

There are numerous antibody labeling protocols in the scientific literature and it can be difficult to determine the best approach in any given situation. This guide will give you a good understanding of the standard chemical approaches to conjugation, and their strengths and weaknesses. We also discuss newer one-step conjugation technologies such as [Lightning-Link®](#) which have made the process of attaching labels to antibodies extraordinarily easy; now anyone able to use a hand-held pipette can make high quality antibody conjugates with less than thirty seconds of effort.

Antibodies and labels

Antibodies are widely used to detect and to quantify antigens in immunoassays such as flow cytometry, ELISA, western blotting, immunohistochemistry and lateral flow. The antibody that recognizes the antigen is referred to as the 'primary' antibody, and it is a very important reagent because it confers *specificity*. The vast majority of immunoassays also incorporate a 'label' whose sole purpose is to provide *measurability*. Generally the label is colored or is able to produce, under the appropriate conditions, a colored substance or light at a characteristic wavelength. Labels include organic dyes, fluorescent proteins, colored particles and enzymes.

Indirect versus direct assay formats

Immunoassays that incorporate a label can be set up in one of two possible formats. With *direct* detection of antigen, the label is attached via a covalent bond directly to the primary antibody. With an *indirect* assay approach, the label is covalently attached to a secondary reagent, often an anti-species antibody, which is capable of binding non-covalently to the unlabeled primary antibody.

Assays based on the principle of indirect detection comprise two distinct parts: first, a period of incubation (which can range from 15 minutes to overnight) with the *unlabeled* primary antibody, during which a fraction of the antibody binds to the antigen (assuming that the antigen is present). Excess unbound primary antibody is washed away (typically 3-5 washes) and in the second part of the assay a labeled secondary reagent is added. After a period of incubation (typically one hour), excess reagent is washed away and the amount of label associated with the primary antibody (i.e. indirectly via the secondary reagent) is quantified.

In the case of direct detection, the covalent attachment of a label to the primary antibody means that only a single incubation step with the antigen-containing sample is required. Moreover, there is only one round of wash steps (as opposed to two rounds of incubation and wash steps with indirect detection). The assay simplification that is afforded by direct detection tends to reduce assay variability and to improve data quality. While covalent modification of primary antibodies can result in altered antibody affinity, the number of such occurrences is insignificant when compared with the frequency of problems caused by non-specific binding of labeled secondary antibodies. The differences between the two approaches and potential problems are outlined schematically in Figure 1.

The indirect approach is particularly challenging in multiplexed assays because the simultaneous measurement of five antigens, as depicted in Figure 1 (left panel), requires five good quality primary antibodies, which *must* be from five different species, together with five species-specific labeled secondary antibodies. Often otherwise excellent primary antibodies cannot be used simply because another primary antibody from the same species has already been selected.

If any of the secondary reagents show any 'crossover' onto the wrong primary antibody the assay results will be invalidated. In Figure 1, the apparent level and/or distribution of the red and green antigens will be wrong, as there is crossover with the primary antibody that binds to the green antigen. In a real-life situation with a patient's sample, the crossover of the antibody with a yellow label in this example may even lead to a misdiagnosis, as the detection of the yellow label on the cell surface would be taken to mean that the yellow antigen is present, when in fact it is not there at all.

Figure 1



The figure depicts a cell surface and the different surface antigens to be measured are color-coded, as are the labels on the secondary antibodies (indirect method) or primary antibodies (direct method). Each primary antibody is considered to be completely selective for its antigen. If the secondary antibodies bind to their correct primary antibodies the colors of the labels and antigens will match. The antigen with a 'dotted' outline is not present on this particular cell surface; if present, it would be detected through the binding of the secondary antibody with the yellow label.

For the reasons given above, direct detection is far simpler and more reliable; moreover the best quality primary antibodies can always be used, whether from one species or a combination of species. In Figure 1 above (right-hand panel), the absence of secondary antibodies means that the presence or absence of antigens is correctly reported.

