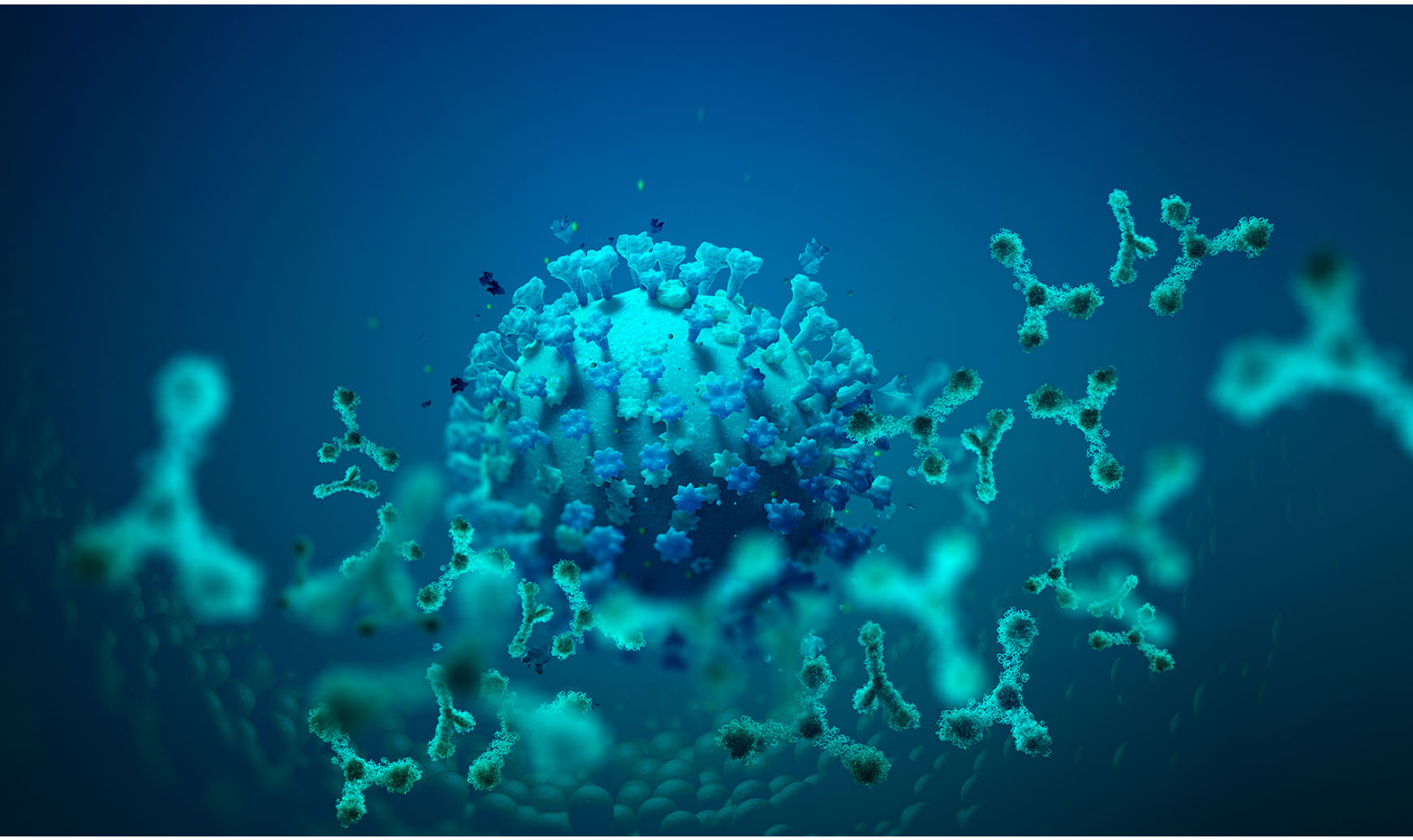
How to Choose Secondary Antibodies



Creative Diagnostics

Secondary Antibody Selection Guide

Creative Diagnostics provides a Secondary Antibody Selection Guide to support your research.

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01 What Is a Secondary Antibody?

The secondary antibody refers to the antibody that can bind to the antibody, that is, the anti-antibody. Its main function is to detect the presence of the antibody and amplify the signal of the primary antibody.

