Antibody Labeling

Overview

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.

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.

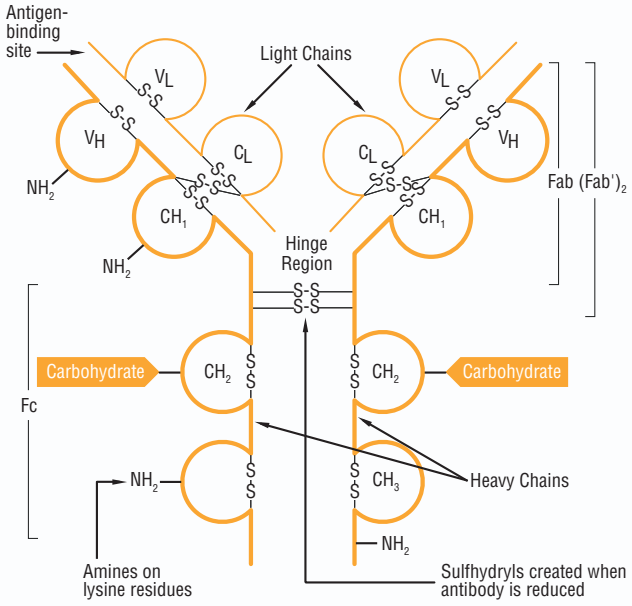

Figure 24. Antibody labeling sites.

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.

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, along with their respective advantages, disadvantages and recommended applications.

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

Enzyme	Advantages	Disadvantages	Substrate Signal	Recommended Applications
Horseradish Peroxidase	Good substrates for Western blotting, ELISA, immunohistochemistry and 1:1 conjugates Fast signal generation	High endogenous levels in blood cells	Soluble-TMB, ABTS, OPD QuantaBlu™ Fluorogenic Substrate SuperSignal® ELISA Substrate	ELISA
			Insoluble-Chloronaphthol, DAB/cobalt, TMB-blotting, SuperSignal® West Substrate	Western blotting Immunohistochemistry
Alkaline Phosphatase	Good substrates for Western blotting, ELISA and immunohistochemistry	Endogenous activities – some in tissues Large conjugates	Soluble-PNPP	ELISA
			Insoluble-NBT/BCIP, Fast Red TR/AS-MX	Western blotting

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 12. Comparison of horseradish peroxidase and alkaline phosphatase physical properties.

	Horseradish Peroxidase	Alkaline Phosphatase
Size	40 kDa	140 kDa
Price	Relatively Inexpensive	Relatively Expensive
Stability (Storage)	Stable at <0˚C	Unstable at <0˚C
Number of Substrates	Many	Few
Kinetics	Rapid	Slower
pH optimum	5-7	8-10

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 $F(ab')_2$ 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 ($-SO_3^-$) 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.

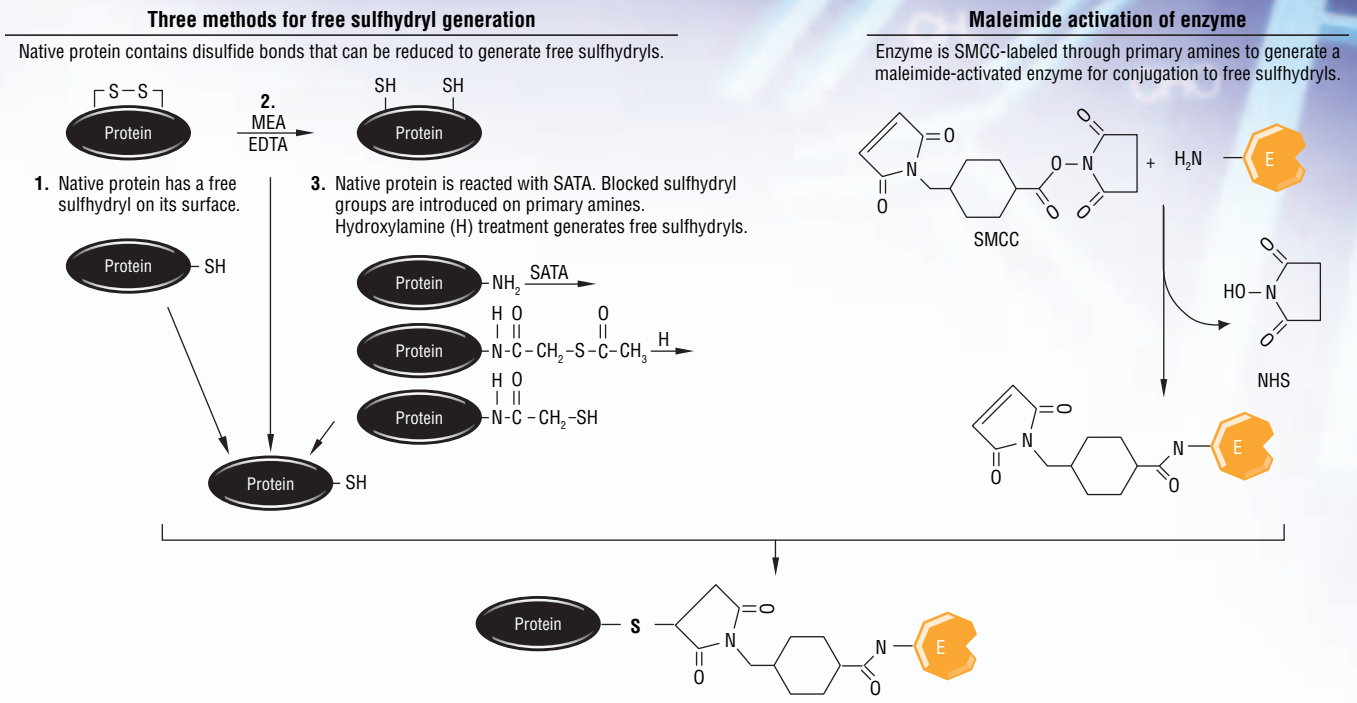

Figure 25. Three strategies for maleimide-mediated conjugation of enzymes.

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

Cordell, J.L., *et al.* (1984). *J. Histochem. Cytochem.* **32**, 219-229.

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Porstmann, B., *et al.* (1985). *J. Immunol. Methods* **79**, 27-37.

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Yolken, R.H. (1982). *Rev. Infect. Dis.* **4(1)**, 35-68.

Ordering Information

Product #	Description	Pkg. Size
31490	ImmunoPure® Horseradish Peroxidase	10 mg
31491	ImmunoPure® Horseradish Peroxidase	100 mg

** SuperSignal® Technology is protected by U.S. patent # 6,432,662.*

Alkaline Phosphatase

A highly sensitive enzyme for ELISA and immunohistochemical applications.

Highlights:

- Purified form – ready to conjugate without prior dialysis
- Activity is not affected by exposure to antibacterial agents, such as sodium azide or thimerosal
- Specific activity >2,000 units/mg
- One unit is defined as the amount that will hydrolyze 1.0 µmole of *p*-nitrophenyl phosphate per minute at 37°C in 1.0 M diethanolamine, 0.5 mM $MgCl_2$, pH 7.8

Specific Activity per mg Protein

Buffer	25°C	37°C
0.1 M Glycine, 1.0 mM $ZnCl_2$, 1.0 mM $MgCl_2$, 6.0 mM *p*-Nitrophenyl phosphate, pH 10.4	>500	>1,000
1.0 M Diethanolamine, 0.5 mM $MgCl_2$, 15 mM *p*-Nitrophenyl phosphate, pH 9.8	>1,000	>2,000

References

Bulman, A.S. and Heyderman, E. (1981). *J. Clin. Pathol.* **34**, 1349-1351.
Cordell, J.L., *et al.* (1984). *J. Histochem. Cytochem.* **32**, 219-229.
Yolken, R.H. (1982). *Rev. Infect. Dis.* **4**, 35-68.

Ordering Information

Product #	Description	Pkg. Size
31391	ImmunoPure® Alkaline Phosphatase Calf intestinal. Supplied in Tris Buffer, pH ~7 Triethanolamine, 1 mM $MgCl_2$, 3 M NaCl, pH 7.6	20 mg
31392	ImmunoPure® Alkaline Phosphatase	100 mg

EZ-Link® Maleimide Activated Alkaline Phosphatase and Horseradish Peroxidase

Make quick and easy enzyme conjugates.

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 Peroxidase References

Choi, J.Y., *et al.* (2002). *J. Biol. Chem.* **277**, 21630-21638.
Seo, Y.R., *et al.* (2002). *PNAS* **99**, 14548-14553.
Yoo, J.H., *et al.* (2004). *J. Biol. Chem.* **279**, 848-858.