There is a widely-held belief that there is amplification of the assay signal when using the indirect method, because more than one molecule of secondary antibody can bind to each primary antibody. Conceptually this is easy to understand and it is probably true that more than one secondary antibody can be accommodated. However, the off-rate of the primary antibody and its impact on the performance of an indirect assay is rarely known or even considered. The concentration of primary antibody in solution in the second phase of the indirect assay i.e. the incubation with the secondary reagent, is zero. As the primary antibody does not have an infinitely high affinity, the bound antibody begins to dissociate

and the system relaxes to a new equilibrium, with a proportion of the previously antigen-bound antibody returning to solution. Further relaxation occurs during the second series of wash steps, when again the concentration of primary antibody in solution is zero. Thus what is actually amplified is a *diminishing amount of primary antibody* and the same result, or an even better result, can be obtained far more easily with direct detection.

Figure 2

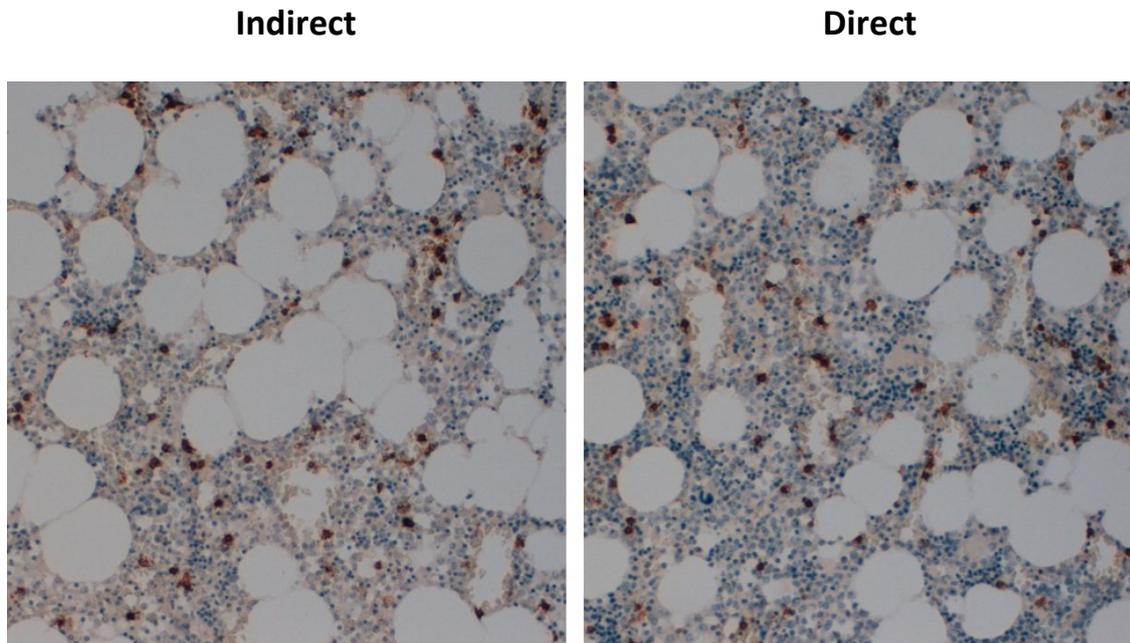


Figure 2 shows immunohistochemical staining of CD20 in formalin-fixed, paraffin-embedded sections of human tonsil. The left-hand image shows indirect labeling with clone L26 in combination with a high sensitivity poly-HRP detection system, and the right-hand image shows clone L26 directly conjugated to HRP. Magnification x20.

As we can see from the discussion so far, making primary antibody labeling easier makes assay development work easier too. Despite the advantages of direct detection, many assays are still run in an indirect format because the necessary labeled primary antibodies are not commercially available. There is also a perception, perhaps, that the production of conjugates is quite difficult. This may have been the case in the past but by the time you reach the end of this guide you should be confident enough to make your own conjugates.

Antibody labeling methods

Some of the most useful techniques for labeling of antibodies are presented below. We do not dwell on chemical structures and full chemical names; this detail is largely irrelevant. You need to understand only the basic chemical principles (i.e. which groups react with what) and, within each class of conjugation chemicals, which is the best reagent to use. With this information you can tackle almost any antibody conjugation project.