Secondary antibodies use the antigenic properties of macromolecular proteins to immunize heterogeneous animals. The immunoglobulin produced by the immune system of the heterogeneous animal is directed against this antibody. Secondary antibodies are reactive against all antibodies (such as IgG, IgM or IgA) of a specific species (such as mouse).

What Is the Difference Between Secondary Antibodies and Primary Antibodies?

The primary antibody is commonly known as an antibody that can specifically bind to an antigen. The secondary antibody can bind to the antibody, i.e., the antibody of the antibody. It is mainly used to detect the presence of antibodies. The primary antibody is an antibody directed against the antigen, and the secondary antibody is an antibody directed against the primary antibody. This means that antibodies can also serve as antigens to stimulate the body to produce antibodies. When an antigen enters the body, it stimulates the body's immune system to produce an immune response. B cells can produce special proteins to specifically bind to the corresponding antigen. Both primary and secondary antibodies are groups that can specifically bind to other substances, and primary antibodies can bind to at least two other groups (substrates and secondary antibodies).

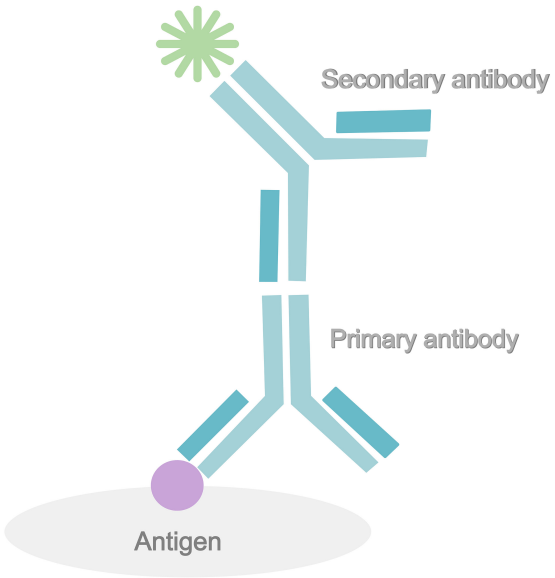

Figure 1. Secondary Antibodies and Primary Antibodies.

In immunodetection (indirect detection), ideally, the secondary antibody specifically binds to the primary antibody of a specific species and class (isotype). Please note that the secondary antibody does not directly bind to the epitope of the target protein and does not have epitope specificity (this is actually determined by the primary antibody). The labeled secondary antibody determines the detection method.

Antibody Structure

Antibodies are Y-shaped molecules composed of three regions of equal size. A flexible hinge region connects the antibody handle (Fc) to the antibody arm (Fab). The two arms are used for antigen binding, while the handle determines the type and functional properties of the antibody. Each arm consists of a heavy chain and a light chain. In addition, these heavy and light chains are composed of a variable region (VL or VH) and a constant region (CL or CH) respectively. The variable regions (V) of the heavy and light chains differ between different antibodies, which determines the specificity with which an antibody recognizes its cognate antigen. The change in the Fc handle is small but important for the interaction of effector molecules and cells.

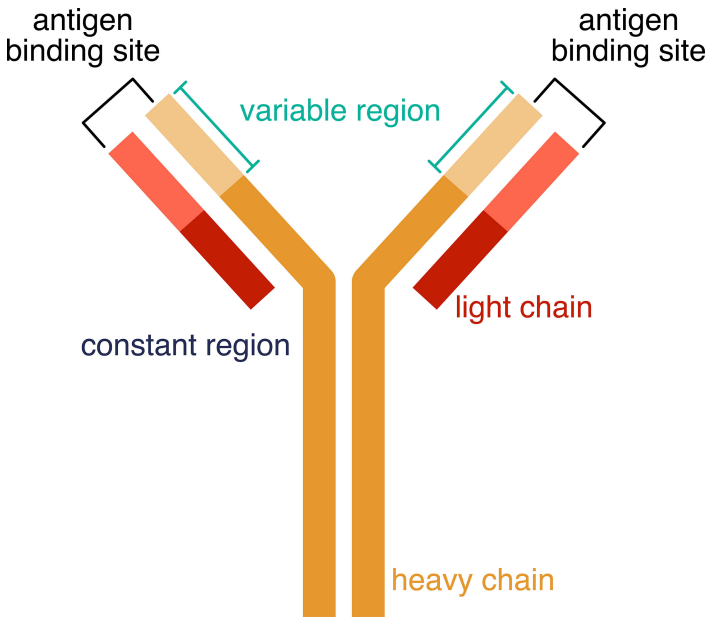

Figure 2. Antibody structure.

