of the primary antibody.

### Marker Selection for Different Applications

Markers such as fluorescent dyes, proteins, enzymes, and biotin can be combined with secondary antibodies to visualize the presence of target proteins. The selection of these markers depends on the specific application.

**Table 1. Different applications require the selection of differently labeled secondary antibodies.**

| Secondary antibodies | Enzyme | Fluorochrome |
|---|---|---|
| IHC | HRP, HRP polymer, biotin | 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 | - |
| Flow cytometry or FACS | - | Alexa Fluor®, Cy® dyes, FITC, PE |

### Cross-Reactions Should Be Avoided

When selecting a secondary antibody, you need to ensure that it will not cross-react with non-specific proteins in the sample. You can choose to use pre-adsorbed secondary antibodies or F(ab) antibody fragments to minimize cross-reactivity between species.

### Pre-adsorbed Secondary Antibodies

Pre-adsorbed secondary antibodies are very useful for multi-channel experiments. In multi-channel experiments, several primary antibodies and corresponding secondary antibodies are used simultaneously. Pre-adsorbed secondary antibodies can eliminate species cross-reactivity. Preadsorption is a method that introduces an additional purification step to improve antibody specificity. The preadsorption process reduces cross-reactivity between secondary antibodies and endogenous

immunoglobulins.

### Fragments F(ab) and F(ab′)2

Nonspecific binding between the Fc portion of the antibody and Fc receptors on the cell can be eliminated using the antibody F (ab) ) and F(ab′)2 fragments. Since the antibody fragments do not contain an Fc moiety, they do not interfere with Fc-mediated detection. Additionally, F(ab) and F(ab′)2 fragments are smaller and penetrate tissues more easily.

### Double Labeled Antibodies

Double immunostaining of cell cultures or tissues requires the use of two primary antibodies obtained from different species and two secondary antibodies that specifically recognize one species. To avoid cross-reactivity, you can choose pre-adsorbed anti-IgG secondary antibodies. Alternatively, directly conjugated primary antibodies can be used.

## Primary Antibody Selection

All key factors to consider for successful validation of antibody specificity are explored here.

Research shows that the verification of antibodies mainly focuses on three key properties of antibodies, namely:

Specificity: Verifying the ability of the antibody to distinguish between various antigens.

Affinity: Demonstrating the binding strength between an antibody and its corresponding antigen-binding site.

Reproducibility: Evaluating whether the experimental data obtained can be reproduced in other experimental settings.

### Points to Consider When Validating Antibody Specificity

- Selection and Preparation of Positive and Negative Controls

The selection and preparation of appropriate positive and negative controls is critical to the successful validation of antibody specificity.

Positive controls should include relevant cell lines or tissue samples that strongly express the target protein of interest to confirm antibody selective binding.

Negative controls should include cell lines or tissue samples that do not express the protein of interest to provide data on the non-selective binding properties of the antibody. If true negative controls are not available, samples expressing low levels of the protein of interest may be considered an acceptable alternative.

- Protocol

Be sure to use an optimized protocol to maximize the success of your antibody validation.

- Buffer Selection

Most antibody tests typically use two types of buffers, PBS or TBS. You need to determine the most appropriate buffer for your experiment, taking into account factors that can affect buffer performance, such as pH.

## Design Models for Positive and Negative Controls

Cell lines or tissues that endogenously express the protein of interest or do not express the protein of interest can be used as positive and negative controls. You can use multiple cell lines with different protein expression levels to provide a range of different levels of control. Alternatively, appropriate positive and negative controls can be designed by using various approaches such as knockout models, siRNA knockdown, or cell treatments.

## Validating Antibody Specificity in Different Applications

There are several methods you can use to verify the specificity of your antibody. Here is an overview of some common applications and their advantages and limitations.

| Validation | Benefits | Limitations |
| --- | --- | --- |
| Mass spectometry/ Immunoprecipitation - Mass spectometry (IP-MS) | • Suitable for high-throughput validation.<br>• Uses standardized techniques to estimate the potential level of target antibody binding to the target protein.<br>• Ability to identify all bound protein subtypes with antibody.<br>• Ability to identify post-translational modifications, interacting partners and complexes.<br>• Confirms specificity by analysis of digested protein fragments. | • Excessive IP washes may result in the removal of low or medium-affinity binders.<br>• It's important to note that not every antibody is compatible with immunoprecipitation (IP).<br>• Distinguishing partner proteins pulled down within a complex from non-specific binding can pose a significant challenge.<br>• Interpreting the data can be complex, as the highest enrichment score doesn't always indicate the antibody's preferential target. |
| Western blot | • Valuable in assessing antibody specificity against the target protein by considering molecular weight.<br>• Well-suited for the detection of proteins that have been reduced or denatured.<br>• Primarily a qualitative assay. | This assay requires a significant amount of time to complete and is less amenable to automation than certain other applications. |
| Immunocytochemistry (ICC) | • Validates the accuracy of antibody recognition by assessing its alignment with the protein's cellular location.<br>• Ensures specificity by testing in both target protein-expressing and non-expressing cells.<br>• Primarily a qualitative assay. | • It is challenging to discern whether an antibody exhibits non-specific recognition of other proteins that share the same cellular localization. |

