Secondary Antibodies

Secondary Antibodies
Secondary antibodies are polyclonal or monoclonal antibodies that bind to primary antibodies or antibody fragments, such as the Fc or Fab regions. They are typically labeled with probes that make them useful for detection, purification or sorting applications. Aladdin's polyclonal secondary antibodies are produced from the serum of host animals such as rabbit, goat and sheep, whereas, monoclonal secondary antibodies are produced from primarily mouse hybridoma clones. Secondary antibodies are used in many applications including ELISA, immunopurification, Western Blotting, IHC, IF, flow cytometry and more.

The specific utility of a secondary antibody depends upon its conjugated probe(s). Probes are molecules that support various detection technologies. The most common detection systems for conjugated secondary antibodies are colorimetric or fluorescent. Colorimetric assays are typically based on the use of alkaline phosphatase (AP) or horseradish peroxidase (HRP) or its derivatives. The biotin avidin (streptavidin) conjugate binding system is often used to amplify the colorimetric signal for AP or HRP. The most common fluorescent assays utilize Fluorescein (FITC), Rhodamine or its derivative, TRITC, Cyanine (Cy3), or Phycoerythrin (R-PE).

Antibody Specificity
Secondary antibodies have been raised to immunoglobulins of various species to provide reagents for visualizing unconjugated primary antibodies bound to antigens in immunoassays. Antibodies to IgG that are whole molecule specific or Fab specific will usually react with all Ig classes, whereas heavy chain specific and Fc specific antibodies will react only with the indicated Ig class. The end user will need to determine which specificity is best suited to his/her work. The assay system and the presence of extraneous protein targets that could bind the antibody and give rise to false positive results or high background will affect the choice of reagents. Table A summarizes the various types of secondary antibodies and their uses.

Conjugates
Alkaline Phosphatase Conjugates
Alkaline phosphatase (AP) is an intestinal enzyme that dephosphorylates alcohols, phenols and amines at alkaline pH. It is a 140 kDa homodimer. The optimal pH range for activity is 9.5-10.5.

Alkaline phosphatase conjugates are widely used in immunoassays such as ELISA, immunohistochemistry and immunocytochemistry, and immunoblotting. AP conjugates are useful in tissues where endogenous peroxidase activity may generate high background staining with peroxidase conjugates. They are usually more sensitive than peroxidase conjugates, allowing use of higher dilutions, or detection of lower signals, in ELISA or blotting assays. Endogenous alkaline phosphatase activity in tissue sections may be blocked by adding levamisole to the substrate buffer. Levamisole inhibits all alkaline phosphatase isoenzymes with the exception of intestinal alkaline phosphatase.

Alkaline phosphatase substrates are available to form either soluble or insoluble products. Alkaline phosphatase conjugates are not recommended for use with intestinal tissue sections or extracts because of endogenous intestinal alkaline phosphatase activity.
References