How Are Secondary Antibodies Produced?

Since the Fc segment of antibodies of the same subtype from the same source is conserved, a secondary antibody is designed based on this principle. The secondary antibody is related to the Fc segment subtype of the primary antibody.

Secondary antibodies are produced and collected in animals by immunizing them with antibodies from another animal. The characteristics of the antibody used for immunization (such as species, subclass, and fragment) determine the specificity of the secondary antibody. For example, rabbit anti-rat IgG2b (H+L) secondary antibody is produced by immunizing rabbits with the heavy and light chains of the rat IgG2b immunoglobulin molecule.

The Role of Labeled Secondary Antibodies

The primary antibody can specifically bind to the substrate, which means it recognizes what we want to detect. Whether or not the primary antibody binds to the substrate cannot be seen with the naked eye. The secondary antibody can be combined with the primary antibody and has a label that can be detected (such as fluorescent, radioactive, chemiluminescent or chromogenic groups), and is used to detect the primary antibody. If the primary antibody itself has a detectable label (such as a fluorescent, radioactive, chemiluminescent or chromogenic group), a secondary antibody is not required. However, this is very costly, because a primary antibody only recognizes one substrate, which is expensive and labor intensive. Therefore, today's design generally uses a secondary antibody with a detectable label to

detect the primary antibody and the antigen. In this way, when the primary antibody binds to the antigen, it can be detected by the secondary antibody.

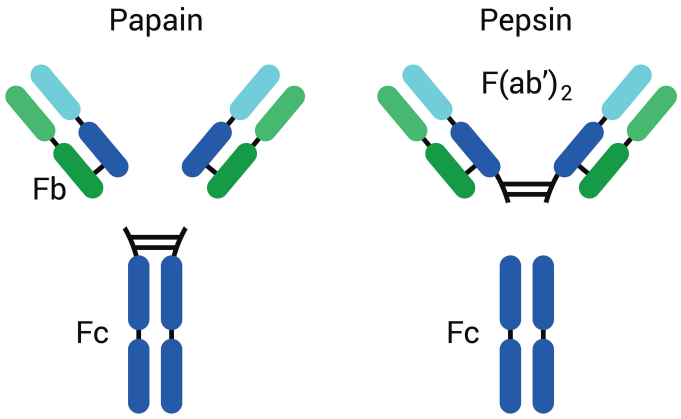

Figure 3. Antibody fragments.

Common Abbreviations for Secondary Antibodies

Abbreviation	Definition	Description
Ig	Immunoglobulin	Also called antibodies
F(ab)	Antigen Binding Fragment	A constant region and a variable region of the heavy and light chains (one arm of the antibody)
F(ab')	Antigen Binding Fragment	F(ab) fragment plus hinge region
$F(ab')_2$	Antigen Binding Fragment	The entire F(ab) region (the two arms of the antibody) plus the hinge region, excluding the Fc region
F_c	Crystallizable Fragment	The heavy chain that forms the handle and hinge of the antibody
C_L	Light Chain Constant Region	
C_H	Heavy Chain Constant Region	
V_L	Light Chain Variable Region	Contains antigen-binding sites that confer specificity to antibodies
V_H	Heavy Chain Variable Region	Contains antigen-binding sites that confer specificity to antibodies

(H+L)	Heavy Chain + Light Chain	Whole immunoglobulins (heavy and light chains)
α	α heavy chain	IgA Class
δ	δ heavy chain	IgD Class
ε	ε Heavy Chain	IgE Class
γ	γ Heavy Chain	IgG Class
μ	μ Heavy Chain	IgM Class
κ	κ Light Chain	
λ	λ Light Chain	

02 Secondary Antibody Selection

Factors Involved in Secondary Antibody Selection

Secondary antibodies bind directly to the primary antibody linked to the target antigen. After the primary antibody binds to the antigen, the labeled secondary antibody specifically binds to the Fc end of the primary antibody. This interaction is used to allow the secondary antibody to indirectly detect and purify the target protein (antigen). Typically, there are several secondary antibodies to choose from in a specific experiment. The selection of the most suitable secondary antibody for this experiment requires a comprehensive consideration of the following aspects:

- Host Source

The selection of secondary antibodies is primarily based on the primary antibody and should match the species source of the primary antibody and needs to be from different species. For example, if the primary antibody is rabbit, then the corresponding secondary antibody should be anti-rabbit, which can be goat anti-rabbit or mouse anti-rabbit, that is the species of the primary antibody determines the reactivity of the secondary antibody.

