Overview

Often it is useful to study or make use of the activity of one portion of an immunoglobulin without interference from other portions of the molecule. It is possible to selectively cleave the immunoglobulin molecule into fragments that have discrete characteristics. Antibody fragmentation is accomplished using proteases that digest or cleave certain portions of the immunoglobulin protein structure. Although fragmentation of all immunoglobulin classes is possible, only procedures for fragmentation of mouse, rabbit and human IgG and IgM have been well-characterized.

Antibody Fragmentation

The two groups of antibody fragments of primary interest are antigen-binding fragments such as Fab and nonantigen-binding, class-defining fragments such as Fc. More than one type of antigen-binding fragment is possible, but each contains at least the variable regions of both heavy and light immunoglobulin chains (VH and VL, respectively) held together (usually by disulfide bridges) so as to preserve the antibody-binding site. Fc fragments consist of the heavy chain constant region (Fc region) of an immunoglobulin and mediate cellular effector functions.

Antibody fragmentation is somewhat laborious, requires optimization of enzyme-mediated digestion of the protein and necessitates an ample supply (e.g., 10 mg) of antibody to make it reasonably efficient. For these reasons, fragmentation is usually performed only when the antibody of interest is available in large quantity and the particular application demands it.

Advantages of Antibody Fragments

Antibody fragments offer several advantages over intact antibody as reagents in an immunochemical technique:

• Using antigen-binding regions that have been separated from the Fc region reduces nonspecific binding that results from Fc interactions (many cells have receptors for binding to the Fc portion of antibodies).
• Small antigen-binding fragments generally provide higher sensitivity in antigen detection for solid-phase applications as a result of reduced steric hindrance from large protein epitopes.
• Because they are smaller, antibody fragments more readily penetrate tissue sections, resulting in improved staining for immunohistochemical applications.
• Antibody fragments are the best choice for antigen-antibody binding studies in the absence of Fc-associated effector functions (e.g., complement fixation, cell membrane receptor interaction).

Types of Antibody Fragments

F(ab’)2, Fab, Fab’ and Fv are antigen-binding fragments that can be generated from the variable region of IgG and IgM. These antigen-binding fragments vary in size (MW), valency and Fc content. Fc fragments are generated entirely from the heavy chain constant region of an immunoglobulin. The structures of these antibody fragments are illustrated in schematic form in Figure 19 and summarized below. In addition, several unique fragment structures can be generated from pentameric IgMs, including an “IgG”-type fragment, an inverted “IgG”-type fragment and a pentameric Fc fragment. IgM fragmentation is discussed in detail on pages 48-49.

F(ab’)2

F(ab’)2 (110,000 dalton IgG fragment, 150,000 dalton IgM fragment) fragments contain two antigen-binding regions joined at the hinge through disulfides. This fragment is void of most, but not all, of the Fc region.

Fab’

Fab’ (55,000 dalton IgG, 75,000 dalton IgM) fragments can be formed by the reduction of F(ab’)2 fragments. The Fab’ fragment contains a free sulfhydryl group that may be alkylated or utilized in conjugation with an enzyme, toxin or other protein of interest. Fab’ is derived from F(ab’)2; therefore, it may contain a small portion of Fc.

Fab

Fab (50,000 daltons) is a monovalent fragment that is produced from IgG and IgM, consisting of the VH, CH1 and VL, CL regions, linked by an intrachain disulfide bond.
Antibody Fragmentation

Fv

Fv (25,000 daltons) is the smallest fragment produced from IgG and IgM that contains a complete antigen-binding site. Fv fragments have the same binding properties and similar three-dimensional binding characteristics as Fab. The \( \gamma \) and \( \delta \) chains of the Fv fragments are held together by noncovalent interactions. These chains tend to dissociate upon dilution, so methods have been developed to cross-link the chains through glutaraldehyde, intermolecular disulfides or a peptide linker.

"r IgG"

"r IgG" (80,000 daltons) is a reduced form of IgG composed of one complete light chain and one complete heavy chain. It is essentially one-half of an intact IgG molecule and it contains a single antigen-binding site. "r IgG" fragments are formed by the selective reduction of disulfide bonds in the hinge region of an antibody.

Fc

Fc (50,000 daltons) fragments contain the C\(_2\) and C\(_3\) region and part of the hinge region held together by one or more disulfides and noncovalent interactions (Figure 19). Fc and Fc5\(_\mu\) fragments are produced from fragmentation of IgG and IgM, respectively. The term Fc is derived from the ability of these antibody fragments to crystallize. Fc fragments are generated entirely from the heavy-chain constant region of an immunoglobulin. The Fc fragment cannot bind antigen, but it is responsible for the effector functions of antibodies, such as complement fixation.