Ordering Information

Product #	Description	Pkg. Size
31486	EZ-Link® Maleimide Activated Alkaline Phosphatase	2 mg
31493	EZ-Link® Maleimide Activated Alkaline Phosphatase Kit	Kit
	Includes: EZ-Link® Maleimide Activated Alkaline Phosphatase	2 mg
	Conjugation Buffer	20 ml
	BupH™ Tris Buffered Saline	2 packs
	BupH™ Phosphate Buffered Saline	1 pack
	Polyacrylamide Desalting Column	1 x 10 ml
	Mercaptoethylamine•HCl	6 mg
	SATA	2 mg
	Hydroxylamine	5 mg
	DMF	1 ml
	Column Extender	
31485	EZ-Link® Maleimide Activated Horseradish Peroxidase	5 mg
31494	EZ-Link® Maleimide Activated Horseradish Peroxidase Kit	Kit
	Includes: EZ-Link® Maleimide Activated Horseradish Peroxidase	5 mg
	Conjugation Buffer	20 ml
	2-Mercaptoethylamine•HCl	6 mg
	SATA	2 mg
	Dimethylformamide	1 ml
	Hydroxylamine•HCl	5 mg
	Polyacrylamide Desalting Column	1 x 10 ml

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, cleaving 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.

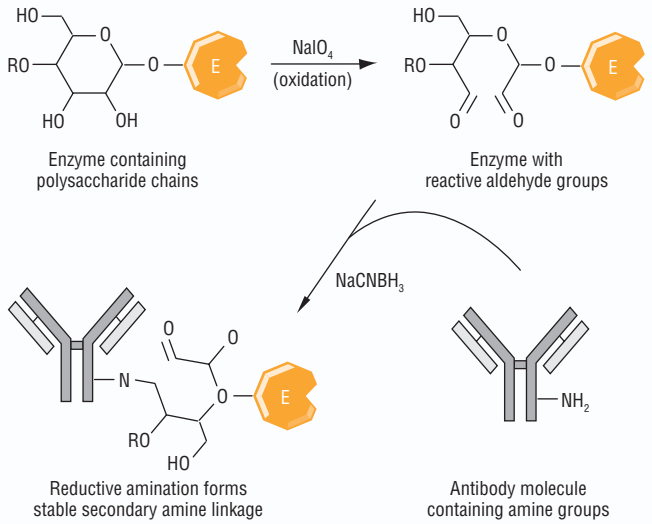

Figure 26. Conjugation scheme for periodate oxidation and subsequent reductive amination.

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 microplate format, where high sensitivity is important and washing off excess conjugate is not a problem.

Glutaraldehyde

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.

EZ-Link® Activated Peroxidase References

Sandt, C.H. and Hill, C.W. (2001). *Infect. Immun.* **69**, 7293-7303.

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EZ-Link® Plus Activated Peroxidase References

Glover, L., (2002). *Eur. J. Biochem.* **269**, 4607-4616.

Nawa, M., *et al.* (2000). *Clin. Diagn. Lab. Immunol.* **7**, 774-777.

Völkel, T., *et al.* (2001). *Protein Eng.* **14**, 815-823.

Ordering Information

Product #	Description	Pkg. Size
31487	**EZ-Link® Plus Activated Peroxidase** (Periodate Activated)	1 mg
31488	**EZ-Link® Plus Activated Peroxidase** (Periodate Activated)	5 x 1 mg
31489	**EZ-Link® Plus Activated Peroxidase Kit** (Periodate Activated) Includes: EZ-Link® Plus Activated Peroxidase Sodium Cyanoborohydride Solution Quenching Buffer BupH™ Phosphate Buffered Saline Pack BupH™ Carbonate Buffer Pack	Kit 5 x 1 mg 1 x 0.5 ml 25 ml 500 ml 500 ml
31496	**EZ-Link® Activated Peroxidase** (Glutaraldehyde Activated)	1 mg
31495	**EZ-Link® Activated Peroxidase** (Glutaraldehyde Activated)	5 mg
31497	**EZ-Link® Activated Peroxidase Antibody Labeling Kit** (Glutaraldehyde Activated) Includes: EZ-Link® Activated Peroxidase Conjugation Buffer Lysine Immobilized Protein A/G Column Gentle Ag/Ab Binding Buffer Gentle Ag/Ab Elution Buffer	Kit 5 mg 50 ml 250 mg 0.5 ml 200 ml 200 ml
20504	**Sodium Meta-Periodate**	25 mg

Purifying Antibody-Enzyme Conjugates

Antibody-enzyme conjugates, prepared using cross-linking reagents, are essential tools in immunoassays and immunodetection systems. Complete and efficient cross-linking is more likely to be obtained when excess enzyme is used in the conjugation reaction. This, however, results in free enzyme in the sample. The excess unconjugated enzyme has no advantage in the immunoassay procedure and often leads to high backgrounds. Removal of the non-conjugated enzyme is advantageous in lowering background and improving signal:noise ratios with fewer washes. Most commercially available secondary antibody-enzyme conjugates have not been purified to eliminate the free enzyme carried over from the conjugation reaction.

Several methods have been used to eliminate the free enzyme. The most effective method for removing unconjugated enzyme is affinity purification. Immunoaffinity techniques allow strong binding of the antibody conjugate but have the disadvantage of requiring harsh conditions that can destroy or severely damage the antibody or enzyme components of the conjugate. Gel filtration has also been used to separate the conjugate from the free enzyme. This technique, however, is ineffective when large aggregates of the enzyme are created, such as applications in which glutaraldehyde is used as the conjugation reagent. The large size of alkaline phosphatase also poses a problem with this method.

Metal chelate affinity chromatography is a powerful purification technique whereby antibodies and histidine-tagged proteins can be separated based upon their ability to form coordination complexes with immobilized metal ions without the harsh conditions associated with other affinity purification methods. Antibody conjugates form coordination complexes with immobilized Ni^{2+}/IDA, but the free enzyme does not form complexes. The conjugate is eluted with EDTA, and a blue band appears as the elution buffer front travels down the column. The blue color is formed when EDTA complexes the Ni^{2+}. This releases both the Ni^{2+} and the conjugate from the column. The blue band containing the conjugate also allows identification of the fraction to collect. The conjugate can then be desalted to remove the Ni^{2+} and EDTA and to exchange the buffer into the proper storage buffer conditions. Pierce offers an Immobilized Iminodiacetic Acid (IDA) column activated with nickel ions (Ni^{2+}) for conjugate purification (FreeZyme® Conjugate Purification Kit, Product # 44920).

An immobilized Protein A/G column can also be used to separate IgG conjugated to HRP from free HRP. Purifying the enzyme-labeled IgG from the free enzyme should eliminate background caused by contamination with the free enzyme. Because it has the binding properties of both Protein A and Protein G, IgG from most species should bind Protein A/G. (For more information on Protein A, G or A/G, please see the Antibody Production and Purification Sections.) Enzyme-labeled antibody does not lose its ability to bind immobilized Protein A/G. Therefore, when a reaction mixture containing labeled antibody and free enzyme is passed through such a column, the free enzyme will be eliminated by the wash procedure, and the labeled antibody will bind to the column. Pierce offers a neutral pH elution buffer for eluting the bound labeled IgG (ImmunoPure® Gentle Elution Buffer, Product # 21027). The neutral pH elution buffer does not affect the catalytic activity of the HRP.

Storing Enzyme Conjugates

Pierce provides a variety of reagents to help preserve enzyme conjugate activity. Typically, conjugates are aliquoted in 50-100 µl increments using purified ethylene glycol (Product # 29810) as a preservative for -20°C storage. Conjugates can maintain activity for up to two years. An alternative to aliquoting is to use SuperFreeze™ Peroxidase Conjugate Stabilizer (Product # 31503), diluting the conjugate 1:1 in the stabilizer and storing it at -20°C for up to one year as a stock solution. Guardian® Peroxidase Stabilizer/Diluents (Product #s 37548 and 37552) allow peroxidase conjugates to be reconstituted and stored at 4°C as a 1:1,000 dilution or a 1:100,000 dilution stock solution.

Conjugate Stabilizers

Ordering Information

Product #	Description	Pkg. Size
37548	Guardian® Peroxidase Conjugate Stabilizer/Diluent	200 ml
37552	Guardian® Peroxidase Conjugate Stabilizer/Diluent	1 L
31503	SuperFreeze™ Peroxidase Conjugate Stabilizer	25 ml
29810	Ethylene Glycol (50% aqueous solution)	200 ml

Pierce also offers other alternatives for storing enzyme conjugate solutions. The Guardian® Peroxidase Conjugate Stabilizer/Diluent (Product # 37552) and SuperFreeze™ Peroxidase Conjugate Stabilizer (Product # 31503) were developed to provide optimum protection for preparations of horseradish peroxidase, along with conjugates. These products are multicomponent cryoprotectants that provide a buffered anti-freeze environment for storage of these enzymes and their conjugates. The SuperFreeze™ Conjugate Stabilizer allows freezer storage of enzyme-antibody or enzyme-protein conjugates, ensuring that they will remain stable and in liquid form. The Guardian® Stabilizer allows peroxidase-conjugates to be stored at room temperature or 4°C without loss of enzyme activity even when diluted to 10 ng/ml.