- Secondary Antibody Format

Choosing the appropriate and correct secondary antibody format is also a key to experimental success. Three secondary antibody formats: Whole IgG, F(ab')2, and Fab.

- Antibody Specificity

Be sure to distinguish the specificity and form of the secondary antibody. Specificity refers to the different primary antibody subtypes or a special region recognized by the secondary antibody. The specificity of antibodies is mainly based on the following three forms:

1. Light chain specificity
2. Fc-terminal specificity
3. Subtype specificity

- Subtype

The selection of the class or subtype of the primary antibody: The secondary antibody needs to match the class or subtype of the primary antibody. This is typically for monoclonal antibodies. Polyclonal antibodies are mainly IgG immunoglobulins, so the corresponding secondary antibody is an anti-IgG antibody.

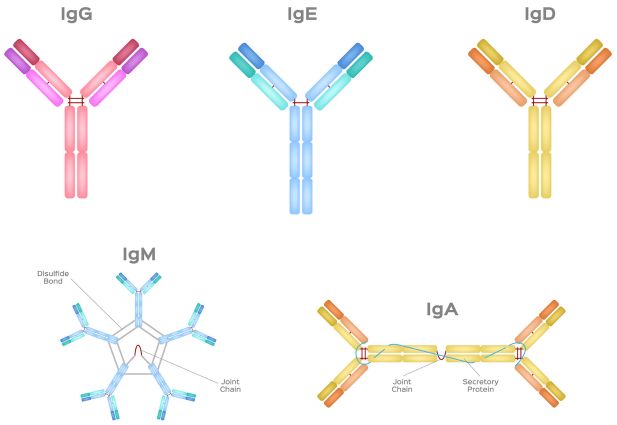

Figure 4. Antibody subtypes.

- Species Source

Species source of secondary antibodies: With the exception of double-labeling experiments, generally speaking, different species sources are not necessarily related to the quality of secondary antibodies. Secondary antibodies derived from goats or donkeys are the same for general experiments. There is no clear difference.

- Coupling Tag

Coupling labels of secondary antibodies: Choice of different labels for different experimental methods. Generally speaking, the probes coupled to the secondary antibodies mainly include enzymes (horseradish peroxidase HRP and alkaline phosphatase AP or their derivatives APAAP, PAP), fluorescent groups (FITC, Rhodamine, Texas Red, PE, Rhodamine, Dylight, etc.), biotin, and gold particles. Usually, HRP-labeled secondary antibodies are recommended for WB, ELISA, IHC and other experiments, and FITC-labeled antibodies are recommended for IF, FC, etc.

Tips: The choice of marker usually depends on the application. Fluorescence detection assays (such as flow cytometry, immunocytochemistry, and immunofluorescence) require secondary antibodies conjugated to fluorescent dyes. The excitation and emission spectra of each fluorescent dye should be considered in the design of each experiment. Commonly used fluorescent dyes include FITC, PE, APC, DyLight™, AlexaFluor™ or Atto dyes. Enzymatic detection requires secondary antibodies conjugated to horseradish peroxidase (HRP), alkaline phosphatase (AP), or biotin. Avidin and streptavidin can combine with biotin and form a complex to achieve signal amplification independent of the secondary antibody host species. HRP is more economical and stable than AP, so HRP is more commonly used in chemiluminescent detection systems. However, AP has a higher sensitivity than HRP and is therefore

more commonly used in chromogenic detection.

- Pre-adsorption

Selection of pre-adsorbed secondary antibodies: For tissues and cells rich in immunoglobulins, it is usually recommended to use pre-adsorbed serum secondary antibodies for Western blotting applications. Pre-adsorbed secondary antibodies are less likely to interact with endogenous immunoglobulins, thus reducing non-specific background.