Antibodies, like all proteins, are composed of amino acids; the side chain of lysine which terminates in a primary amine ($-NH_2$) is invariably used to attach labels covalently to

antibody molecules. Despite the many labeling procedures in the scientific literature and hundreds of chemical modification reagents, there are, in reality, just a handful of important methods and reagents.

Five common lysine-based conjugation procedures are summarized below. For each approach we discuss the advantages and disadvantages and we provide some tips on the best reaction conditions and on what to avoid.

1. NHS (succinimidyl) ester method.

This approach is often used with fluorescent dyes, which can readily be purchased with a reactive NHS group. After reaction with the antibody any surplus reactive dye is removed using chromatography, which separates the conjugate from free dye on the basis of *size*. The chromatography is carried out using disposable columns, which are allowed to run under gravity, or using spin columns which contain exactly the same type of chromatography matrix in a tube that fits into a micro centrifuge. These purification steps result in some losses of conjugate but they are obligatory if high backgrounds are to be avoided.

This approach to conjugation is relatively simple but its major limitation is that NHS esters are very sensitive to moisture; when pots of NHS esters are opened and/or stored for long periods in fridges/freezers the reactive groups gradually disappear. It is therefore necessary to carry out trial conjugations periodically to check the degree of dye incorporation. All NHS esters must be dissolved in a dry organic solvent (e.g. DMSO), and preferably at a high concentration to limit the amount of solvent added to the antibody. As solvents are never truly dry, the NHS ester must be used quickly and cannot be stored. Another possible disadvantage of NHS ester dyes (unless you are labeling a very large amount of antibody) is that it is not possible to weigh out accurately the amount of dye that is required, which is often a tiny fraction of 1mg.

NHS ester reactions should be carried out at pH 7.2-8.0 in a primary amine-free buffer. A good choice is 100mM sodium phosphate buffer. The rate of reaction increases with increasing pH because lysines are more reactive in their deprotonated state (i.e. $-\text{NH}_2$) than in the protonated form ($-\text{NH}_3^+$). The rate of ester decomposition (hydrolysis) also increases with increasing pH. Tris buffer should not be used as it contains a primary amine and will react with the dye and thus compete with lysine residues in the antibody.

2. Carbodiimide method.

Carbodiimide reagents ('EDC' is one very common example) are used to create covalent links between amine- and carboxyl-containing molecules. Carbodiimides activate carboxyl groups, which then react with an amine (e.g. a lysine residue in an antibody). EDC is an unusual linker in that it leaves no trace of itself in the final conjugate; for this reason EDC is often referred to as a 'zero-length crosslinker'.

Carbodiimides are mainly used to conjugate antibodies to carboxylated *particles* and to other carboxylated surfaces, such as microwell plates or chip surfaces. Carbodiimides are

never used to attach protein labels to antibodies because the two molecules both have amine (lysine) and carboxyl groups (i.e. aspartate, glutamate). The addition of EDC inevitably results in an ill-defined mixture of aggregated products.

Carbodiimide reactions are carried out in a primary amine- and carboxyl-free buffers. The best choice is MES buffer. A pH of 4.7 is often recommended in the literature but MES has a pKa of 6.1 and is not an effective buffer at pH 4.7. A reaction pH of around pH 5.5 with MES buffer is a better starting point, which is a compromise between the optimum pH for carboxyl activation with EDC (~pH 3.5) and the pH optimum for lysine reactions (i.e. > pH 7). Phosphate buffer is also commonly recommended for use in carbodiimide reactions; it is acceptable but not the best choice, as phosphate reacts with carbodiimides and reduces reaction efficiency.

Hundreds of carbodiimides are known but as almost all of them are insoluble in aqueous conditions they are used mainly in synthetic organic chemistry. One such application of carbodiimides is the production of NHS-activated dyes, which we discussed earlier. The only carbodiimide of any real significance for antibody/protein work is EDC (also called EDAC), which is usually purchased as the hydrochloride salt.

3. Sodium periodate method.

Sodium periodate (a strong oxidizer) is used to activate the glycoprotein horseradish peroxidase (HRP), the most popular enzyme used in diagnostic tests. The periodate molecule reacts with carbohydrate chains to create aldehyde groups, which can react with lysine residues in antibodies. HRP is unusual in having very few lysines itself, which is helpful in limiting unwanted self-polymerization, especially if the activation pH is below 7. You will probably never actually need to use sodium periodate because pre-activated aldehyde HRP is commercially available.