F(ab\(^{\prime}\))\(_2\), Fab\(^{\prime}\), Fab and Fv fragments produced from IgM function in much the same way as F(ab\(^{\prime}\))\(_2\), Fab\(^{\prime}\), Fab and Fv fragments from IgG. However, compared to those in IgG, individual antigen-binding sites in IgM generally have lower binding affinities, which are compensated in the complete IgM by its pentameric form. The increased binding valency of F(ab\(^{\prime}\))\(_2\) may make it preferable to Fv and Fab fragments.

F(ab\(^{\prime}\))\(_2\) fragments are divalent, and they may be a superior alternative to Fab fragments for antibodies with low affinity. The F(ab\(^{\prime}\))\(_2\) fragments have higher avidity than the Fab and Fab\(^{\prime}\) fragments. F(ab\(^{\prime}\))\(_2\) fragments can precipitate antigen. Fab and Fab\(^{\prime}\) are univalent molecules that cannot precipitate antigen. Fab and Fab\(^{\prime}\) fragments have a decreased binding strength, and normally stable antigen-antibody complexes may dissociate during washes in certain applications.

Cloning of IgG

The hinge region of an immunoglobulin monomer (IgG) is readily accessible to proteolytic attack by enzymes. Cleavage at this point produces F(ab\(^{\prime}\))\(_2\) or Fab fragments and the Fc fragment. The Fc fragment may remain intact or become further degraded, depending upon the enzyme and conditions used. Proteolytic IgG fragmentation using three different enzymes is discussed below and summarized in Figure 20. Traditionally, proteolysis was accomplished in solution using free enzyme. Pierce has developed immobilized enzyme products that enable better control of the digestion and separation of reaction products from the enzyme.

Immobilized Papain

Papain is a nonspecific, thiol-endopeptidase that has a sulfhydryl group in the active site, which must be in the reduced form for activity. When IgG molecules are incubated with papain in the presence of a reducing agent, one or more peptide bonds in the hinge region are split, producing three fragments of similar size: two Fab fragments and one Fc fragment. When Fc fragments are of interest, papain is the enzyme of choice because it yields a 50,000 dalton Fc fragment.

Papain is primarily used to generate Fab fragments, but it also can be used to generate F(ab\(^{\prime}\))\(_2\) fragments. To prepare F(ab\(^{\prime}\))\(_2\) fragments, the papain is first activated with 10 mM cysteine. The excess cysteine is then removed by gel filtration. If no cysteine is present during papain digestion, F(ab\(^{\prime}\))\(_2\) fragments can be generated. These fragments are often inconsistent, and reproducibility can be a problem. If the cysteine is not completely removed, overdigestion can be a problem.
Crystalline papain is often used for the digestion of IgG; however, it is prone to autodigestion. Mercuripapain, which is less prone to autodigestion than crystalline papain, can be used; however, both of these non-immobilized enzymes require an oxidant to terminate digestion. Immobilized papain is the preferred reagent because it allows for easy control of the digestion reaction, as well as separation of enzyme from the crude digest. There is no need to develop an ion exchange method for separating the fragments from the enzyme. The use of immobilized papain will also prevent formation of antibody-enzyme adducts, which can occur when using the soluble form of sulfhydryl proteases (such as papain). These adducts can be detrimental to fragments in the presence of reductants.

Immobilization also increases stability of the enzyme against heat denaturation and autolysis and results in longer maintenance of activity. Regeneration of the papain is often possible after immobilization, resulting in decreased costs. Cleavage can be regulated by digestion time or flow rate through a column, yielding reproducible digests. Pierce Immobilized Papain (Product # 20341) offers all the advantages of immobilized enzyme supports (Figure 21). Pierce ImmunoPure® Fab Preparation Kit (Product # 44885) has been optimized for human IgG digestions. It also has been used successfully for mouse and rabbit digestions, and suggestions on how to vary the protocols for other species’ IgG are provided with the kit. The procedure required that the IgG is able to be bound by Protein A, which is used to separate Fc from Fab fragments.

**Figure 20. Use of papain, pepsin and ficin for IgG fragmentation.**

**Figure 21. Preparation and isolation of Fab and Fc fragments with Immobilized Papain.**

**Preparation and Isolation of Fab and Fc Fragments**

1. Equilibration of Immobilized Papain
   a) Add 0.5 ml of Immobilized Papain slurry + 4 ml of digestion buffer to the tube as in A.
   b) Mix the tube contents as in B.
   c) Compress the gel as in C and discard the buffer.
   d) Repeat steps a, b and c.
   e) Resuspend the gel in 0.5 ml buffer.

2. Addition of IgG sample to Immobilized Papain
   Add IgG in 1.0 ml digestion buffer to tube containing Immobilized Papain.