Certain additives, such as glycerol, DMSO or ethylene glycol, are used to stabilize enzymes and other proteins during freezer storage by lowering the freezing point of the aqueous sample and inhibiting the formation of ice crystals. These materials are usually of undetermined purity and have not been tested for the presence of contaminants that will affect enzyme activity. Pierce offers purified Ethylene Glycol (Product # 29810) that is suitable for enzyme storage because impurities have been removed during the manufacturing process. Because it is not a potential oxidant, ethylene glycol is preferable to DMSO. In addition, ethylene glycol does not support growth of microorganisms, making it preferable to glycerol.

Biotin Labeling

The highly specific interaction of avidin with the small vitamin biotin can be a useful tool in designing assays, detection and targeting systems for biological analytes. The extraordinary affinity of avidin for biotin allows biotin-containing molecules in a complex mixture to be discretely bound with avidin conjugates.

Avidin is a glycoprotein found in the egg white and tissues of birds, reptiles and amphibia. This protein contains four identical subunits having a combined mass of 67,000-68,000 daltons. Each subunit consists of 128 amino acids and binds one molecule of biotin. The extent of glycosylation is very high; carbohydrate accounts for about 10% of the total mass of avidin. Avidin has a basic isoelectric point (pI) of 10-10.5 and is very soluble in water and aqueous salt solutions. Avidin is stable over a wide range of pH and temperature. Extensive chemical modification has little effect on the activity of avidin, making it useful for detection and protein purification.

Streptavidin is another biotin-binding protein that is isolated from *Streptomyces avidinii* and has a mass of 60,000 daltons. In contrast to avidin, streptavidin has no carbohydrate and has an acidic pI of 5. Pierce products use a recombinant form of streptavidin with a mass of 53,000 daltons and a near-neutral pI. Streptavidin is much less soluble in water than avidin and can be crystallized from water or 50% isopropyl alcohol. There are considerable differences in the composition of avidin and streptavidin, but they are remarkably similar in other respects. Streptavidin is also a tetrameric protein, with each subunit binding one molecule of biotin with a similar affinity to that of avidin. Guanidinium chloride will dissociate avidin and streptavidin into subunits, but streptavidin is more resistant to dissociation.

NeutrAvidin™ Biotin Binding Protein has a mass of 60,000 daltons and is a deglycosylated form of avidin. As a result, lectin binding is reduced to undetectable levels, yet biotin-binding affinity is retained because the carbohydrate is not necessary for this activity. NeutrAvidin™ Protein offers the advantages of a neutral pI to minimize nonspecific adsorption, along with lysine residues that remain available for derivatization or conjugation. NeutrAvidin™ Protein yields the lowest nonspecific binding among the known biotin-binding proteins.

Biotin, a 244 dalton vitamin found in tissue and blood, binds with high affinity to avidin, streptavidin and NeutrAvidin™ Protein. Since biotin is a relatively small molecule, it can be conjugated to many proteins without significantly altering the biological activity of the protein. A protein can be reacted with several molecules of biotin that, in turn, can each bind a molecule of avidin. This greatly increases the sensitivity of many assay procedures. The avidin-biotin interaction is the strongest known noncovalent biological interaction ($K_\alpha = 10^{15}\ M^{-1}$) between protein and ligand. The bond formation between biotin and avidin is very rapid and, once formed, is unaffected by most extremes of pH, organic solvents and other denaturing agents. The avidin-biotin complex can withstand up to 3 M guanidine•HCl. Biotin can be released by 8 M guanidine•HCl at pH 1.5 or by autoclaving. Avidin can be considered to have essentially nonreversible biotin-binding properties because bound biotin can be released only by denaturing the subunits of the proteins.

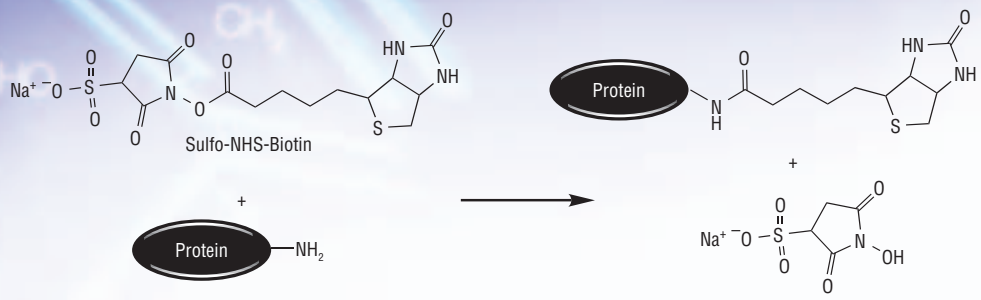


Figure 27. The reaction of Sulfo-NHS-Biotin (water-soluble) with a primary amine.

Amine-Specific Biotinylation Reagents

The most common target for labeling antibodies is the amine group, which is present as lysine side chain ε-amines and N-terminal α-amines. Based on water solubility, amine-reactive biotinylation reagents can be divided into two groups. NHS esters of biotin are essentially water-insoluble, while sulfo-NHS esters are readily dissolved in aqueous buffers. The reaction chemistries of NHS and sulfo-NHS esters are essentially identical. An amide bond is formed, and NHS or sulfo-NHS are leaving groups in the reaction (Figure 27).

Because the target for the ester is the deprotonated form of the primary amine, the reaction becomes significant at neutral pH values and above when the amine is able to react with the ester by nucleophilic attack. Hydrolysis of the NHS ester is a major competing reaction in aqueous solution, and the rate of hydrolysis increases with increasing pH. NHS- and sulfo-NHS esters have a half-life of hydrolysis of 2-4 hours at pH 7. This half-life decreases to just a few minutes at pH 9.

There is considerable flexibility in the actual conditions used for conjugating NHS esters (or sulfo-NHS esters) to primary amines. Incubation temperatures range from 4-37°C; reaction mixture pH values range from 7-9; and incubation times range from a few minutes to overnight. Buffers containing amines (such as Tris or glycine) should be avoided because they compete with the reaction. In preparing an NHS ester biotin conjugate, a particular set of conditions will result in a conjugate with optimum properties for a specific application. Preparing an ideal conjugate is largely dependent on the degree of incorporation of the biotin.

Sulfo-NHS-Biotin (Product # 21217), Sulfo-NHS-LC-Biotin (Product # 21335), Sulfo-NHS-LC-LC-Biotin (Product # 21338) and Sulfo-NHS-SS-Biotin (Product # 21331) are water-soluble NHS esters of biotin. Their water solubility results from the presence of the sulfonate ($-SO_3^-$) group on the *N*-hydroxysuccinimide ring and eliminates the need to dissolve the reagent in an organic solvent prior to use. Sulfo-NHS-LC-Biotin has a long-chain spacer arm of 22.4 Å. The long-chain spacer arm reduces steric hindrance associated with binding multiple biotinylated molecules on one avidin and results in enhanced detection sensitivity. Sulfo-NHS-LC-Biotin usually is a first-choice biotinylation reagent because of its long-chain spacer arm and its reactivity toward primary amines. Sulfo-NHS-LC-LC-Biotin may be used when steric hindrance is a major concern and the longest possible spacer arm is required.

Pierce also supplies the two most popular biotinylation reagents, Sulfo-NHS-Biotin and Sulfo-NHS-LC- Biotin, as complete labeling kits (Product #s 21420, 21430). These kits provide all of the required labeling reagents along with an optimized protocol that yields a high labeling efficiency.

NHS-PEO_4-Biotin (Product #s 21330, 21329) is another water-soluble, amine-reactive biotinylation reagent. The solubility results from its spacer arm containing polyethylene oxide (PEO). This reagent has the advantage of conferring increased solubility to the labeled protein molecule. Unlike sulfo-NHS esters, which lose their solubility-enhancing sulfonate group during the labeling reaction, reagents containing a PEO spacer arm transfer their high solubility when coupled to a protein.

NHS-Biotin (Product # 20217), NHS-LC-Biotin (Product # 21336) and NHS-LC-LC-Biotin (Product # 21343) are water-insoluble NHS esters of biotin. These compounds will biotinylate in aqueous solutions if they are first dissolved in an organic solvent (typically DMSO or DMF), then diluted into the aqueous reaction mixture. The solvent acts as a carrier for the biotinylation reagent, forming a microemulsion in the aqueous phase and allowing the biotinylation reaction to proceed.