An advantage of the periodate approach to HRP conjugation is that antibodies in their *native* form can be added directly to periodate-activated HRP; this is very rarely the case with protein labels (see, for example, the 'two-tag' methods below), though it is possible with modern methods of conjugation (see later).

Conjugations with aldehyde-HRP are carried out at high pH (>9) in amine-free buffers. The resulting bonds formed between lysines and aldehydes are reversible unless stabilized by reduction with sodium cyanoborohydride. This is a disadvantage of the periodate method, particularly as sodium cyanoborohydride is very hazardous.

4. Isothiocyanate method.

This method is worthy of a special mention because of the historical significance of FITC (fluorescein isothiocyanate). FITC has been used for many years to make fluorescein derivatives of antibodies and proteins. Isothiocyanate analogues of other dyes are also commercially available (e.g. TRITC), but NHS ester derivatives are far more common.

Isothiocyanates are more stable than NHS esters which is both an advantage (less prone to decomposition on storage) and a disadvantage (less reactive with antibodies). As with NHS

esters, excess dye reagent has to be removed by chromatography before the conjugate can be used. FITC is water-insoluble and needs to be dissolved in an organic solvent.

Isothiocyanate reactions should be carried out at pH 9.5 or greater, otherwise the reaction with lysines will be very slow. Sodium bicarbonate/sodium carbonate buffer (100mM) is most commonly used. Some monoclonal antibodies may be damaged at high pH values, which is a possible limitation of the method.

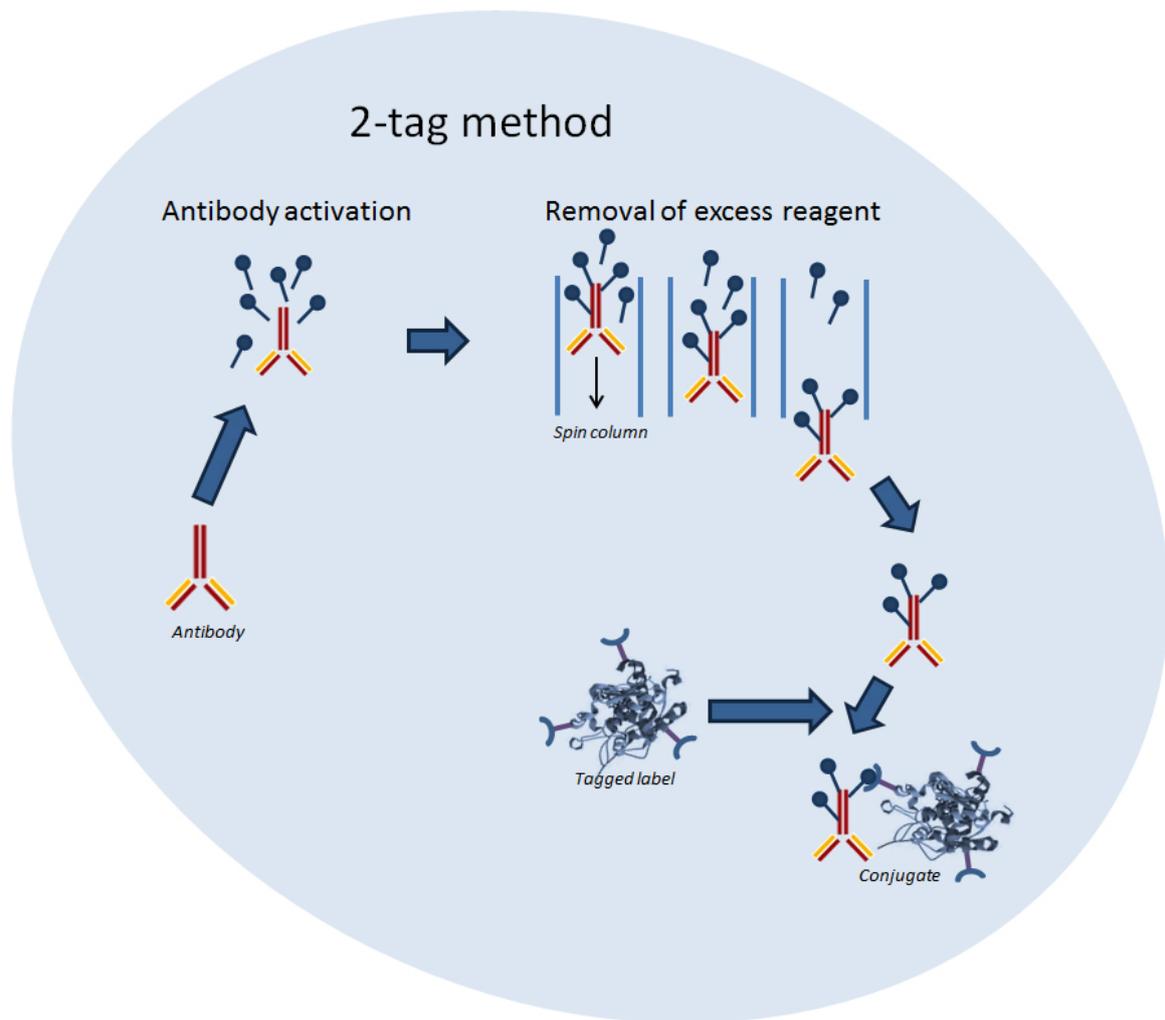
5. 'Two-tag' methods (requiring the antibody to be 'tagged' before a label can be attached).