3. Digestion of IgG
   Incubate in a shaker water bath at 37°C for the appropriate amount of time.

4. Separation of crude digest
   Compress gel with separator and pour crude digest into clean tube.

5. Washing of Immobilized Papain
   a) Add 1.5 ml of Binding Buffer to gel as in A. Mix as in B.
   b) Add wash to rest of crude digest.

6. Addition of crude digest to Immobilized Protein A column
   Add crude digest to an equilibrated Immobilized Protein A Column. Collect column eluate.

7. Collection of Fab fragments
   a) Add 6 ml ImmunoPure® IgG Binding Buffer to Protein A column and collect eluate. Dialyze against PBS, pH 7.4 overnight.
   b) Wash column with additional 3 ml binding buffer. Discard wash.

8. Collection of Fc and undigested IgG
   a) Transfer Protein A column to another tube.
   b) Elute Fc and undigested IgG from column with 6 ml ImmunoPure® IgG Elution Buffer.
**Immunoblot Pepsin**

Pepsin is a nonspecific endopeptidase that is active only at acid pH. It is irreversibly denatured at neutral or alkaline pH. Digestion by the enzyme pepsin normally produces one Fab'2 fragment and numerous small peptides of the Fc portion (Figure 20). The resulting Fab'2 fragment is composed of two disulfide-connected Fab units. The Fab fragment is extensively degraded, and its small fragments can be separated from Fab by dialysis, gel filtration or ion exchange chromatography.

Fab'2 can be separated by mild reduction into two sulphydryl-containing, univalent Fab fragments. The advantage of Fab' fragments is that they can be conjugated to detectable labels directly through their sulphydryl groups, ensuring that the active binding site remain unhindered and active. Pierce offers 2-Mercaptoethanol-HCl (2-MEA, Product # 20408) for mild reduction of Fab'2 fragments. For alternative labeling protocols, the free sulphydryl may be blocked with an alkylating reagent, such as N-Ethylmaleimide (NEM, Product # 23030).

Immunoblot Pepsin (Product # 20343) can be substituted for free pepsin in any application. Immunoblot pepsin is advantageous because of its ability to immediately stop the digestion process, yielding reproducible digests. Immunoblotting of the enzyme allows for easy separation of the enzyme from the crude digest, eliminating the need to develop an ion exchange method for separating the fragments from the enzyme. Also, immobilization increases the stability of the pepsin against heat denaturation and autolysis, resulting in longer maintenance of activity. ImmunoPure® Fab'2 Preparation Kit (Product # 44888) has been optimized for human IgG digestions. The kit also has been used successfully for rabbit and mouse IgG digestions. Suggestions on how to vary protocols for other species' IgG (IgG must bind to Protein A) are provided.

**Immunoblot Ficin**

Ficin is a thiol protease that can digest mouse monoclonal IgG into either Fab'2 or Fab fragments, depending on the concentration of cysteine used. Ficin will generate Fab'2 in the presence of 1 mM cysteine. Fab fragments will be generated with ficin in the presence of 10 mM cysteine (Figure 20).

Ficin cleavage produces Fab'2, fragments of nearly identical size to those obtained from IgG by pepsin but with immunoreactivities and affinities comparable to those of intact IgG antibody. By increasing the concentration of cysteine activator, Fab antigen-binding fragments can be generated. The integrity of the resultant antigen-binding fragments is aided by the neutral pH conditions of the ficin digestion. The difficulties of using pepsin in this application makes ficin digestion the preferred method for producing Fab'2 fragments from murine monoclonal IgG. Although Fab'2 fragments have been generated from an IgG antibody using preactivated pepsin, stable, consistent product by pepsin is often difficult to obtain.

Immobilized Ficin (Product # 44881) enables better control of the digestion reaction than free ficin, resulting in antibody fragments that are free of autodigestion products (Figure 22). In addition, the use of Immobilized Ficin eliminates the incorporation of ficin into antibody fragments. The ImmunoPure® IgG, Fab and Fab'2 Preparation Kit (Product # 44880) was developed to allow gentle production and purification of both Fab and Fab'2 fragments from intact murine IgG antibodies. Immobilized Ficin can be used repeatedly to cleave an IgG subclass antibody, yielding either Fab or Fab'2 fragments. The type of fragment produced is controlled by the specific concentration of cysteine activator used during the digestion.