Table A

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>Description</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-IgG (whole molecule)</td>
<td>Anti-IgG (whole molecule) antibodies have been generated using the intact IgG molecule as immunogen. Antibodies are developed to all portions of the IgG molecule that are different from the host’s own IgG. Some of the epitopes recognized may also be found on Igs of other species. This means that antibodies to IgG, whole molecule have a very broad specificity and may cross-react not only with other Ig classes such as IgA and IgM, but also with IgG from other species. For instance, anti-goat IgG, whole molecule, may react with human IgG.</td>
<td>Use when the presence of multiple Ig classes is not a concern, when it is desirable to detect all Ig present, regardless of subclass, or to give a strong signal. They are also a good choice when the antibody Ig class is not known or when detecting total Ig in cell or tissue samples.</td>
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<tr>
<td>Anti-IgG (Fab specific)</td>
<td>Fab specific antibodies are raised by immunizing the host animal with Fab fragments generated by papain digestion of IgG. Since Fab fragments contain epitopes common to all Ig classes, these antibodies can recognize all Ig classes. They do not bind to the class-specific Fc region. As with anti-IgG, whole molecule, these antibodies may react with Igs from other species via epitopes shared among species.</td>
<td>Use when binding to the Fc region is either not possible or undesirable, such as when the target Ig Fc is bound by Protein A, anti-Fc specific antibodies, or Fc receptors on cell surfaces.</td>
</tr>
<tr>
<td>Anti-IgG heavy chain specific (alpha, gamma, mu)</td>
<td>Heavy chain specific antibodies are prepared from anti-whole molecule antibodies that have been adsorbed to remove essentially all cross-reactivity to epitopes on the Ig molecule that are not unique to the Ig class of the immunogen. Adsorption is performed as described below in Anti-IgG, Adsorbed with Various Species, using adsorbants containing immunoglobulins of the same species but different Ig class than the immunogen. Antibodies that recognize epitopes on the adsorbant Ig bind to the adsorbant. The heavy-chain specific antibodies are collected in the unbound portion. The resultant antibodies will only recognize a single Ig class. The specificity is indicated by a Greek letter corresponding to the class-specific determinants in the Fc region of the heavy chain: alpha-chain - IgA specific, gamma-chain - IgG specific, mu-chain - IgM specific.</td>
<td>Use when the target contains multiple Ig classes, but only one class is of interest. A common application is detection of individual Igs on tissue sections or in cell preparations for flow cytometry using anti-IgG, IgA, or IgM, heavy chain specific, conjugated to a fluorochrome to determine the level of an individual Ig on a particular cell type. They are also useful for double labeling, such as the localization of two different antigens in a single tissue section using an IgG monoclonal antibody and an IgM monoclonal antibody and detecting bound antibody with anti-mouse IgG, gamma-chain specific, and anti-mouse IgM, mu-</td>
</tr>
<tr>
<td><strong>Anti-IgG (Fc specific)</strong></td>
<td>Fc specific antibodies are raised by immunizing the host animal with Fc fragments generated by papain digestion of IgG (see the Antibody Fragmentation section). These antibodies are highly specific for IgG, and do not recognize IgA or IgM. Note: Fc specific and gamma-chain specific antibodies have essentially the same specificity.</td>
<td>Same as for Anti-Ig, Heavy-Chain Specific, but for IgG only.</td>
</tr>
<tr>
<td><strong>Anti-IgG, Adsorbed</strong></td>
<td>These antibodies are prepared by adsorption with IgG from species other than the species of the target IgG to remove cross-reactivity to those species. This cross-reactivity arises because immunoglobulins contain epitopes that are common to several species. Adsorption is accomplished by incubating the antibodies with IgG from the other species attached to solid-phase supports (adsorbants). Antibodies that recognize epitopes on IgG from those species bind to the adsorbant. The species-specific antibodies are collected in the unbound portion. The resultant antibodies retain good reactivity with IgG from the target species, but reactivity with the adsorbant species is minimized. Adsorbed secondary antibodies are useful for reducing background in immunoassays caused by the secondary antibody binding to the protein or tissue being assayed in addition to the primary antibody.</td>
<td>Use when the target IgG is attached to a matrix that contains potential targets from other species. For example, when detecting primary antibodies on human cells or tissues in immunohistochemistry, choose a secondary antibody that has been adsorbed with human serum proteins.</td>
</tr>
</tbody>
</table>

**Peroxidase Conjugates**

Horseradish peroxidase (HRP) is an enzyme that specifically reduces hydrogen peroxide in the presence of a proton-donor. HRP is a 40 kDa glycoprotein. The optimal pH range for activity is 6.0-6.5. HRP exhibits good thermal stability (up to 60°C) and pH stability (4-10). It is inhibited by azide.