NHS-Iminobiotin (Product # 21117) is the guanido analog of NHS-Biotin. NHS-Iminobiotin can be used for applications that require mild dissociation conditions from avidin and for those that cannot tolerate the reducing conditions required to break the disulfide bond of a cleavable biotinylation reagent. At pH 9.5 or greater, avidin binds tightly to iminobiotin; complete dissociation of the complex occurs at pH 4. There are two possible explanations for this binding phenomenon. One reasoning deals with the ionizing character of the cyclic guanido group of 2-iminobiotin. With increasing pH, the affinity for avidin increases. The cyclic guanido group of 2-iminobiotin has a pKa of 11.5 to 12, so avidin may bind iminobiotin only as the deprotonated form. However, the reduced binding affinity at pH values below 6 suggests that an ionizable group on avidin may also be involved. Regardless of the exact mechanism of binding, the pH-dependent avidin-binding property of iminobiotin derivatives provides a gentle, reversible option for employing the avidin-biotin system.

Pierce researchers recently devised an innovative strategy for IgG biotinylation that speeds and simplifies the process. The strategy involves first immobilizing the antibody to a metal-chelated affinity support, then biotinylating the immobilized antibody and finally eluting the biotin-labeled antibody. This approach obviates the need for dialyzing or desalting to remove reaction byproducts and can be completed in about one hour. The Solid-Phase Biotinylation Kits are available in a column format for labeling 1-10 mg of IgG (Product # 21440) and in a spin format for labeling 0.1-1 mg of IgG (Product # 21450).

Sulfhydryl-Specific Biotinylation Reagents

Targeting a sulfhydryl group for biotinylation can be used as a method for preserving the biological activity of a molecule when amines are found at the site of biological activity (e.g., antigen-binding site of an antibody). Modification of these amines may make these macromolecules inactive, but this complication can be avoided by using derivatives of biotin that react with sulfhydryls. Reactions with these biotinylation reagents should be performed in buffers free of extraneous thiols. Therefore, substances such as 2-mercaptoethanol, dithiothreitol and mercaptoethylamine should be removed prior to biotinylation.

Antibodies to be biotinylated by sulfhydryl-reactive reagents must have a free sulfhydryl group available. Sulfhydryls can be generated from disulfides by incubation with a reducing agent. Mercaptoethylamine•HCl (Product # 20408) can be used with IgG or $F(ab')_2$ molecules to cleave the disulfides between the heavy chains, while maintaining both the disulfide linkages between the heavy and light chains and the activity of the antibody. When doing mercaptoethylamine cleavage, EDTA should be included for its antioxidative effect. EDTA chelates trace amounts of metals in solution that promote disulfide bond formation. Free sulfhydryl groups from the hinge region of IgG molecules are fairly stable in the presence of EDTA. Using nitrogen-purged buffers is an additional precaution used to prevent reoxidation of the free sulfhydryls to disulfides.

Iodoacetyl-LC-Biotin (Product # 21333) and PEO-Iodoacetyl-LC-Biotin (Product # 21334) are sulfhydryl-reactive derivatives of biotin. Iodoacetyl-LC-Biotin is water-insoluble and should be dissolved in a solvent prior to use in an aqueous reaction mixture. The PEO-Iodoacetyl-LC-Biotin is water-soluble by virtue of its polyethyleneoxide (PEO) spacer arm and may be dissolved directly in aqueous solution. The iodoacetyl group reacts mainly with thiol groups at pH 7.5-8.5, resulting in a stable thioether bond. Unless precautions are taken, iodoacetyl groups may not be specific for sulfhydryls. The reaction can be directed toward sulfhydryl groups by limiting the molar ratio of Iodoacetyl-Biotin to protein, such that the

concentration of biotin is present at a small excess over the sulfhydryl content. Also, the reaction pH should be maintained in the range of 7.5-8.5. Below pH 9, the reactivity with amine, thioether and imidazole groups is minimized. Therefore, maintaining a lower pH ensures the modification of sulfhydryl groups and not amino groups. If there are no cysteines available, the reaction can be directed at imidazoles by adjusting the pH to 6.9-7.0. However, the incubation time must be increased to a week. Histidyl side chains and amino groups react in the unprotonated form and may take part in reactions above pH 5 and pH 7, respectively, although this reaction is much slower than that for sulfhydryls.

Biotin-BMCC (Product # 21900) is a sulfhydryl-reactive, water-insoluble biotinylation reagent. Biotin-BMCC has a long spacer arm of 32.6 Å, often enhancing the sensitivity of detection. Maleimide-PEO_2-Biotin (Product # 21901) is another sulfhydryl-reactive biotinylation reagent, but because of its PEO spacer arm it is water-soluble. These compounds contain a maleimide functional group, which distinguishes them from Iodoacetyl-LC-Biotin. The maleimide reaction is more specific for a free -SH group than the iodoacetyl reaction is. At pH 7 the maleimide group is 1,000 times more reactive toward a free sulfhydryl than toward an amine. The reaction of these reagents with free thiols is carried out at pH 6.5-7.5 since reactivity toward primary amines can occur at higher pH values. Hydrolysis of the maleimide group also increases at higher pH values.

Pierce researchers recently devised an innovative strategy for IgG biotinylation that speeds and simplifies the process. The strategy involves first immobilizing the antibody to a metal-chelated affinity support, then reducing and biotinylating the immobilized antibody, and finally eluting the biotin-labeled antibody. This approach obviates the need for dialyzing or desalting to remove reaction byproducts and can be completed in about two hours. The Solid-Phase Biotinylation Kits are available in a column format for labeling 1-10 mg of IgG (Product # 21920) and in a spin format for labeling 0.1-1 mg of IgG (Product # 21930).

Carbohydrate-Reactive Biotinylation Reagents

Mild oxidation of an immunoglobulin with sodium periodate (Product # 20504) will produce reactive aldehydes from *cis*-diols on the carbohydrate moieties of the Fc portion, which then can be labeled. This approach is advantageous for use with antibodies because they become biotinylated in a manner that maintains immunological reactivity. This is an ideal method for biotinylating polyclonal antibodies because they are heavily glycosylated. Monoclonal antibodies may be deficient in glycosylation and success with this method will depend on the extent of glycosylation for a particular antibody.

Temperature, pH of oxidation and the periodate concentration all affect the reaction with hydrazide derivatives of biotin. Since glycosylation varies with each antibody, optimal conditions must be determined empirically. Tris, or other primary amine-containing buffers, are not recommended for use in either the oxidation or biotinylation steps since these buffers react with aldehydes, quenching their reaction with hydrazides.

Biotin Hydrazide (Product # 21339), Biotin-LC-Hydrazide (Product # 21340) and Biocytin Hydrazide (Product # 28020) bind to oxidized carbohydrates through the hydrazide group (–NH-NH_2), forming a hydrazone linkage (Figure 28). Biotin Hydrazide and Biotin-LC-Hydrazide are soluble in aqueous buffers up to about 5 mM, and Biocytin Hydrazide is a more soluble derivative. They can also be dissolved first in DMSO (at concentrations up to 50 mM), then diluted into aqueous reaction mixtures. It should be noted that Biotin-Hydrazide and Biotin-LC-Hydrazide are poorly soluble in DMF (less than 5 mM). Hydrazides react spontaneously with oxidized carbohydrates to yield stable hydrazone bonds.

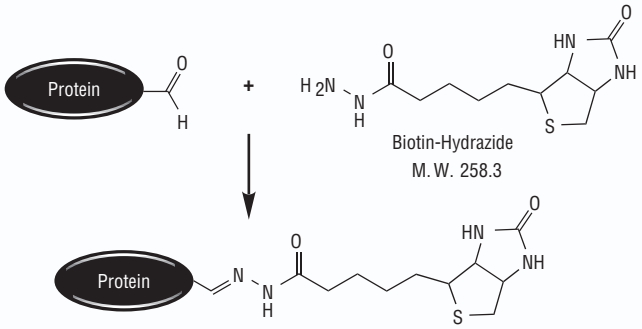


Figure 28. Reaction of biotin hydrazide with an aldehyde.

Table 13. Biotinylation reagent selection guide.