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Figure 22. Selective preparation of monovalent or bivalent antigen-binding fragments from IgG. Figure 22A. Digestion products of mouse anti-avidin IgG monoclonal on immobilized ficin. 5 hours, 37°C, 10 mM cysteine. Ficin: MAB = 11:1 (mole:mole). Figure 22B. Size exclusion profile of fraction not bound to Protein A column. Component composition: 4% Fab', 96% Fab. Total Recovery: 47% Fab. Figure 22C. Digestion products of mouse anti-avidin IgG monoclonal on immobilized ficin. 20 hours, 37°C, 1 mM cysteine. Ficin: MAB = 11:1 (mole:mole). Figure 22D. Size exclusion profile of fraction not bound to Protein A column. Component composition: 98% Fab'2, 2% Fab. Total Recovery: 95% Fab'2.

References
Table 10. Enzymes used for antibody digestion.

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<tr>
<th>Enzyme</th>
<th>Molecular Weight</th>
<th>Ext. Coefficient A280 of 1%</th>
<th>Type</th>
<th>Specificity</th>
<th>pH Optimum (Enhancers)</th>
<th>Activators</th>
<th>Imm. Enzyme pl</th>
<th>pH &gt; 6, epoxides</th>
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<td>Pepsin</td>
<td>35,000</td>
<td>14.7</td>
<td>Acid</td>
<td>Broad – prefer</td>
<td>1 (1-5)</td>
<td>cysteine, sulfide, sulfi</td>
<td>heavy metals, carbonyl, N-ethyl maleimide (NEM), p-chloromercuro-benzoate</td>
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<td></td>
<td>Pre, Met Leu, Tryp bonds</td>
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<td>Papain</td>
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<td>25</td>
<td>Thiol</td>
<td>Broad – prefer</td>
<td>6.5 (4-9.5)</td>
<td>cysteine, sulfide, sulfi</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arg, Lys, His, Gly, Tyr bonds</td>
<td></td>
<td>sulfite, cyanide, (EDTA) (NBS) (acridine dye)</td>
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<tr>
<td>Ficin</td>
<td>26,000</td>
<td>21</td>
<td>Thiol</td>
<td>Uncharged or aromatic amino acids</td>
<td>6.5 (4-9.5)</td>
<td>cysteine, sulfide, sulfi</td>
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<td>Trypsin</td>
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<td>14.3</td>
<td>Serine</td>
<td>Arg, Lys</td>
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<td>Ca2+ acts as a stabilizer</td>
<td>organophosphorous compounds, DFP*, benzimidine</td>
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*NBS = N-bromosuccinimide **DFP = diisopropyl fluorophosphate

Immunopure® Fab Preparation Kit

The easiest, most convenient way to generate Fab fragments from IgG.

Pierce has developed a complete kit to digest human or mouse IgG molecules into Fab fragments and Fc fragments by using immobilized papain. After digestion, the fragments are purified on an immobilized Protein A column provided in the kit. Detailed instructions allow for flexibility in the protocol for hard-to-digest antibodies.

**Highlights of Fab Fragments:**
- Will not be affected by Fc receptors on cells such as macrophages, B cells, T cells, neutrophils and mast cells
- Will not precipitate antigen
- Easier to make and purify than F(ab')2 fragments
- More rapid clearance of radiolabeled fragments from normal tissue than whole IgG conjugates
- Reduced immunogenicity (as a result of Fc region absence), minimizes human anti-mouse immunoglobulin (HAMA) response
- Fragments are less susceptible to phagocytosis

**Antibody Fragment Applications:**
- Immunohistochemistry
- Immunoassays, including in vitro diagnostic assays
- Radioimmunoassay for detection and radiotherapy
- Immunostaining through the use of fragment conjugates as immunotoxins
- Crystallographic study of antibody-binding sites
- Study of Fc-binding proteins and effector functions

**Highlights of Fc Fragments:**
- Useful for studying effector functions of IgG without interference from antigen-binding sites
- Fc fragments can be used as blocking agents for histochemical staining

**Ordering Information**

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Problems with digesting mouse monoclonal IgG1 can now be overcome by using immobilized Ficin. Ficin cleavage produces F(ab′)2 fragments of nearly identical size to those obtained from IgG by pepsin, but with immunoreactivities and affinities comparable to those of the intact IgG1 antibody. Similarly, by increasing the concentration of cysteine activator in the digestion buffer, Fab fragments can be created from the original IgG.

Highlights:
• Can generate both Fab and F(ab′)2 fragments from mouse IgG1
• Reaction can be easily controlled
• Antibody fragments are free of autodigestion products
• Ficin contamination into antibody fragments is eliminated

References
Fragmentation of IgM

IgM is an extremely large molecule that has a tendency to interact with other molecules and matrices besides the antigen. The large size of IgM creates difficulties in applications in which IgM is used for in vitro experiments. Intact IgM does not effectively penetrate tissues for immunohistochemical studies; it is necessary to produce smaller, active fragments for these studies. Also, because IgM molecules have difficulty permeating cell membranes, they are not ideal for use in vivo. Fragments are cleared more rapidly than intact IgM.