The 'two-tag' description helps us to classify a subset of protein-antibody conjugation methods that are often chemically dissimilar but conceptually identical; they share a common set of advantages and limitations. These methods can be associated with long chemical names and jargon, but the basic principles are easy to understand.

With protein labels (e.g. alkaline phosphatase, phycoerythrin) the antibody labeling procedure is complicated by the fact that both antibody and label have multiple lysine residues; many reactions other than the one intended can occur. A strategy to avoid unwanted reactions is to modify some of the lysines on one molecule (e.g. the antibody) to introduce a reactive tag ('X') and some of the lysines on the protein label to introduce another reactive tag ('Y'). These tags may also be referred to as 'linkers' or the actual name of the particular chemical groups may be used instead. You can often purchase labels with pre-attached 'Y' tags, but you will usually need to add the 'X' tag to the antibody yourself, or pay for someone else to do it.

The two-tag approach is shown schematically in Figure 3 below.

Figure 3. The two-tag approach to conjugation.



The two-tag approach is often referred to as a ‘heterobifunctional (HBF) conjugation strategy’ because HBF reagents are needed to add the tags or linkers to the antibody. HBF reagents are chemicals that have a lysine-reactive group on one end of the molecule (i.e. an NHS ester) and a tag on the other. The tag therefore becomes attached to lysine residues on the antibody.

After addition of the ‘X’ tags to the antibody (and ‘Y’ tags into label, if required) the excess HBF reagent is removed using desalting columns or spin columns which we discussed earlier. After purification, the antibody with its ‘X’ tags can then be reacted with the ‘Y’ groups on a label to create a heterodimeric conjugate (i.e. antibody connected to a label).

One advantage of the two-tag approach with protein labels is that the formation of heterodimers over homodimers is favored. The major disadvantage of ‘two-tag’ methods is the need to incorporate an ‘X’ tag into the antibody followed by separation steps, *before* the label can be attached.

Pre-conjugation separation steps are immensely disadvantageous in conjugation work as they lead to losses of valuable antibody reagent, uncertainty over the amount of antibody recovered, batch-to-batch variation of conjugates, and lack of scalability of the conjugation process.

Examples of two-tag methods include 'click chemistry' which is a hot topic in the field of organic synthesis. For application to antibodies it is limited, as all tag methods are, by the need to attach a 'click' tag to the antibody (and of course a complementary click tag to the label). Similar limitations apply to 'hydrazone' chemistry, which involves the use of an aromatic aldehyde tag in combination with a hydrazide tag. Other pairs of tags/linkers are also known, but the basic principle remains the same: after adding a tag to the antibody using an unstable NHS ester, the purified antibody derivative then can be conjugated to the subset of labels that have the complementary tag. You cannot mix and match tags, as the chemistries are often completely different, only certain pairings are allowed.