Product #	Description	Chemical Reactivity	Water-Soluble	Spacer Arm Length	Cleavable	Membrane-Permeable*	Pkg. Size	U.S. Price
21217	Sulfo-NHS-Biotin	Primary Amine	Yes	13.5 Å	No	No	50 mg	$126
21420	Sulfo-NHS-Biotinylation Kit	Primary Amine	Yes	13.5 Å	No	No	Kit	$244
21335	Sulfo-NHS-LC-Biotin	Primary Amine	Yes	22.4 Å	No	No	100 mg	$162
21430	Sulfo-NHS-LC-Biotinylation Kit	Primary Amine	Yes	22.4 Å	No	No	Kit	$262
21338	Sulfo-NHS-LC-LC-Biotin	Primary Amine	Yes	30.5 Å	No	No	50 mg	$140
21330	NHS-PEO_4-Biotin	Primary Amine	Yes	29 Å	No	No	25 mg	$190
21362	NHS-PEO_4-Biotin	Primary Amine	Yes	29 Å	No	No	50 mg	$249
21329	Pre-Measured NHS-PEO_4-Biotin	Primary Amine	Yes	29 Å	No	No	8 x 2 mg	$139
21440 21450	NHS-PEO Solid-Phase Biotinylation Kit	Primary Amine	Yes	32 Å	No	No	Column Kit Spin Kit	$245 $350
20217	NHS-Biotin	Primary Amine	No	13.5 Å	No	Yes	100 mg	$ 64
21336	NHS-LC-Biotin	Primary Amine	No	22.4 Å	No	Yes	50 mg	$100
21343	NHS-LC-LC-Biotin	Primary Amine	No	30.5 Å	No	Yes	50 mg	$140
21117	NHS-Iminobiotin	Primary Amine	No	13.5 Å	No	Yes	100 mg	$162
21901	Maleimide PEO_2-Biotin	Sulfhydryl	Yes	29.1 Å	No	No	50 mg	$162
21902	Pre-Measured Maleimide PEO_2-Biotin	Sulfhydryl	Yes	29.1 Å	No	No	8 x 2 mg	$145
21920 21930	Maleimide-PEO Solid-Phase Biotinylation Kit	Sulfhydryl	Yes	29.1 Å	No	No	Column Kit Spin Kit	$299 $399
21900	Biotin-BMCC	Sulfhydryl	No	32.6 Å	No	Yes	50 mg	$162
21334	PEO-Iodoacetyl Biotin	Sulfhydryl	Yes	24.7 Å	No	No	50 mg	$162
21333	Iodoacetyl-LC-Biotin	Sulfhydryl	No	27.1 Å	No	Yes	50 mg	$162
28020	Biocytin Hydrazide	Carbohydrate/RNA/DNA	Yes	19.7 Å	No	No	25 mg	$162
21339	Biotin Hydrazide	Carbohydrate	No	15.7 Å	No	Yes	100 mg	$162
21340	Biotin-LC-Hydrazide	Carbohydrate	No	24.7 Å	No	Yes	50 mg	$162

** Membrane permeability is implied due to a molecule's hydrophobic/hydrophilic nature.*
*** When used with EDC (Product # 22980, 22981).*
For more complete information on individual biotin-labeling reagents, please consult a current Pierce catalog or visit our web site (www.piercenet.com).

EZ™ Biotin Quantitation Kit

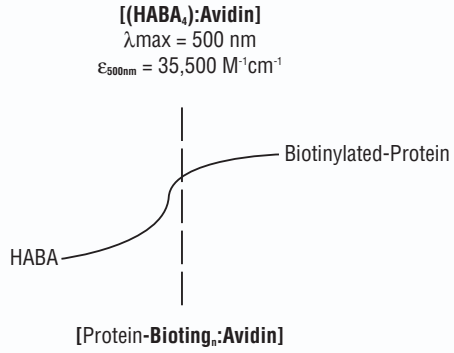
A convenient, accurate method for determining the degree of biotinylation.

Determine the molar ratio of biotin incorporated into an antibody using the HABA-Avidin method. The HABA dye (2-hydroxyazo-benzene-4′-carboxylic acid) binds to avidin to produce a yellow-orange colored complex that absorbs at 500 nm. Free biotin present in solution with this avidin-HABA complex will displace the HABA dye and cause the absorbance to decrease.

The amount of biotin present can be calculated directly from the decreased absorbance at 500 nm. The EZ™ Biotin Quantitation Kit contains pre-measured doses of the Avidin-HABA mixture and Biotinylated HRP positive control to simplify reagent preparation and minimize the amount of waste generated. The convenient assay can be performed either in a cuvette or in a microplate, and the math is simplified using a calculator on the Pierce web site. ImmunoPure® Avidin and Biotinylated HRP are also available separately in larger quantities.

How does this biotinylation assay work?
The $HABA_4$:Avidin complex is at the core of this displacement assay that can estimate the extent of protein biotinylation. HABA dye binds to avidin to form a complex that absorbs strongly at 500 nm with an extinction at that wavelenth of 35,000 $M^{-1}cm^{-1}$.

The assay is based on the **decrease** in absorbance of the $[(HABA)_4$:Avidin] complex when HABA is displaced from the complex by biotin.



Highlights:

- HABA-avidin complex can be used over a wide range of pH and salt concentrations
- Amount of biotin can be calculated directly from the decreased absorbance at 500 nm complexing with the HABA dye

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Ordering Information

Product #	Description	Pkg. Size
28005	EZ™ Biotin Quantitation Kit Includes: No-Weigh™ HABA-Avidin Premix Biotinylated HRP	24 assays 24 tubes 5 mg
28010	ImmunoPure® HABA (2-[4′-Hydroxyazobenzene]-benzoic acid)	10 g
28050	ImmunoPure® HABA	50 g
21121	ImmunoPure® Avidin	10 mg
29129	ImmunoPure® D-Biotin	1 g

Fluorescent Labeling

Antibody molecules can be labeled with any of a number of different fluorescent probes currently available from commercial sources. Each probe option has its own characteristic spectral signals of excitation (or absorption) and emission (or fluorescence). Many derivatives of these fluorescent probes possess reactive functional groups convenient for covalently linking to antibodies and other molecules. Each of the main fluorophore families contains at least a few different choices in coupling chemistry to direct the modification reaction to selected functional groups on the molecule to be labeled. These choices include amine-reactive, sulfhydryl-reactive and aldehyde-reactive.

In addition to the wide range of commercial probes obtainable, many other fluorescent molecules have been synthesized and described in the literature. Only a handful, however, are generally used to label antibody and other protein molecules. Perhaps the most common fluorescent tags with application to immunoassays include derivatives of fluorescein, rhodamine, amino-methylcoumarin (AMCA) and phycoerythrin. Figure 29 shows the reaction of fluorescein isothiocyanate (FITC), one of the most common fluorescent probes, with an antibody molecule.

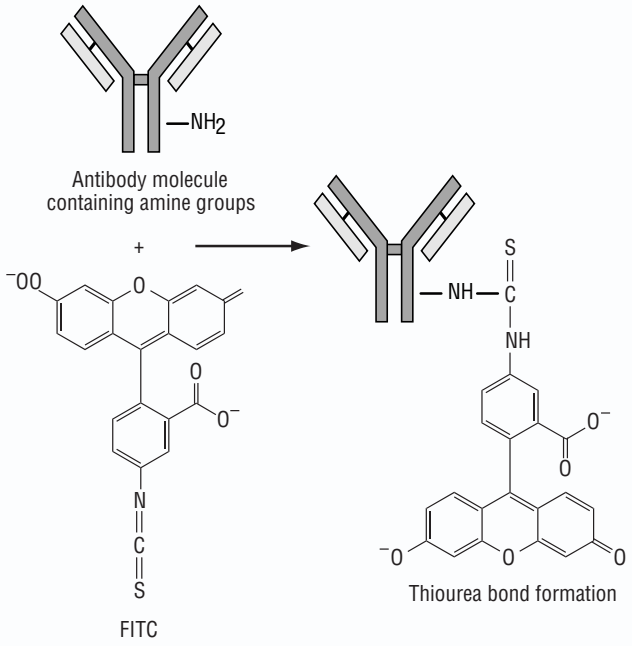

Figure 29. FITC reaction with a primary amine.

Industrial standardization has occurred for the use of these four probes due to the large literature documentation available on their successful application to antibody-based assays. As a result of this, instrumentation has become widely available for measuring the fluorescent response of any of these probes, including standard filter selections that match their excitation and emission patterns. Such fluorescently labeled antibodies can be used in immunohistochemical staining, in flow cytometry or cell sorting techniques, for tracking and localization of antigens, and in various double-staining methods.

In choosing a fluorescent tag, the most important factors to consider are good absorption; stable excitation; and efficient, high-quantum yield of fluorescence. Some fluorophores, such as fluorescein, exhibit fluorescence quenching, which lowers the quantum yield over time. Up to 50% of the fluorescent intensity observed on a fluorescein-stained slide can be lost within one month of storage. AMCA, by contrast, has better stability, but all fluorophores lose some intensity upon exposure to light and upon storage.

In some cases, the preparation of a fluorescently labeled antibody is not even necessary. Particularly, if indirect methods are used to detect antibody binding to antigen, then preparing a fluorescently labeled primary antibody is not needed. Instead, the selection from a commercial source of a labeled secondary antibody having specificity for the species and class of primary antibody to be used is all that is required. However, if the primary antibody needs to be labeled and it is not available commercially, then a custom labeling procedure will be required.

Generalized protocols for the attachment of these fluorophores to protein molecules, including antibodies, can be found in the literature. The main consideration for modifying immunoglobulins is to couple these probes at an optimal level to allow good detectability without substantially decreasing the antibody activity or increasing background. Too low a substitution level and the response of the fluorophore will yield low signal strength and poor sensitivity. Too high a substitution level and the fluorophore may decrease the antibody's ability to bind target molecules by blocking the antigen-binding sites or cause nonspecific interactions, resulting in high background or noise levels. In some cases, trial and error will be required to optimize this labeling process.