Each species of IgM reacts differently to enzymatic cleavage and reduction. For example, the relative structure of mouse and human IgM differ in the manner in which the monomers are linked to give the pentameric form, primarily as a result of differences in the location of disulfides between the monomers. Oligosaccharides components, which may hinder enzymatic cleavage, also vary between species. Therefore, optimal digestion and reduction conditions for one species may prove ineffective for another.

Fragmentation of IgM by proteolytic enzymes proceeds differently from IgG fragmentation. These changes are related to differences in structure. The heavy (µ) chains are folded into multiple globular domains, and IgM has an actual domain (Ç2) in place of a hinge region. Ç2 lacks the proline-rich sequence that is found in the hinge region of IgG. This proline sequence makes the hinge more susceptible to cleavage. Also, IgM has a large carbohydrate portion in the Ç2, which may sterically interfere with the action of proteolytic enzymes.

Enzymes and Reagents for IgM Fragmentation

Papain is a nonspecific, thiol-endopeptidase that has a sulfhydryl group in the active site, which must be in the reduced form for activity. Papain has been shown to produce heterogeneous fragments from IgM. Oligosaccharides in the hinge region of IgM interfere with papain digestion, causing a cleavage shift of 3-5 amino acids in either direction.

Pepsin is a nonspecific endopeptidase that is active only at an acid pH, and it is irreversibly denatured at neutral or alkaline pH. It is possible to produce Fab(')2, F(ab')2 and Fv fragments using pepsin to digest IgM (Figure 23). Many methods have been developed that use pepsin to produce different IgM fragments from different species.

Trypsin is a serine protease that reacts optimally at pH 8.0. In general, increasing the enzyme/substrate ratio and/or temperature will increase the rate of digestion. Trypsin can generate Fab(')2, Fab, IgG-type and Fc5µ fragments from IgM (Figure 23). Trypsin digestion of several species of IgM was studied using trypsin with and without urea pretreatment. Urea alters the susceptibility of the domains to digestion and produces different fragments than those digested in aqueous buffer. Many other procedures have been developed to digest IgM using trypsin.

Mild reduction can be achieved using 2-Mercaptoethanolamine-HCl (Product # 20408). Reduction will vary among IgM species, but an IgG-type and/or reduced IgG ("rIgG") should be formed in varied proportions, depending upon reduction time and/or temperature (Figure 23). Fragmentation of mouse IgM also produces an inverted IgG-type fragment.
IgM Fragmentation Kit

Pierce has developed the ImmunoPure® IgM Fragmentation Kit (Product # 44887) to allow quick, easy production of fragments from mouse and human IgM. This kit can be used for species other than human and mouse; however, the protocols have not been optimized for all species. The kit contains everything needed to produce IgM fragments using trypsin, pepsin and 2-MEA protocols. The trypsin and pepsin are supplied in immobilized forms, eliminating the need to separate enzyme from the IgM fragments. The IgM is digested as it passes through the prepacked immobilized enzyme columns. The enzyme remains bound to the support matrix, ensuring there is no autodigested enzyme to contaminate the IgM fragments. Also, the immobilized enzymes offer increased stability against heat denaturation and autolysis. Regeneration of the immobilized enzymes makes the process more cost-efficient. The cleavage of IgM is easily regulated by adjustment of incubation times. Sample concentrators are included in the kit for easy fragment separation and concentration. Complete instructions and protocols are also included.

References

The large size of IgM creates difficulties in applications in which IgM is used for in vitro experiments. Intact IgM does not effectively penetrate tissues for immunohistochemical studies, therefore it is necessary to produce smaller, active fragments for in vitro or in vivo studies. Pierce created a kit combining immobilized trypsin and pepsin to easily generate a variety of IgM fragments (Figure 23).

Highlights:
- Immobilized trypsin can generate F(ab')2, Fab, “IgG”-type and Fc(5µ) fragments from IgM
- Immobilized pepsin can produce F(ab')2, Fab and Fv fragments from IgM
- Complete kit, including detailed instructions, to digest and purify IgM fragments
- Immobilized enzymes prevent enzyme contaminants in final fragment preparation

Immunopure® IgM Fragmentation Kit

Makes IgM fragmentation easy!

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Antibody Modification Sites

Understanding the functional groups available on an antibody is the key to choosing the proper method for modification.

For example:

**Primary amines** (–NH\(_2\)) are found on lysine residues and the N-terminus. These are abundant and distributed over the entire antibody.

**Sulfhydryl groups** (–SH) are found on cysteine residues and are formed by selectively reducing disulfide bonds in the hinge region of the antibody.

