Technical Info

Well advised with Roth.

Selection, Application, and Validation of Antibodies

A guide for the targeted use of primary and secondary antibodies

Contents

I. Antibody Selection and Application

In addition to confirming that an antibody has sufficient specificity, several other aspects should be taken into account when selecting one for an experiment. The criteria listed below are relevant for most common antibody-based applications.

1. Species Reactivity

Primary antibodies recognize proteins from only certain species. This typically refers to the species from which the immunizing antigen originally originated, as well as closely related species. Check whether the antibody has been validated for your species. Highly conserved proteins may also be recognized in untested species.

2. Application Compatibility

Not all antibodies work in all techniques (e.g., IHC vs. Western blot). This is usually due to differences in sample preparation between methods, which affect how antigens are presented or made accessible. Therefore, the suitability of a chosen antibody for the intended application must be verified and validated.

3. Clonality

Clonality reflects how the antibody was produced and how many epitopes it targets.

- **Polyclonal:** recognizes multiple epitopes; stronger signal; suitable when target protein levels are low.
- **Monoclonal:** specific and reproducible; suitable for sandwich ELISAs or long-term experiments.

4. Target Epitope Location

The location of the antibody's binding site can be crucial. For FACS, for example, it must recognize an extracellular domain because the membrane cannot be crossed. In cases of alternative splice variants or knockout models, the epitope location can also influence interpretability. The epitope is determined by the immunogen, which may consist of all or part of the protein; details can be found in the product datasheet.

5. Carrier Proteins and Preservatives

Antibodies are typically stored in PBS with carrier proteins such as BSA and preservatives such as glycerol or sodium azide to ensure stability and prevent bacterial growth. However, these additives can interfere with experiments: BSA can reduce the efficiency of conjugation with fluorophores or enzymes, and sodium azide is cytotoxic. For conjugation or work with live cells, additive-free antibodies are preferred.

6. Antibody Concentration

The optimal concentration depends on affinity, application, and sample. Dilution series help determine the best signal-to-noise ratio.

7. Direct vs. Indirect Detection

Once an antibody has bound to its target protein, its binding must be visualized. This can be done either directly or indirectly (Figure 1).

- **Direct:** Primary antibody conjugated to a marker.
- **Indirect:** Secondary antibodies detect primary antibodies; signal amplification is possible, but background may be higher.

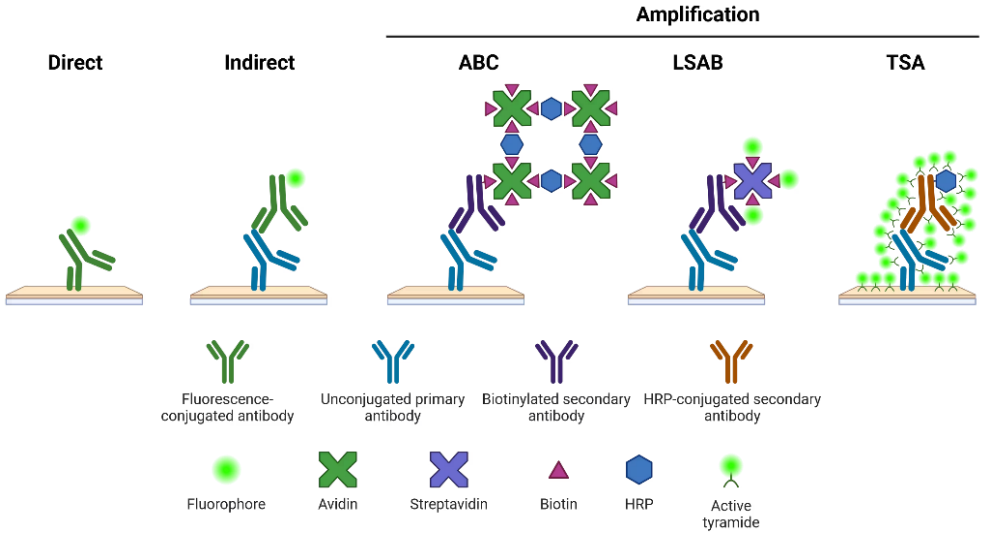

Figure 1: Schematic representation of various methods for target detection. Amplification methods include the avidin-biotin complex (ABC), labelled streptavidin-biotin (LSAB) and tyramide signal amplification (TSA) systems. Created with BioRender from antibodies

8. Amplification

In addition to indirect detection, signal strength can be increased through additional amplification steps (Figure 1). Secondary antibodies conjugated to biotin are commonly used; biotin is bound by multiple avidin or streptavidin molecules. These, in turn, carry several fluorophores or enzymes, thereby amplifying the signal originating from a single secondary antibody.
Another amplification method is tyramide signal amplification (TSA), which is based on the enzymatic activity of horseradish peroxidase (HRP). HRP converts inactive, fluorophore-labeled tyramide into a reactive radical that binds to nearby tyrosine residues, making the region around the antibody binding site visible.