Pierce supplies a variety of reactive fluorescent molecules for labeling different functional groups with the most poplar fluorescent dyes. Five complete kits are also available for labeling antibodies with FITC (Product # 53004), NHS-Fluorescein (Product # 53000), NHS-Rhodamine (Product # 53002), DyLight™ 547 NHS-Ester (Product # 53009) and DyLight™ 647 NHS-Ester (Product # 53015). These kits provide all of the required labeling reagents along with an optimized protocol that yields a higher labeling efficiency than published protocols.

Amine-Reactive Probes

Product #	Probe Name	MW	Excitation Wavelength (nm)	Emission Wavelength (nm)	Comments	Ref. #	Pkg. Size	U.S. Price
33005	AMCA-NHS	330	345	440-460	• Blue fluorescent probe		5 mg	
33010	AMCA-Sulfo-NHS	431	345	440-460	• Blue fluorescent probe • Water-soluble		5 mg	
46110	Fluorescein Isothiocyanate (FITC)	389	494	520	• Soluble in aqueous buffer pH >6 and DMF	1-7	1 g	
46100	NHS-Fluorescein	473	491	518	• Soluble in DMF and DMSO	6	100 mg	
46102	NHS-Rhodamine	527	544	576	• Soluble in DMF and MeCN	6	25 mg	
46112	Tetramethyl-rhodamine-5-(and 6)-isothiocyanate (TRITC)	479*	541	572	• Soluble in DMF	2, 4	10 mg	
46115	Texas Red® Sulfonyl Chloride	625	596	615	• Soluble in DMF and MeCN	2, 8, 9	10 mg	
46200	DyLight™ 547 NHS-Ester	736	557	574	• Replaces Cy®3 Dye in all applications		1 mg	
46205	DyLight™ 647 NHS-Ester	762	652	673	• Replaces Cy®5 Dye in all applications		1 mg	

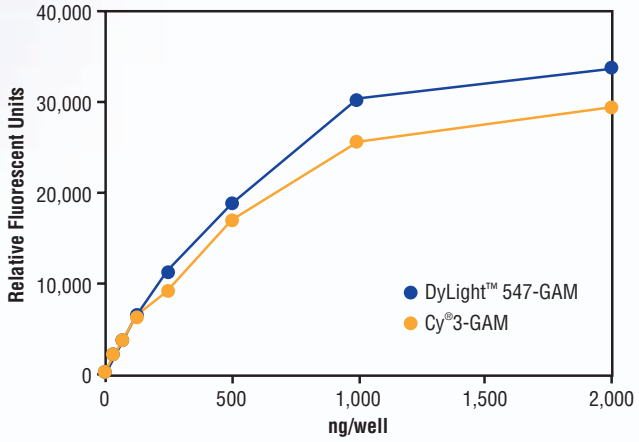

Figure 30. DyLight™ 647 Fluor used for IgG detection in an ELISA format. Goat anti-mouse antibodies were conjugated with DyLight™ 547 and Cy®3 Fluor using the same reaction conditions. Conjugates were then used to detect mouse IgG coated onto a microplate.

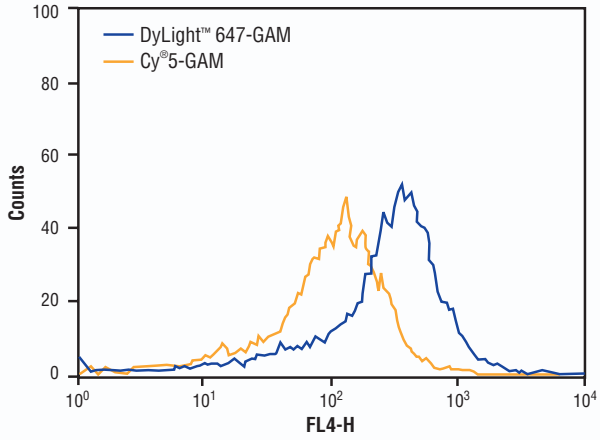

Figure 31. DyLight™ 647 Fluor used in flow cytometry. Goat anti-mouse secondary antibodies were conjugated with DyLight™ 647 and Cy®5 Fluors using the same reaction conditions. Conjugates were then used to detect CD3 receptors on Jurkat cells by flow cytometry.

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Aldehyde/Ketone- and Cytidine-Reactive Probe

Product #	Probe Name	MW	Excitation Wavelength (nm)	Emission Wavelength (nm)	Comments	Pkg. Size	U.S. Price
33015	AMCA-Hydrazide	247	345	440-460	• Soluble in DMF	5 mg	

**Molecular weight based on the chloride salt*

Sulfhydryl-Reactive Probes

Product #	Probe Name	MW	Excitation Wavelength (nm)	Emission Wavelength (nm)	Comments	Ref. #	Pkg. Size	U.S. Price
33020	**AMCA-HPDP**	528.7	345	440-460	• Blue fluorescent probe	5	5 mg	$105
46130	**Fluorescein-5-maleimide**	427	490	515	• Soluble in aqueous buffer >pH 6 and DMF	1	25 mg	$174
46120	**5-Iodoacetamido-fluorescein (5-IAF)**	515	490	520	• Soluble in aqueous buffer >pH 6 and DMF	2-4	25 mg	$255
46300	**DyLight™ 547 Maleimide**	761	557	574	• Replaces Cy®3 Dye in all applications		1 mg	$199
46305	**DyLight™ 647 Maleimide**	787	652	673	• Replaces Cy®5 Dye in all applications		1 mg	$199

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Site-Selective Probes

Product #	Probe Name	MW	Excitation Wavelength (nm)	Emission Wavelength (nm)	Comments	Ref. #	Pkg. Size	U.S. Price
22030	**Biotin-Fluorescein**	732.8	492	510	• Binds to streptavidin • Produces a greenish-yellow signal		5 mg	
46190	**4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)**	350	345	455	• Soluble in H_2O • Fluorescent retrograde tracer of neurons • Binds tightly to tubulin *in vitro* with no interference with microtubule assembly or GTP hydrolysis	1-7	10 mg	

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Phycobiliproteins

Product #	Probe Name	MW	Excitation Wavelength (nm)	Emission Wavelength (nm)	Ref. #	Pkg. Size
46185	**R-Phycoerythrin**	240,000	480, 545 and 565	578	1-6	2 mg

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EZ-Label™ Fluorescent Labeling Kits

Make your own fluorescent-labeled antibody in less than two hours!

EZ-Label™ Kits are designed for labeling sample volumes ranging from 50 µl-1 ml with antibody concentration up to 10 mg/ml for each reaction. EZ-Label™ Kits were specially developed and optimized for the most efficient labeling.

EZ-Label™ Kits contain everything you need to successfully label your antibody:

- Fluorescent dye provided in individual microtubes, eliminating the need to weigh dye
- Conveniently packaged dimethylformamide (DMF) to prepare the fluorescent dye solution
- Pre-made borate and phosphate buffers – just add water to the powder and they are ready-to-use
- Pre-packed, ready-to-use desalting columns for fast buffer exchange when your protein sample volume is greater than 100 µl
- Slide-A-Lyzer® MINI Dialysis Units* for easy buffer exchange when your protein sample volume is less than or equal to 100 µl
- Amber reaction tubes – no handling in the dark required

EZ-Label™ Kit	Excitation Wavelength (nm)	Emission Wavelength (nm)
Fluorescein Protein Labeling Kit	491	518
Rhodamine Protein Labeling Kit	544	576
Fluorescein Isothiocyanate (FITC) Protein Labeling Kit	494	520

These kits contain sufficient reagents to perform five fluorescent labeling reactions, which use up to 10 mg/ml of protein for each reaction (50 µl-1 ml volume of protein).

Ordering Information

Product #	Description	Pkg. Size
53000	**EZ-Label™ Fluorescein Labeling Kit** *Sufficient for five coupling reactions.*	Kit
	Includes: No-Weigh™ Fluorescein	6 x 1 mg
	Dimethylformamide (DMF)	1 ml
	BupH™ Borate Buffer Packs	5 packs
	BupH™ Phosphate Buffered Saline Packs	5 packs
	D-Salt™ Dextran Desalting Columns	5 columns
	Slide-A-Lyzer® MINI Dialysis Unit Pack	5 units
	Reaction Tubes	5 tubes
53002	**EZ-Label™ Rhodamine Labeling Kit** *Sufficient for five coupling reactions.*	Kit
	Includes: No-Weigh™ Rhodamine	6 x 0.5 mg
	Dimethylformamide (DMF)	1 ml
	BupH™ Borate Buffer Packs	5 packs
	BupH™ Phosphate Buffered Saline Packs	5 packs
	D-Salt™ Dextran Desalting Columns	5 columns
	Slide-A-Lyzer® MINI Dialysis Unit Pack	5 units
	Reaction Tubes	5 tubes
53004	**EZ-Label™ FITC Labeling Kit** *Sufficient for five coupling reactions.*	Kit
	Includes: No-Weigh™ FITC	6 x 1 mg
	Dimethylformamide (DMF)	1 ml
	BupH™ Borate Buffer Packs	5 packs
	BupH™ Phosphate Buffered Saline Packs	5 packs
	D-Salt™ Dextran Desalting Columns	5 columns
	Slide-A-Lyzer® MINI Dialysis Unit Pack	5 units
	Reaction Tubes	5 tubes

** U.S. patent # 6,039,871*

DyLight™ 547 and 647 Monoclonal Antibody Labeling Kits

Efficient labeling and exceptional antibody recovery!