**Carbohydrate residues** containing cis-diols can be oxidized (–CHO) to create active aldehydes for coupling. These are localized to the Fc region on antibodies and are more abundant on polyclonal antibodies.

Most antibody labeling strategies use one of three targets (Figure 24). The most common target for antibody labeling is primary amines, which are found primarily on lysine residues. They are abundant, widely distributed and easily modified due to their reactivity and their location on the surface of the antibody. The lone drawback to this labeling strategy is that it often results in a significant decrease in the antigen-binding activity of the antibody. The decrease may be particularly pronounced when working with monoclonal antibodies or when multiple labels are attached to the antibody.

The second common target for labeling antibodies is carbohydrate moieties because antibodies are often significantly glycosylated. Because the glycosylation sites are predominantly found on the Fc portion of the antibody, they can often be modified without significantly reducing the antigen-binding capacity. Labeling carbohydrates requires more steps than labeling amines because the carbohydrates must first be oxidized to create reactive aldehydes; however, it generally results in antibody conjugates with high activity.

The third common target is sulfhydryls that exist in proteins under reducing conditions, but more often are found in oxidized form as disulfide bonds. Disulfide bonds are important contributors to antibody structure as they participate in the tertiary structure of each subunit, covalently connecting the heavy and light chains and connecting the two halves of an antibody in the hinge region. These disulfides in the hinge region are the primary target for sulfhydryl labeling of an antibody because they are easily reduced to sulfhydryls, splitting the antibody into monovalent halves (rIgG) without damaging the antigen-binding sites. Like carbohydrates, they require more steps to label yet they result in high-activity conjugates.

Antibodies, like other proteins, can be covalently modified in many ways to suit the purpose of a particular assay. Many immunological methods involve the use of labeled antibodies, and a variety of reagents have been created to allow labeling of antibodies. Enzymes, biotin, fluorophores and radioactive isotopes are all commonly used to provide a detection signal in biological assays. Covalently attaching such a label to an antibody combines the unique specificity of the antibody with a sensitive means for detection, thus creating an ideal probe molecule. Aside from antibodies, these same labels can be attached to avidin, streptavidin, Fc-binding proteins such as Protein A or G, and many other proteins.

Figure 24. Antibody labeling sites.
Enzyme Labeling

Enzymes offer the advantages of long shelf life, high sensitivity and the possibility of direct visualization. The disadvantages of enzymes as labels include multiple assay steps, some hazardous substrates and the possibility of interference from endogenous enzymes. Enzymes also may give poor resolution in cytochemistry. The use of enzyme labels is recommended for immunohistochemistry, immunoblots and quantitative and qualitative immunoassays.

Using enzymes as labels offers several advantages over fluorescently labeled and radiolabeled substances. Enzyme immunoassay reagents are more stable and do not have the dangers associated with radioisotopic labels. In addition, enzyme assays can be at least as sensitive as radioimmunoassays. Many enzyme detection methods are visual or use a standard spectrophotometer, eliminating the need for expensive, sophisticated equipment.

When detecting cellular antigens to tissue structures, enzyme labels also have advantages. Antibodies (and especially their fragments) conjugated to small enzymes can readily cross cell membranes. It is then possible to localize and observe cellular antigens to tissue structures using light microscopy. In addition, tissue sections that have been developed with the appropriate substrate can be stable for years. This is in stark contrast to most immunofluorescent staining techniques, in which the fluorescent signal rapidly decreases upon exposure to light.

Selection of an enzyme and the appropriate substrate solution are dependent on the application involved (Table 11). Enzyme-labeled antibodies perform well in immunoblotting, histochemical staining and ELISA. They can provide an instant visual result and good sensitivity; however, the rate of the enzyme reaction must be measured to obtain an accurate indication of the amount of bound enzyme, making them difficult to use in quantitative assays. In addition, enzyme-labeled reagents are not homogeneous. In immunohistochemistry, nonspecific staining can present a problem when using enzymes; additional problems include endogenous peroxidase or phosphatase activity. In these cases, a different enzyme may need to be selected. Table 11 compares peroxidase and alkaline phosphatase uses, along with their respective advantages, disadvantages and recommended applications.