| | | |
|---|---|---|
| Immunohistochemistry (IHC) | • Validates the antibody's target protein recognition by assessing its tissue-specific localization.<br>• Confirms specificity through testing in tissue samples that express and do not express the target protein.<br>• Primarily a qualitative assay. | • Difficult to ascertain whether the antibody exhibits non-specific recognition of other proteins sharing identical tissue localization. |
| Protein/peptide array | • Facilitates screening for antibody binding with a wide array of proteins or peptides.<br>• Utilizes a high-throughput screening methodology. | • In the case of protein arrays, it's not possible to screen for post-translationally modified proteins if the arrays consist of proteins synthesized in *E. coli* (bacteria).<br>• Arrays constructed using peptides and denatured proteins exclusively offer linear epitopes for examination. |

It should be noted that these two methods are not sufficient as the sole means to comprehensively test antibody specificity, so we do not recommend using them alone:

- Immune Peptide Blocking

This method can be used to confirm whether an antibody is bound to its immunogen. However, immunogenic peptides can block specific and nonspecific antibodies and therefore cannot be used as a comprehensive method to test antibody specificity.

- Missing Primary Antibody

This method can be used to evaluate secondary antibody or tissue detection reagents, but does not evaluate the specificity of the primary antibody.

## Antibody Storage and Handling

Proper storage and handling are essential to maintain antibody activity over time, often months to years. This section outlines general guidelines for antibody storage and handling, but it's critical to consult the manufacturer's datasheet for specific recommendations.

## General Storage Guidelines

Upon receiving the antibody, it is advisable to centrifuge it at 10,000 x g for 20 seconds to eliminate any solution trapped in the vial threads. The antibody should then be aliquoted into low protein binding microcentrifuge tubes. Aliquoting prevents damage from repeated freeze/thaw cycles, which can denature antibodies and lead to the formation of aggregates, reducing their binding capacity. It also minimizes the risk of contamination from multiple pipetting instances from a single vial. These aliquots should be freeze-thawed only once and the remaining solution should be stored at 4°C. It is generally recommended that antibodies be stored at -20°C, as there is typically no significant advantage to storing them at -80°C. The size of the aliquots should be determined based on your typical usage in experiments, but they should not be smaller than 10 µL. Smaller aliquots are more susceptible to concentration changes due to evaporation and antibody adsorption onto the vial's surface. In most cases,

storing antibodies at 4°C upon receipt is acceptable for one to two weeks, but adherence to the datasheet recommendations is crucial.

### Avoiding Freeze/Thaw Damage

It is imperative not to use a frost-free freezer, as the cycling between freezing and thawing should be avoided. To prevent such damage, please place antibody vials in the freezer area with minimal temperature fluctuations, such as toward the back rather than on a door shelf. Some researchers add the cryoprotectant glycerol to achieve a final concentration of 50% to mitigate freeze/thaw damage, as glycerol lowers the freezing point to below -20°C. However, the use of glycerol should be verified with the manufacturer's recommendations, as it may not be suitable for all antibodies. Storing solutions with glycerol at -80°C is discouraged, as this temperature falls below glycerol's freezing point. It's also essential to ensure the sterility of glycerol or any other cryoprotectant to avoid contamination.

### Storage of Conjugated Antibodies

Conjugated antibodies, whether linked to fluorochromes, enzymes, or biotin, require additional precautions in storage and handling due to their complexity. For instance, they should be stored in dark vials or protected with foil, as exposure to light can compromise the activity of the conjugates. Fluorescent conjugates, in particular, are susceptible to photo-bleaching and should be shielded from light throughout all phases of an experiment.

### Avoiding Contamination with Sodium Azide

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

### When Not to Use Sodium Azide

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

### Protein Concentration and Stability

Proteins are generally more resistant to degradation when stored at higher concentrations, preferably ≥1 mg/mL. Therefore, it is recommended that antibodies not be diluted to working concentrations and stored at 4° C for longer than one day.

### Antibody Formats and Purification

In cases where you receive an unpurified antibody, it may be necessary to perform purification before incorporating it into your experimental protocols. Methods for antibody purification vary from relatively crude to highly specific, and the extent of purification required depends on your intended use of the antibody.

### Antibody Dilution and Titer

When utilizing an antibody for the first time, it's essential to fine-tune its dilution to align with the specific requirements of your experiment and setup. This section explains how to determine the optimal antibody concentration through titration, and provides suggested dilutions for antibodies lacking recommended dilution guidelines in the datasheet.

Table 2. Recommended Antibody Dilutions for Various Applications

| | Tissue culture supernatant | Ascites | Whole antiserum | Purified anti body |
|---|---|---|---|---|
| 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 | - |

## References


1. Nagalingam K, *et al.*; Chromatin immunoprecipitation (ChIP) method for non-model fruit flies (Diptera: Tephritidae) and evidence of histone modifications. *PLoS One*. 2018, 13(3):e0194420.
2. Tony O Hara, *et al.*; Electrochemical Biosensors for Detection of Pesticides and Heavy Metal Toxicants in Water: Recent Trends and Progress. *ACS EST Water*. 2021,1, 462-478.


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