The DyLight™ Monoclonal Antibody Labeling Kits were specifically developed for fast, efficient labeling of 100 µg of monoclonal antibody*. The DyLight™ Dyes are an excellent alternative to Cy®3 and Cy®5 Dyes, providing greater photostability and fluorescence over a broad range of pH values.

Highlights:

- Fast – label and purify protein in approximately one hour
- DyLight™ 547 replaces Cy®3 and DyLight™ 647 replaces Cy®5
- Amine-reactive dyes – label virtually any antibody
- Pre-measured fluorescent dye eliminates wasted time and reagent
- Average antibody recovery of 80% with minimal dilution
- Efficient free dye removal allows accurate determination of antibody-dye ratios
- Spin cleanup step eliminates column preparation, faction screening and slow elutions.

** Alternatively, 100 µg of polyclonal antibody can be labeled with similar efficiency.*

Ordering Information

Product #	Description	Pkg. Size
53009	**DyLight™ 547 Monoclonal Antibody Labeling Kit** *Sufficient reagents to label and purify 5 x 100 µg of IgG.*	Kit
	Includes: DyLight™ 547 NHS Ester	5 x 20 µg
	DMF (dimethylformamide)	2 ml
	Borate buffer	1 ml
	Zeba™ Desalt Spin Columns	10 x 0.5 ml
53015	**DyLight™ 647 Monoclonal Antibody Labeling Kit** *Sufficient reagents to label and purify 5 x 100 µg of IgG.*	Kit
	Includes: DyLight™ 647 NHS Ester	5 x 20 µg
	DMF (dimethylformamide)	2 ml
	Borate buffer	1 ml
	Zeba™ Desalt Spin Columns	10 x 0.5 ml

Iodine Labeling

Radioactive probes are among the most sensitive markers used for biological detection. Iodine isotopes, ^{14}C, ^{32}P, ^{35}S and tritium (^{3}H) are commonly used radiolabels. The iodine isotopes, which are γ emitters, have several advantages, including a relatively short half-life. The maximum specific activity that can be achieved with an isotope is inversely related to its half-life. In addition, γ rays are directly detectable without a scintillation cocktail. Radioimmunoassay (RIA) is a common procedure that utilizes high specific-activity radiolabeled antibodies as tracers. Radioiodination is a common procedure and provides excellent sensitivity in many applications. Iodine-125 is the isotope primarily used in radioimmunoassays because of its high, easily detectable specific activity and low energy γ emission. Iodine-125 has a 60-day half-life, which allows labeled material to be prepared and stored for extended time periods. Iodine-131 is rarely used for radioimmunoassays. Although it has a shorter half-life than ^{125}I, it has several drawbacks. For example, ^{131}I usually is not available with a carrier and its decay cannot be counted as efficiently as ^{125}I. The shorter half-life indicates that labeled compounds must be prepared more often. In addition, the γ rays from ^{131}I are more penetrating than ^{125}I, requiring more precautions and additional protection for those synthesizing or working with the radioactive compound.

Methods for Iodination of Molecules

Radioiodination involves the introduction of radioactive iodine into certain amino acids, usually tyrosines. Iodination takes place at the positions ortho to the hydroxyl group on tyrosine; mono- or di-substitution may occur. Studies on the mechanism of the reaction of iodine with tyrosine and other phenolic groups indicate that it is the phenolic anion that is attacked. Histidine residues are also iodinated by some iodinating methods.

Radioactive ^{125}I can be incorporated into antibodies either by enzymatic or chemical oxidation. In the chemical oxidation method, the oxidizing agent of choice for many years was chloramine-T. The use of chloramine-T as an iodination reagent, however, requires great care because its powerful oxidative properties may destroy the biological activity of the antibody. After oxidation, the reaction is terminated by introduction of a reducing agent that also may affect the antibody.

For maintaining biological activity, the ideal oxidizing agent is one that is mild, generates sufficient reactive iodine and does not require a reduction step. An immobilized oxidant would result in a two-phase system, limiting direct contact of the oxidant with the protein and allowing a slower and more easily-controlled reaction. An immobilized oxidation reagent would also allow the radiolabeled reagent to be easily separated from the reaction mixture. Pierce IODO-BEADS® Reagent and IODO-GEN® Tubes are ready-to-use immobilized oxidizing agents that are ideal for iodination of antibodies.

The IODO-BEADS® Iodination Reagent is a convenient, gentle and efficient method for iodinating soluble and membrane bound proteins. IODO-BEADS® Iodination Reagent (Product # 28665, 28666) is *N*-chloro-benzenesulfonamide (sodium salt) immobilized on nonporous, polystyrene beads (Figure 32). It is useful over a broad pH range, requires 2-15 minutes to complete the iodination step, and it is very easy to use. The reaction mixture can be easily separated from the IODO-BEADS® Reagent by removing the bead or decanting the reaction solution. Radioiodide incorporation as high as 99% can be achieved, while recovering over 90% of the labeled protein. IODO-BEADS® Iodination Reagent is compatible with many common buffer components. Detergents such as SDS, NP-40 and Triton® X-100, and denaturants such as urea, will not harm the reaction and may actually improve incorporation of the radioactive iodine by making tyrosines more accessible. High salt concentrations (1.0 M NaCl) and azide do not inhibit the reaction. Iodinations can be performed in phosphate, Tris, HEPES and other common buffers. These beads are not compatible with reducing agents that counteract the oxidative effect needed to initiate the labeling reaction. It is possible to quantitatively iodinate histidine residues with the IODO-BEADS® Reagent, simply by increasing the pH of the reaction from 7.0 to 8.2.

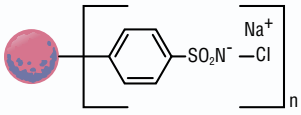


Figure 32. IODO-BEADS® Iodination Reagent *N*-Chloro-benzenesulfonamide (sodium salt) derivatized, uniform, nonporous, polystyrene beads.

There are two distinct disadvantages associated with the IODO-BEADS® Reagent. First, the beads have a relatively short shelf life (approximately 1 year). Second, they are made of polystyrene that can adsorb some proteins, resulting in loss of sample when working with small quantities. Pierce IODO-GEN® Iodination Reagent (Product # 28600) can be a useful alternative to the IODO-BEADS® Reagent. While the IODO-GEN® Reagent is more stable than the IODO-BEADS® Reagent, its use requires more labor since the reagent must be deposited first on a solid surface.

IODO-GEN® Precoated Iodination Tubes (Product # 28601) have been created by immobilizing IODO-GEN® Reagent (Figure 33) on the inside surface of a test tube, creating the simplest, most consistent method for radioiodination. IODO-GEN® Tubes provide a gentle, easy-to-control environment and eliminate any contact with or handling of the iodination reagent.

IODO-GEN® Iodination Reagent 1,3,4,6-Tetrachloro-3α, 6α-diphenylglycouril

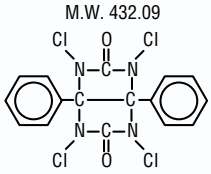


Figure 33. Structure of IODO-GEN® Reagent.

IODO-GEN® Pre-Coated Iodination Tubes

Flexibility and reproducibility for radioiodinations.

Highlights:

- Iodinations can be completed in under 2 minutes
- Eliminates the tedious reagent surface-coating step
- Provides a consistent IODO-GEN® Reagent-coated and flake-resistant surface in the tube
- Allows iodinations to be carried out directly in the tube
- Enables ^{125}I to be pre-activated directly in the tube
- Offers the opportunity to iodinate protein without ever having the protein contact the IODO-GEN® Reagent directly

New ^{125}I pre-activation strategy[2] offers significant benefits, including:

- Elimination of oxidative damage to labile proteins
- No losses from nonspecific protein binding to the surface
- Flexibility to conduct iodinations in a wide variety of vessels (microcentrifuge tubes, tissue culture flasks, silanized tubes, etc.)
- Compatibility with common detergents

References

Chizzonite, R. (1996). Hoffmann LaRoche, Department of Inflammation/ Autoimmune Diseases.