9. Host Species & Multiplexing

The host species of an antibody refers to the animal species in which it was generated. This is especially important for indirect detection methods, as secondary antibodies are designed to recognize the host species of the primary antibody. Primary antibodies should ideally not originate from the same species as the examined sample — for example, mouse antibodies on mouse tissue — because in such cases, secondary antibodies (e.g., goat anti-mouse) may bind not only the primary antibody but also endogenous proteins.
In multiplex IHC, primary antibodies from different host species must be used to allow simultaneous detection of multiple proteins (Figure 2). If two primary antibodies from the same species are present, the secondary antibodies will recognize both, leading to cross-reactivity and complicating visualization. Additional issues may arise when secondary antibodies recognize other secondary antibodies, for example when donkey anti-goat binds both goat primary and goat secondary antibodies.

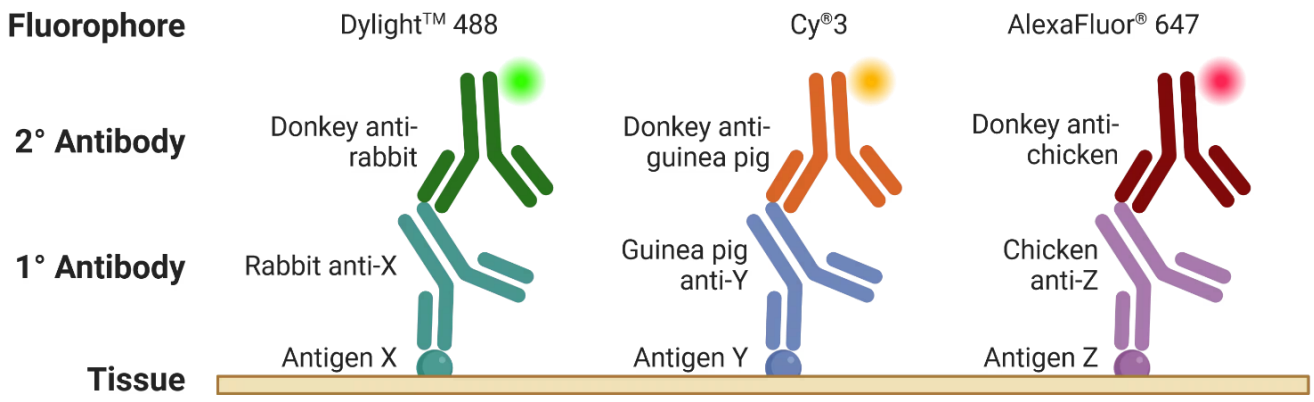

Figure 2: Multiplex staining using IHC. By using primary antibodies from different species, multiple antigens can be detected simultaneously. Secondary antibodies recognize primary antibodies from a specific species and, when conjugated to spectrally distinct fluorophores, can be resolved under a fluorescence microscope. Created with BioRender by antibodies.com.

10. Storage & Freezing

Antibodies in solution are typically shipped on ice, whereas lyophilized antibodies can be shipped at room temperature and reconstituted in water or PBS before use. For short-term storage (1–2 weeks), 4 °C is sufficient. For long-term storage, antibodies should be aliquoted and frozen at –20 °C or –80 °C; frost-free freezers should be avoided. Aliquots reduce contamination and damage caused by repeated freeze–thaw cycles. Aliquot sizes should match typical experimental needs but not be smaller than 10 µl to prevent evaporation. Conjugated antibodies, especially fluorophore- or enzyme-conjugated ones, require special handling: light-sensitive fluorophores must be stored in the dark; enzymes should not be frozen unless the datasheet explicitly allows storage at –20 °C with cryoprotectant. Secondary antibody conjugates often retain activity for months at 4 °C.