- Special Requirements

Secondary antibodies in the form of F(ab')2 fragments can avoid binding to Protein A/G or Fc receptors. The molecules themselves are relatively small, allowing easier penetration into cell tissue structures and higher sensitivity. Secondary antibodies in the form of Fab fragments contain only one binding site and can be used to block endogenous immunoglobulins. Unless otherwise noted, secondary antibodies generally refer to anti-IgG H&L type antibodies, the most commonly used and cost-effective form of secondary antibodies. Antibodies can react with both the heavy and light chains of IgG molecules, and can also react with other immunoglobulin families (IgM, IgA, IgD, IgE) and subfamilies, since all immunoglobulins share the same light chain.

Secondary Antibody Selection Guide

- WB Experiments

For routine WB experiments, based on the principle of correct species origin and reaction species, we generally choose enzyme-labeled or biotin-labeled full-fragment secondary antibodies without special forms.

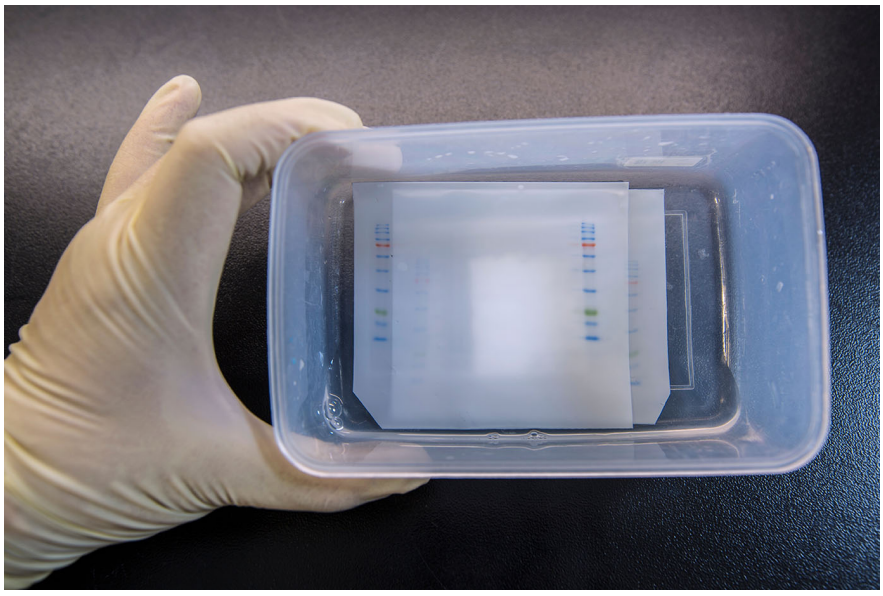

Figure 5. WB Experiments.

- Fluorescence WB Experiment

Unlike the enzyme-labeled secondary antibodies used in chemiluminescent WB experiments, fluorescent WB requires the selection of secondary antibodies with fluorescent groups (e.g., FITC/Dylight/Alexa Fluor fluorescent secondary antibodies). In addition, imaging equipment with appropriate light sources and filters is required.

- Post-IP WB Experiment

In IP or co-IP experiments, the same primary antibody or primary antibody from the same species is usually used in the IP experiment and subsequent WB experiments. In this case, conventional non-specific Whole IgG enzyme-labeled secondary antibodies can recognize both the heavy chain (55kDa) and the light chain (25kDa) produced after denaturation of the IP antibody in WB experiments. However, this non-specific signal often partially or even completely masks the signal of the target protein.

Therefore, when the target protein is located near a molecular weight of 55kDa, it is necessary to select an anti-light chain secondary antibody to avoid interference from the heavy chain; conversely, when the target protein is located near a molecular weight of 25kDa, it is necessary to select an anti-Fc end secondary antibody to avoid interference from the light chain. In addition, if there is an experimental need to remove light chain or heavy chain interference simultaneously, a conformation-specific secondary antibody can be selected.

- IHC/ICC/IF Experiment

When studying certain cells (such as macrophages, B cells, and natural killer cells) and tissues (lymph nodes, spleen, peripheral blood, etc.), these cells or tissues will interact with the Fc of antibody molecules due to an excess of Fc-terminal receptors on their surfaces. This will result in non-specific binding and high background.