Peroxidase conjugates are used in a variety of immunoassays such as ELISA, immunohistochemistry and immunocytochemistry, and immunoblotting. HRP conjugates are useful in tissues such as intestine where endogenous alkaline phosphatase activity may generate high background with alkaline phosphatase conjugates. The selection of available peroxidase substrates is wider than that for alkaline phosphatase, and the colors generated are frequently more intense. Endogenous peroxidase activity may be blocked by treating the tissue with an excess of hydrogen peroxide before addition of the conjugate, or by use of other blocking agents such as phenylhydrazine, azide plus nascent hydrogen peroxide, or periodic acid. A wide variety of substrates are available to form either soluble or insoluble products.

HRP conjugates are not recommended for use with samples such as blood cells and kidney tubules that contain high levels of endogenous peroxidase activity.

**References**

Fluorochrome Conjugates
Fluorophores absorb light at one wavelength, the absorption or excitation wavelength, inducing an excited electronic state. This state is unstable, and the molecule quickly returns to the unexcited, or ground, state by emitting light. Due to energy loss, this light is emitted at a longer wavelength (lower energy), which is termed the emission wavelength. The difference between the excitation and emission wavelengths is unique to each fluorophore, and the intensity of excitation and emission drops quickly as the wavelength varies from the maximum. The Fluorescent Dye Properties Table gives the excitation and emission wavelengths and the fluorescent color for several common fluorophores.

Fluorescent Dye Property Table

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
<th>Color</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine Orange</td>
<td>500</td>
<td>526 (DNA) 650 (RNA)</td>
<td>Green (DNA) Red (RNA)</td>
<td>Nucleic acid stain. Flow cytometry, fluorescence microscopy</td>
</tr>
<tr>
<td>Cy3</td>
<td>552</td>
<td>568-574</td>
<td>Red-orange</td>
<td>Protein conjugation. Flow cytometry</td>
</tr>
<tr>
<td>Cy5</td>
<td>649</td>
<td>670</td>
<td>Red</td>
<td>Protein conjugation. Fluorescence microscopy</td>
</tr>
<tr>
<td>DAPI</td>
<td>360</td>
<td>450</td>
<td>Blue</td>
<td>AT, minor groove dsDNA stain. Flow cytometry, fluorescence microscopy</td>
</tr>
<tr>
<td>DII-CLB(3)</td>
<td>550</td>
<td>565</td>
<td>Red-orange</td>
<td>Membrane stain (LR,CM) - cell fusion/ permeabilization. Flow cytometry</td>
</tr>
<tr>
<td>Fluorescein (FITC)</td>
<td>495</td>
<td>525</td>
<td>Green</td>
<td>Protein conjugation. Flow cytometry, fluorescence microscopy</td>
</tr>
<tr>
<td>Green Fluorescent Protein (GFP)</td>
<td>395 (470)</td>
<td>509</td>
<td>Green</td>
<td>Fusion protein. Recombinant protein expression. Fluorescence microscopy</td>
</tr>
<tr>
<td>Hoechst 33258</td>
<td>360</td>
<td>470</td>
<td>Blue</td>
<td>AT, minor groove dsDNA stain. Flow cytometry, fluorescence microscopy</td>
</tr>
<tr>
<td>R-Phycocerythrin (PE)</td>
<td>488</td>
<td>578</td>
<td>Red-orange</td>
<td>Protein conjugation. Flow cytometry</td>
</tr>
<tr>
<td>PKH2</td>
<td>490</td>
<td>504</td>
<td>Green</td>
<td>Cell membrane stain-cell tracking. Flow cytometry, fluorescence microscopy</td>
</tr>
<tr>
<td>PKH26</td>
<td>551</td>
<td>567</td>
<td>Red-orange</td>
<td>Cell membrane strain-cell tracking. Flow cytometry, fluorescence microscopy</td>
</tr>
<tr>
<td>PKH67</td>
<td>490</td>
<td>502</td>
<td>Green</td>
<td>Cell membrane stain-cell tracking. Flow cytometry, fluorescence microscopy</td>
</tr>
</tbody>
</table>