HBF reagents remain very useful for introducing new reactive groups into various aminated molecules and surfaces, but the use of HBFs for *antibody* modification and conjugation should always be questioned, as the approach has been superseded by far simpler methods that do not require the introduction of tags.

If you are unsure whether a particular literature method or commercial kit uses a two-tag approach to conjugation, check the protocol to see if *anything* first has to be attached to the antibody, followed by a separation step (e.g. a spin column), before the actual label is attached. If so, it is a two-tag method of antibody conjugation.

As we mentioned earlier, NHS ester-based HBF reagents are always used in two-tag methods, so the initial reaction pH is between pH 7 and pH 8. The specific requirements for the subsequent conjugation reaction between the purified tagged antibody and a suitably tagged label will of course depend on the specific types of tags that have been introduced.

Buffer additives

Every antibody will contain, minimally, a buffer and/or salts, and possibly other proteins and additives. Different conjugation methods can be impacted to varying extents by substances commonly found in preparations of antibodies but, as mentioned earlier, the majority of labeling methods exploit lysine residues on antibodies. Thus buffers and other additives with *primary amines* should be avoided in all of the antibody labeling procedures discussed above. Sometimes it may be necessary to re-purify an antibody prior to carrying out the labeling reaction especially if there are stabilizing proteins (e.g. BSA) or low molecular weight substances, such as sodium azide, tris or glycine.

Glycine and tris buffer often originate from procedures used to purify and neutralize the antibody, and azide may be added at a later stage in the process to prevent microbial growth. Citric acid is commonly used at low pH to elute antibodies from antigen affinity columns, and in general is preferred over low pH glycine if purified antibodies are to be labeled. However, citric acid is not without its problems as it causes severe interference in carbodiimide-mediated reactions.

What the above observations tell us is that purified antibodies should be *dialysed extensively* before use in conjugation reactions, and that one should not add substances to

purified antibodies without good reason. Dialysis of antibodies is far more efficient if the buffer is changed two or three times, but you do not have to make two or three times as much buffer! For example, if an antibody is eluted from an affinity column with 50mM glycine and 10ml of the antibody is dialysed against 5L of buffer, the final concentration of glycine is 0.1mM (assuming equilibrium has been reached). This may seem quite a low concentration, but a 1mg/ml solution of antibody (i.e. a typical concentration for conjugation reactions) corresponds to a concentration of 6.7uM. Dialysis against 3 x 1L volumes is far more effective in removing glycine (i.e. final concentration of 0.05uM, instead of 100uM for a single 5L dialysis step).

If additives (e.g. azide) need to be added post-dialysis, use the lowest concentration possible (e.g. 0.02%). It also helps if you store antibodies in a concentrated form. For example, if you have 0.02% azide in 5mg/ml antibody the azide will be reduced to 0.004% if the subsequent conjugation reaction is with 1mg/ml antibody. However, if you add 0.1% azide to a solution of 0.5 mg/ml antibody you can expect significant interference in most conjugation reactions.

Antibody Purity

For labeling reactions, the antibody will need to be reasonably pure (e.g. >90%, preferably >95%) and at a concentration of at least 0.5mg/ml. Many commercially available antibodies are provided in a pure form suitable for labeling, but this is not always so. For example, antibodies are also sold as hybridoma tissue culture supernatant (TCS), ascites fluid, or crude serum. TCS often contains many other proteins and culture nutrients (e.g. amino acids) which are particularly problematic. If TCS is the starting material, then antibody purification (e.g. on protein A columns) is obligatory. Ascites fluids and crude serum have higher concentrations of antibody than TCS, but again these materials are impure and contain high concentrations of interfering substances; further purification of the antibody will generally be required.

Lightning-Link® – making antibody labeling easier

In the discussion so far we have identified the most common traditional conjugation methods and discussed their advantages and limitations. It should be possible for you to carry out almost any type of conjugation project using one of these approaches. However, we will now move on to a more modern method of conjugation (Lightning-Link®) which offers a number of advantages over traditional methods.