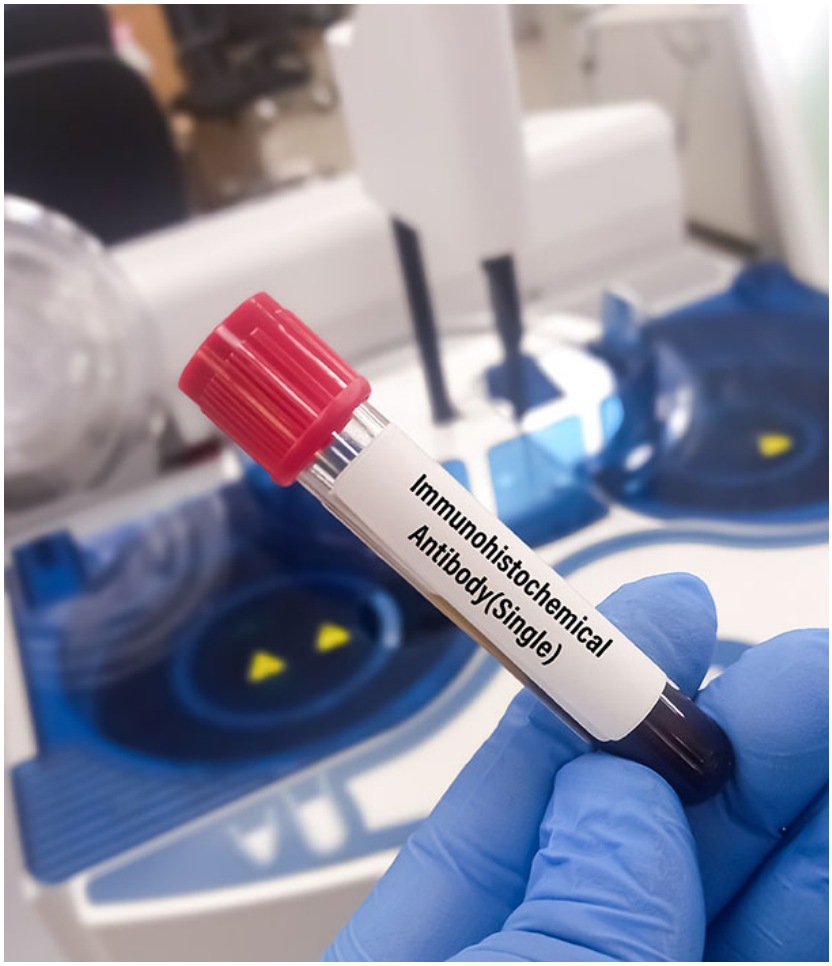

Figure 6. Immunhistochemical(IHC) Antibody.

The use of secondary antibodies in the form of F(ab')2 can avoid non-specific binding to Fc receptors in living cells, and can also avoid non-specific binding to Protein A and Protein G, thereby improving experimental accuracy.

- Multi-label Staining IF/IHC Experiment

To prevent cross-reaction between the secondary antibody and the sample and other primary antibody species, you must first select a pre-adsorbed secondary antibody to reduce cross-reaction when performing multi-labeling experiments.

- Double-stained IF Experiment with the Same Primary Antibody Source

Sometimes, due to limitations in actual experimental conditions, when performing double-stained IF experiments, the primary antibodies are derived from the same species. At this time, you can use Fab fragment secondary antibodies to perform experiments. This is because each Fab fragment secondary antibody only contains one antigen-binding site, so it will not bind to another molecule during the subsequent incubation process.

It is not recommended to use F(ab')2 fragment antibody for double labeling experiments because it is a bivalent molecule and contains two binding sites. After binding to the first primary antibody, the second binding site may remain unoccupied, and this unoccupied binding site can bind to the second antibody, resulting in a false positive reaction .

Tips: Fab fragment secondary antibodies can also be used to block endogenous immune proteins. If the primary antibody is from the same species as the tissue being tested (e.g., mouse primary antibody is used for mouse tissue), Fab fragment secondary antibodies can be used to block endogenous immune proteins to improve the accuracy of the experiment.

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