Fraker, P.J. and Speck, J.C., Jr. (1978). *Biochem. Biophys. Res. Commun.* **80**, 849-857.

Zuk, P.A. and Elferink, L.A. (2000). *J. Biol. Chem.* **275**, 26754-26764.

Ordering Information

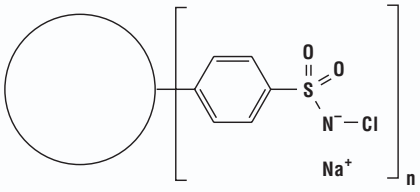
Product #	Description	Pkg. Size
28601	**IODO-GEN® Pre-Coated Iodination Tubes** Contains: 50 µg IODO-GEN® Iodination Reagent evaporated from 100 µl volume in 12 mm x 75 mm glass test tubes	10/pkg.

IODO-BEADS® Iodination Reagent

A convenient and effective method for iodinating proteins – including histidine-tagged proteins.

Highlights:

- Derivatized, uniform, nonporous polystyrene beads
- Remarkably reproducible iodinations
- Radioiodide incorporation as high as 99%; labeled protein recovery >90%
- Iodinates in the presence of azides, detergents, urea and high salt
- Allows efficient iodination of cell membrane surface proteins
- More gentle method for iodination than soluble chloramine-T because there is no contact between the protein and the immobilized oxidizing agent[1]
- Reaction stopped by simply removing beads from reaction mixture with tweezers or Pasteur pipet; no reducing agent necessary to terminate reaction



IODO-BEADS® Iodination Reagent

Recommended IODO-BEADS® Reaction Conditions	
Protein or Peptide	5-500 µg of tyrosine-containing antibody per bead
Beads	One or more; specific activity can be conveniently and reproducibly controlled by changing the number of beads
Reaction Volume	100-1,000 µl per bead; smaller volumes are possible using polypropylene Eppendorf tubes
Iodination Buffer	100 mM phosphate or Tris buffer. Solvents that dissolve the polystyrene (such as DMSO or DMF) are incompatible
pH	5.5-7.0; IODO-BEADS® Reagent functions best at pH 6.5
Temperature	Functions over a wide range of temperatures
Time	2-15 minutes

References

Markwell, M.A. (1982). *Anal. Biochem.* **125**, 427-432.

Tsomides, T.J., *et al.* (1991). *Proc. Natl. Acad. Sci. USA* **88**, 11276-11280.

Ordering Information

Product #	Description	Pkg. Size
28665	**IODO-BEADS® Iodination Reagent***	50 beads
28666	**IODO-BEADS® Iodination Reagent*** *(N-Chloro-benzenesulfonamide)* Bead Diameter: 1/8 inch (3.175 mm), nonporous polystyrene Oxidative Capacity: 0.55 ± 0.05 µmoles/bead	250 beads

**U.S. patent #'s 4,448,764 and 4,436,718*

IODO-GEN® Iodination Reagent

A solid-phase iodinating reagent for gentle iodinations.

Highlights:

- Solubility in chloroform permits "plating" aliquots of the reagent onto the walls of the glass or plastic iodination vessels
- Side reactions are negligible
- No reducing reagent is required because reactions are terminated by simply decanting the reaction solution away from the plated IODO-GEN® Reagent
- More efficient than chloramine-T or lactoperoxidase[1]
- Iodinate phenolic groups on cross-linking reagents

References

1. Salacinski, P.R., *et al.* (1981). *Anal. Biochem.* **117**, 136-146.
Fraker, P.J. and Speck, J.C., Jr. (1978). *Biochem. Biophys. Res. Commun.* **80(4)**, 849-857.
Kleene, R., *et al.* (2000). *Biochemistry* **39**, 9893-9900.
McClard, R.W. (1981). *Anal. Biochem.* **112**, 278-281.
Millar, W.T. and Smith, J.F.B. (1983). *Int. J. Appl. Radiat. Isot.* **34(3)**, 639-641.
Piatyszek, M.A., *et al.* (1988). *Anal. Biochem.* **172**, 356-359.
Tolan, D.R., *et al.* (1980). *Anal. Biochem.* **103**, 101-109.

Comparison of IODO-BEADS® and IODO-GEN® Iodination Reagents

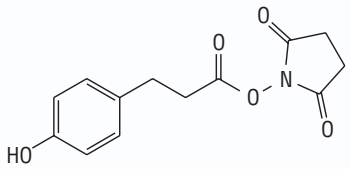
	IODO-BEADS® Reagent	IODO-GEN® Pre-Coated Iodination Tubes	IODO-GEN® Reagent
Detergent-Compatible	Yes	Yes	Yes
Denaturant-Compatible	Yes	Yes	Yes
pH Range	4-8.5 (5-6.5 optimum)	4.4-9 (8-9 optimum)	4.4-9 (8-9 optimum)
Stability	Short – one year	Stable indefinitely	Stable indefinitely
Ease of Use	Easy	Easy	Requires good plating technique

Ordering Information

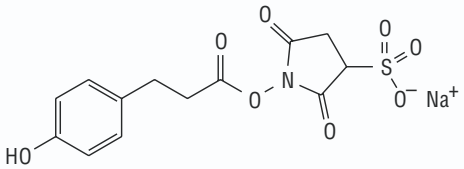
Product #	Description	Pkg. Size
28600	**IODO-GEN® Iodination Reagent** (1,3,4,6-Tetrachloro-3α,6α-diphenylglycoluril)	1 g

Bolton-Hunter Reagent

Increases ^{125}I label on proteins.



Bolton-Hunter Reagent (SHPP)
M.W. 263.25



Water-Soluble Bolton-Hunter Reagent (Sulfo-SHPP)
M.W. 365.29

Highlights:

- Attaches tyrosine-like residues to primary amines to increase the yield of a subsequent iodination
- Reacts with *N*-terminal amino groups optimally at pH 8.5
- Iodinate before or after coupling to the molecule of interest
- Introduces tyrosyl moieties with a neutral linkage, through *N*-terminal α-amino groups or ε-amino groups of lysine
- Preserves tyrosines that might affect function or immunogenicity

Reference

Bolton, A.E. and Hunter, W.M. (1973). *Biochem. J.* **133**, 529-539.
Thirkell, D., *et al.* (1989). *Infect. Immun.* **57**, 1697-1701.
Thompson, J.A., *et al.* (1987). *Biochem.* **26**, 743-750.

Ordering Information

Product #	Description	Pkg. Size
27710	**Bolton-Hunter Reagent (SHPP)** (*N*-Succinimidyl-3-[4-hydroxyphenyl]propionate)	1 g
27712	**Water-Soluble Bolton-Hunter Reagent (Sulfo-SHPP)** (Sulfosuccinimidyl-3-[4-hydroxyphenyl]propionate)	100 mg

Structure courtesy of: Crystal Structure At 2.7A Resolution Of A Complex Between A Staphylococcus Aureus Domain and A Fab Fragment Of A Human Igm Antibody; PDB: 1DEE; Citation: Graille, M., Stura, E. A., Corper, A. L., Sutton, B. J., Taussig, M. J., Charbonnier, J. B., Silverman, G. J.: Crystal Structure of a Staphylococcus Aureus Protein a Domain Complexed with the Fab Fragment of a Human Igm Antibody: Structural Basis for Recognition of B-Cell Receptors and Superantigen Activity *Proc.Nat.Acad.Sci.USA* 97 pp. 5399 (2000).

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How do you purify your protein out of a universe of possibilities?

Use UltraLink® Supports.

- Ultra-low nonspecific binding – less unwanted protein in your final sample*
- Ultra-high coupling capacity – up to 35 mg of protein can be bound per ml of base support
- High flow rates – up to 3,000 cm/hour for fast purification
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*Supported by independent, published research [Ju, *et al.* (2002) *J. Biol. Chem.* **277**, 178-186]

Pre-conjugated supports to purify antibodies and biotin-tagged proteins:

- Protein A, G and A/G
- Mannan Binding Protein (MBP)
- Streptavidin
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- Monomeric Avidin

Activated supports to create your own affinity resin:

- Amine-reactive
- Sulfhydryl-reactive
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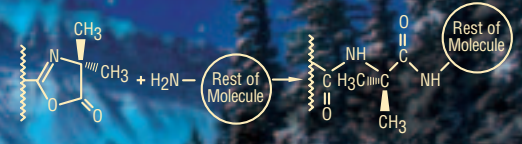
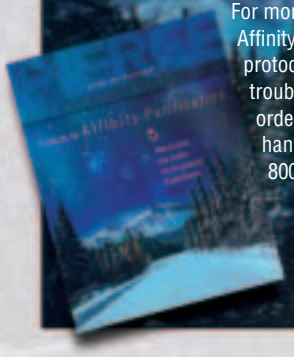
- Carboxyl-reactive



UltraLink® *bis*-acrylamide Support with Azlactone Groups | Amine Containing Ligand | Amide Bond Formation with Ring Opening



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