Table 11. Comparison of horseradish peroxidase and alkaline phosphatase uses.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Substrate Signal</th>
<th>Recommended Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horseradish Peroxidase</td>
<td>Good substrates for Western blotting, ELISA, immunohistochemistry and 1:1 conjugates, Fast signal generation</td>
<td>High endogenous levels in blood cells</td>
<td>Soluble-TMB, ABTS, OPD</td>
<td>ELISA</td>
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<td></td>
<td></td>
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<td>QantaBlue™ Fluorogenic Substrate</td>
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<td>SuperSignal® ELISA Substrate</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Insoluble-Chloronaphthol, DAB/cobalt, TMB-blotting, SuperSignal® West Substrate</td>
<td>Western blotting Immunohistochemistry</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>Good substrates for Western blotting, ELISA and immunohistochemistry</td>
<td>Endogenous activities – some in tissues, Large conjugates</td>
<td>Soluble-PNPP</td>
<td>ELISA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Insoluble-Net/BCIP, Fast Red TRVAM-MX</td>
<td>Western blotting</td>
</tr>
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</table>
Alkaline phosphatase, a 140 kDa protein that is generally isolated from calf intestine, catalyzes the hydrolysis of phosphate groups from a substrate molecule, resulting in a colored or fluorescent product or the release of light as a byproduct. AP has optimal enzymatic activity at a basic pH (pH 8-10) and can be inhibited by cyanides, arsenate, inorganic phosphate and divalent cation chelators, such as EDTA. As a label for Western blotting, AP offers a distinct advantage over other enzymes. Because its reaction rate remains linear, detection sensitivity can be improved by simply allowing a reaction to proceed for a longer time period.

Horseradish peroxidase is a 40 kDa protein that catalyzes the oxidation of substrates by hydrogen peroxide, resulting in a colored or fluorescent product or the release of light as a byproduct. HRP functions optimally at a near-neutral pH and can be inhibited by cyanides, sulfides and azides. Antibody-HRP conjugates are superior to antibody-AP conjugates with respect to the specific activities of both the enzyme and antibody. In addition, its high turnover rate, good stability, low cost and wide availability of substrates make HRP the enzyme of choice for most applications.

<table>
<thead>
<tr>
<th>Table 12. Comparison of horseradish peroxidase and alkaline phosphatase physical properties.</th>
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<tbody>
<tr>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>Size</td>
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<tr>
<td>Price</td>
</tr>
<tr>
<td>Stability (Storage)</td>
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<tr>
<td>Number of Substrates</td>
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<tr>
<td>Kinetics</td>
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<tr>
<td>pH optimum</td>
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Maleimide activation

The heterobifunctional cross-linker SMCC (Product # 22360) and its water-soluble analog Sulfo-SMCC (Product # 22322) have more general utility in preparing immunologically active HRP or AP conjugates. They are most useful when preparing conjugates of reduced IgG and (Ab′), because these methods involve the initial step of preparing a maleimide-activated (sulfhydryl-reactive) enzyme derivative. Studies have shown that the two-step maleimide method is superior to glutaraldehyde or metaperiodate methods for enzyme conjugation (Figure 25). The maleimide method gives higher yields with less polymerization, producing a conjugate preparation with superior immunoassay characteristics.

Maleimide-activated enzymes can be prepared using the heterobifunctional cross-linker Sulfo-SMCC. This reagent contains an N-hydroxy-sulfosuccinimide (Sulfo-NHS) functional group and a maleimide functional group and it is water-soluble due to the presence of the sulfonate (–SO3-) group on the N-hydroxysuccinimide ring. The sulfonate group also contributes to the stability of the molecule in aqueous solution. A study of the hydrolysis rate of the maleimide functional group from Sulfo-SMCC showed that it is less prone to hydrolysis to the maleamic acid than the non-sulfonated SMCC. The maleimide groups of Sulfo-SMCC exhibit no decomposition at pH 7 at 30°C within 6 hours. The Sulfo-NHS ester group reacts with primary amines on the enzyme surface to form a stable amide bond. After this first step of conjugation, the enzyme will have maleimide groups on its surface that react optimally toward sulfhydryl groups between pH 6.5 and 7.5 to form stable thioether bonds. Maleimide-mediated conjugation strategies are summarized in Figure 25.
Three methods for free sulfhydryl generation

1. Native protein has a free sulfhydryl on its surface.

2. MEA

3. Native protein is reacted with SATA. Blocked sulfhydryl groups are introduced on primary amines. Hydroxylamine (H) treatment generates free sulfhydryls.

Native protein contains disulfide bonds that can be reduced to generate free sulfhydryls.

Enzyme is SMCC-labeled through primary amines to generate a maleimide-activated enzyme for conjugation to free sulfhydryls.

Figure 25. Three strategies for maleimide-mediated conjugation of enzymes.