Fluorescent dyes are used to detect molecules in a variety of biological applications. Unlike many visible dyes, fluorescent dyes generally may be used under physiological conditions and allow staining of living cells and tissues. There is comparatively little overlap between emission wavelengths of many fluorophores, allowing staining with two or more fluorescent probes on one sample. However, this would require that the excitation wavelengths be very similar and the emission wavelengths be considerably different. Tandem dyes such as Quantum Red™, which is a conjugate of two dyes in which the emission wavelength of one dye matches the excitation wavelength of another, can supply an additional color using the same lamp. Some fluorescent dyes stain cell structures directly, such as acridine orange, DAPI, and Hoechst 33258, which stain nucleic acids. Others, such as fluorescein, rhodamine, and phycocerythrin, are conjugated to antibodies, lectins, nucleotides or other biological probes for localization of specific cell or tissue targets.

Fluorochrome conjugates should be protected from light during storage and use.

References

Biotin Conjugates
Biotin (Vitamin H) is a cofactor that binds with high affinity (Ka = 1015) to avidin at a ratio of 4:1. The strength of the binding results in an essentially irreversible interaction. This interaction has been exploited for immunolabeling of antigens in histochemical, blotting, and multwell assays. Biotinylated antibody probes bind to targets on
tissue samples, microtiter plates, or membranes. Avidin, conjugated to enzyme, fluorochrome, or colloidal metal binds to multiple sites on the biotinylated probes. Thus the avidin amplifies the signal, resulting in greater sensitivity than that achieved with an antibody-enzyme or antibody-fluorochrome conjugate alone. Visualization may be accomplished by detection of fluorescence, by the colorimetric or chemiluminescent end product of substrate conversion by the attached enzyme, or by microscopic examination. The avidin-biotin system has been used in immunohistology and immunocytology, immunoblotting, and ELISA. Some tissues, such as liver and kidney, contain endogenous biotin which can lead to high background staining when using the biotin-avidin system.

References

Avidin, ExtrAvidin and Streptavidin Reagents
Avidin has been reported to exhibit non-specific binding to membranes and tissues. For applications where nonspecific binding of avidin is a problem, Aladdin offers Streptavidin and ExtrAvidin. Avidin, ExtrAvidin and Streptavidin are available unconjugated or conjugated to enzymes, fluorochromes and colloidal gold for use in immunoassays. Avidin is also available immobilized on agarose for immunoprecipitation or affinity purification procedures.

Avidin
Avidin is a 65 kDa protein found in egg whites. It consists of 4 identical subunits, each with a high-affinity binding site for biotin (Vitamin H). The strength of the binding (Ka = 1015) results in an essentially irreversible interaction. This interaction has been exploited for immunolabeling of antigens in histochemical, blotting, and multwell assays. Biotinylated probes, which may be secondary antibodies, lectins, or other bioactive compounds, bind to targets on tissue samples, microtiter plates, or membranes. Avidin conjugated to an enzyme, fluorochrome, or other label binds to the biotinylated probes for visualization, either by detection of fluorescence or enzymatic conversion of substrate to produce a visible end product. In this system avidin serves as a secondary probe, attaching to several sites on the primary biotinylated probe and amplifying the signal. Use of an avidin-enzyme conjugate provides further amplification by conversion of substrate by the enzyme, which will continue to produce a visible product until the substrate is exhausted or the reaction is stopped.

ExtrAvidin
ExtrAvidin is a modified form of egg white avidin that retains the high affinity and specificity of avidin for biotin, but does not exhibit the nonspecific binding at physiological pH that has been reported for avidin.

Streptavidin
Streptavidin is a form of avidin produced by Streptomyces avidinii that exhibits somewhat less non-specific binding than egg white avidin, although background staining may still sometimes be a problem. Streptavidin is a homotetrameric protein of approximately 60 kDa composed of four identical subunits of approximately 15 kDa each. One molecule of streptavidin binds four molecules of biotin by a non-covalent interaction that is essentially irreversible.

References

For more information and a complete list of the related products, please click: Aladdin