11. Additives

To prevent bacterial contamination, antibodies may contain sodium azide at a final concentration of 0.02 % (w/v). The datasheet indicates whether sodium azide has been added. When working with live cells, sodium azide should be avoided because it is cytotoxic. It also inhibits HRP activity, making it unsuitable for HRP-conjugated antibodies. Glycerol acts as a cryoprotectant, lowering the freezing point below –20 °C, and thus enables storage in a freezer without actual freezing. This preserves antibacterial effects and activity while preventing damage from repeated freeze–thaw cycles. At –80 °C, however, glycerol does freeze, so glycerol-containing stocks should be stored at –20 °C. Glycerol is typically used at a final concentration of 50 %; however, some antibodies are unstable under these conditions, so the product datasheet should be consulted.

II. Selecting a Secondary Antibody

Secondary antibodies are immunoglobulins used in research to bind primary antibodies that recognize specific target molecules. They are usually conjugated to fluorophores or enzymes, enabling highly sensitive detection tailored to both the species and isotype of the primary antibody.

Choosing the appropriate secondary antibody is crucial for the success of experiments requiring indirect antigen detection. This guide summarizes the key properties and selection criteria that should be considered.

Our checklist provides a concise overview to support rapid selection of the appropriate secondary antibody.

1. Definition and Function

Secondary antibodies bind to primary antibodies that recognize target molecules. They are typically coupled to fluorophores or enzymatic reporters, enabling highly sensitive signal amplification (Figure 3) and flexible use across different applications.

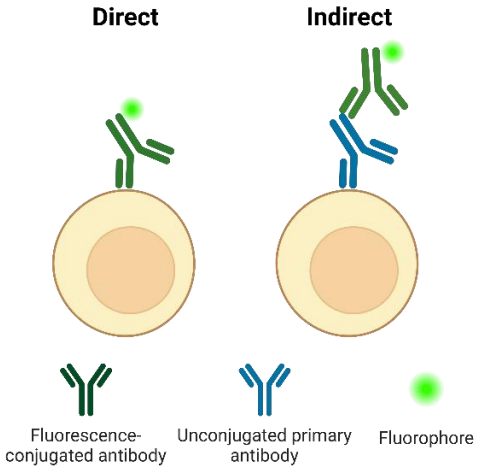

Figure 3 Antigens can be detected directly using conjugated primary antibodies or indirectly using secondary antibodies.:

Advantages of using secondary antibodies

- Signal amplification: Multiple secondary antibodies per primary antibody increase signal intensity.
- Flexibility: One secondary antibody can detect many primary antibodies from the same host species.
- Expanded conjugate options: Reporters or fluorophores are more widely available for secondary antibodies than for primary ones.
- Broader applicability: A single primary antibody can be used in different techniques.
- Disadvantage: potential for increased background signal.

2. Selection Criteria

a. Host species of the primary antibody

Secondary antibodies must be specific to the host species of the primary antibody.

b. Host species of the secondary antibody

Typically goat or donkey. When multiplexing, choose different hosts to avoid cross-reactivity.

c. Isotype and format of the primary antibody

Secondary antibodies are specific to isotypes (IgG, IgM, etc.) and specific regions (H, L, or H+L chains). Choosing appropriately reduces off-target binding and enables multiplexing.

d. Secondary antibody format

- Whole IgG antibodies: high sensitivity, more reporters attached.
- Fragments (Fab, $F(ab')_2$): improved tissue penetration, avoid Fc-mediated off-target effects.

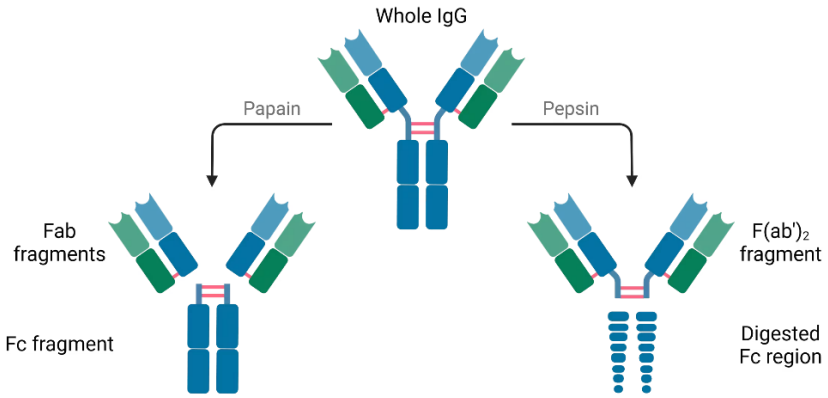

Figure 4: Antibody fragments generated by enzymatic digestion using papain or pepsin.

e. Conjugate

- Fluorophores: for immunofluorescence and spatial resolution.
- Enzymes (HRP, AP): for chromogenic or chemiluminescent assays (ELISA, Western blot, IHC).
- Biotin: for signal amplification via avidin/biotin systems.
  Selection depends on application, detection method, multiplexing, amplification needs, and availability.

f. Cross-adsorption

Reduces off-target binding in closely related species or multiplex experiments.
Use only when necessary, as it may slightly reduce epitope recognition.