Horseradish Peroxidase

*Its high-specific enzyme activity makes it the enzyme of choice.*

Highlights:

- Superior to alkaline phosphatase and β-galactosidase conjugates due to the higher specific enzyme activity
- Small size (40 kDa) allows excellent cellular penetration
- Variety of substrates available
- Ideal in blotting and cytochemistry applications
- Used as the reporter enzyme for SuperSignal® Chemiluminescent Substrates*

References


Ordering Information

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* SuperSignal® Technology is protected by U.S. patent # 6,432,662.
Two reagents, Mercaptoethylamine•HCl (Product # 20408) and SATA (Product # 26102), are available to produce free sulfhydryls on macromolecules for conjugation to the maleimide-activated enzymes. For labeling antibody molecules, mild reduction with Mercaptoethylamine•HCl (MEA) results in two half-antibody fragments containing free sulfhydryl groups in the hinge region. Labeling in this area is advantageous because it directs the modification away from the antigen-binding region. Native proteins lacking a free sulfhydryl on their surface can be reacted with SATA to generate blocked sulfhydryl groups. The SATA molecule reacts with primary amines via its NHS ester end to form stable amide linkages. The acetylated sulfhydryl group (blocked) is stable until treated with hydroxylamine to generate the free sulfhydryls.

Pierce offers stable, preactivated enzyme derivatives that are reactive toward sulfhydryl (-SH) groups, EZ-Link® Maleimide Activated Alkaline Phosphatase (Product # 31486) and Horseradish Peroxidase (Product # 31485). These products eliminate the first step of the two-step maleimide method, simplifying and facilitating the conjugation protocol, while saving several hours. They can be used to prepare enzyme conjugates directly from proteins, peptides or other ligands that contain a free-SH group. Two reagents, SATA and mercaptoethylamine-HCl, are also included in the kit formats to produce free sulfhydryls on macromolecules for conjugation.

### EZ-Link® Maleimide Activated Alkaline Phosphatase and Horseradish Peroxidase

Make quick and easy enzyme conjugates.

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### EZ-Link® Maleimide Activated Peroxidase References


### References

Periodate
Glycoproteins such as HRP, glucose oxidase and most antibody molecules can be activated for conjugation by treatment with periodate. Oxidizing polysaccharide residues in a glycoprotein with sodium periodate provides a mild and efficient way of generating reactive aldehyde groups for subsequent conjugation with amine- or hydrazide-containing molecules via reductive amination (Figure 26). Some selectivity of monosaccharide oxidation may be accomplished by regulating the concentration of periodate in the reaction medium. In the presence of 1 mM sodium periodate, sialic acid groups are specifically oxidized at adjacent hydroxyl residues, clearing off two molecules of formaldehyde and leaving one aldehyde group. At higher concentrations of sodium periodate (10 mM or greater), other sugar residues will be oxidized at points where adjacent carbon atoms contain hydroxyl groups. This reaction should be performed in the dark to prevent periodate breakdown and for a limited period of time (15-30 minutes) to avoid loss of enzymatic activity.

Another method for conjugation uses glutaraldehyde, one of the oldest homobifunctional cross-linking reagents used for protein conjugation. It reacts with amine groups to create cross-links by one of several routes. Under reducing conditions, the aldehydes on both ends of glutaraldehyde will couple with amines to form secondary amine linkages. The reagent is highly efficient at protein conjugation but has a tendency to form various high-molecular weight polymers, making results difficult to reproduce.

Glutaraldehyde

Cross-linking with an amine-containing protein takes place under alkaline pH conditions through the formation of Schiff base intermediates. These relatively labile intermediates can be stabilized by reduction to a secondary amine linkage with sodium cyanoborohydride. Reductive amination has been done using sodium borohydride or sodium cyanoborohydride; however, cyanoborohydride is the better choice because it is more specific for reducing Schiff bases and will not reduce aldehydes. Small blocking agents such as lysine, glycine, ethanolamine or Tris can be added after conjugation to quench any unreacted aldehyde sites. Ethanolamine and Tris are the best choices for blocking agents because they contain hydrophilic hydroxyl groups with no charged functional groups.

The pH of the reductive amination reaction can be controlled to affect the efficiency of the cross-linking process and the size of the resultant antibody-enzyme complexes formed. At physiological pH, the initial Schiff base formation is less efficient and conjugates of lower molecular weight result. At more alkaline pH (i.e., pH 9-10), Schiff base formation occurs rapidly and with high efficiency, resulting in conjugates of higher molecular weight and greater incorporation of enzyme when oxidized enzyme is reacted in excess. Low molecular weight conjugates may be more optimal for immunohistochemical staining or blotting techniques in which penetration of the complex through membrane barriers is an important consideration. Washing steps also more effectively remove excess reagent if the conjugate is of low molecular weight, thus maintaining low background in an assay. By contrast, conjugates of high molecular weight are more appropriate for ELISA procedures in a microparticle format, where high sensitivity is important and washing off excess conjugate is not a problem.

EZ-Link® Activated Peroxidase References

EZ-Link® Plus Activated Peroxidase References

Ordering Information

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