3. Multiplexing

Simultaneous detection of multiple target antigens requires primary antibodies from different host species to avoid cross-reactivity. Secondary antibodies should carry spectrally distinct conjugates (see Figure 2).

4. Secondary Antibody Checklist

- Determine the host species of the primary antibody
- Choose the host species of the secondary antibody
- Identify isotype and format of the primary antibody
- Select the secondary antibody format (IgG, Fab, $F(ab')_2$)
- Choose an appropriate conjugate (fluorophore, enzyme, biotin)
- Check for cross-adsorption and use when necessary

5. Example of Nomenclature

Goat anti-rabbit IgG H&L (HRP), cross-adsorbed:

- Goat: host of the secondary antibody
- Anti-rabbit IgG: species and isotype of the primary antibody

- H&L: recognizes heavy and light chains
- HRP: conjugate
- Cross-adsorbed: reduced off-target binding

III. Antibody Applications and Validation

Antibodies are versatile tools in research and enable investigation of numerous protein-related questions, including abundance, modifications, cellular localization, and molecular interactions. Before using an antibody, its ability to bind specifically to its target must be verified to ensure reliable and reproducible results.

1. Major Applications of Antibodies

- **ELISA:** Quantifies target antigens in liquid samples such as serum. Common formats include direct, indirect, sandwich, and competitive ELISA. Sandwich ELISA is often the most specific because it uses two complementary antibodies.
- **Flow Cytometry (FACS):** Analyzes cells or particles in suspension by measuring light scattering and fluorescence from labeled antibodies. Allows identification of cell types, quantification of frequencies, and assessment of activation status or protein expression.
- **Immunohistochemistry (IHC), Immunocytochemistry (ICC), and Immunofluorescence (IF):** Detect proteins in tissues or cells using enzyme-based or fluorescent readouts. IF facilitates multiplexing. After fixation, primary antibodies bind to the target protein, followed by direct or indirect visualization. IHC is widely used in pathology for diagnostics and prognostics.
- **Immunoprecipitation (IP) and Co-IP:** Antibody-coated beads isolate proteins from complex samples; Co-IP additionally captures interacting partners. Isolated proteins can be analyzed by ELISA, Western blot, or mass spectrometry.
- **Chromatin Immunoprecipitation (ChIP):** Isolates DNA-bound proteins to analyze target sequences and chromatin structure. RIP is analogous but investigates protein–RNA interactions.
- **Western Blot:** Detects specific proteins separated by SDS-PAGE, providing information on quantity, molecular weight, and post-translational modifications.

2. Antibody Specificity and Validation

Antibodies typically bind their targets with high specificity but may also recognize off-target proteins with similar epitopes. Validation is therefore essential to ensure accurate research results.

- **Knockout (KO) validation:** The gold standard. Compares wild-type and KO samples lacking the target protein. A specific antibody yields no signal in the KO sample. KO cells or organisms can be generated via homologous recombination, TALENs, or CRISPR-Cas9. Typically assessed by ICC or Western blot.

- **Protein array validation:** Genome-wide protein arrays test antibody specificity against >19,000 proteins, detecting both on-target and off-target binding. Signals are visualized using fluorophore-labeled secondary antibodies.
- **Biophysical validation:** Ensures purity and specificity. SDS-PAGE is common, but SEC-HPLC is frequently used to detect and quantify aggregates. All antibodies.com biosimilar antibodies are tested by SEC-HPLC and functional assays.
- **Additional methods:** Orthogonal validation, independent antibody validation, tagged protein expression, and immunocapture mass spectrometry (IMS).

3. Controls

- **Positive control:** Sample with high target expression; antibody should produce a strong signal.
- **Negative control:** Sample lacking the target; antibody should produce no signal.
- **Technique-specific controls** and alternative approaches such as knockdown or overexpression provide additional reliability.

List of Figures

References

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