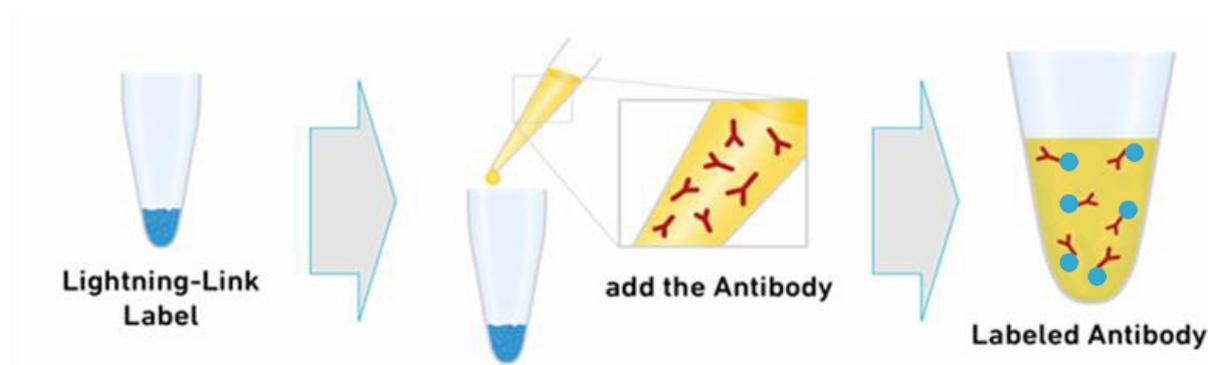
Lightning-Link® technology greatly simplifies the antibody labeling process by eliminating all separation steps (i.e. there are no desalting columns or spin columns). The removal of the pre-conjugation separation steps found in the two-tag methods is especially significant. The simplification of the conjugation process effectively circumvents issues that beset other procedures - losses of material, sample dilution during separation, batch-to-batch variation and difficulties in scaling up.

Lightning-Link® is also remarkably tolerant of common additives and works with azide and tris at modest levels, though additive-free formulations of antibodies are always preferred for the best possible conjugate performance. If your antibody is too impure for conjugation

work (see the advice in the sections on ‘Antibody purity’ and ‘Buffer additives’) a range of AbSelect™ purification kits is available from Innova Biosciences that can address every conceivable starting position (e.g. ascites fluid, tissue culture supernatant, glycine contamination). All of the AbSelect™ components are compatible with the range of Lightning-Link® kits and you can conjugate your antibody immediately after purification without the need for dialysis steps.

The Lightning-Link® process is summarized in *Figure 4*. The antibody to be labeled is simply added to a vial of lyophilized mixture containing the particular label of interest. Dissolution of the vial contents activates the chemicals that mediate the antibody labeling reaction.

Figure 4. Lightning-Link® antibody labeling process



The byproducts of the reaction are completely benign and in the absence of separation steps, either before or after the conjugation reaction, all of the antibody is recovered. The hands-on time is less than thirty seconds. You can see a timed demonstration ([Lightning-Link® video](#)) of the antibody labeling process.

Although the antibody labeling procedure from a user’s perspective is incredibly easy, the chemistry is very sophisticated and allows the formation of high quality antibody conjugates at neutral pH without the complexities of the two-tag methods.

As Lightning-Link® technology targets lysine residues, the method is applicable to all antibodies (and many other proteins too). Furthermore enzymes, dyes, biotin, streptavidin and other proteins can all be attached to antibodies using *exactly the same chemistry*; it is not necessary to use different chemical approaches with different labels.

Another great advantage of Lightning-Link® is that the scale of conjugation can be reduced to low microgram quantities, greatly facilitating conjugate optimization and thus development of assays exhibiting the best possible performance. For example, the smallest standard pack size allows a trial conjugate to be made with just 10ug of antibody. Reactions at this scale are simply not possible with any method that requires separation steps. Even at 100ug scale, substantial amounts of valuable antibody will be lost if a separation step is used.

The scale can be increased easily with Lightning-Link® when required, by orders of magnitude if necessary, and yet the hands-on time remains the same i.e. less than 30 seconds.

Figure 5 below illustrates that it is possible to scale up or scale down without any significant change in conjugate performance.

Figure 5 ELISA of antibodies labeled at various scales with Lightning-Link®

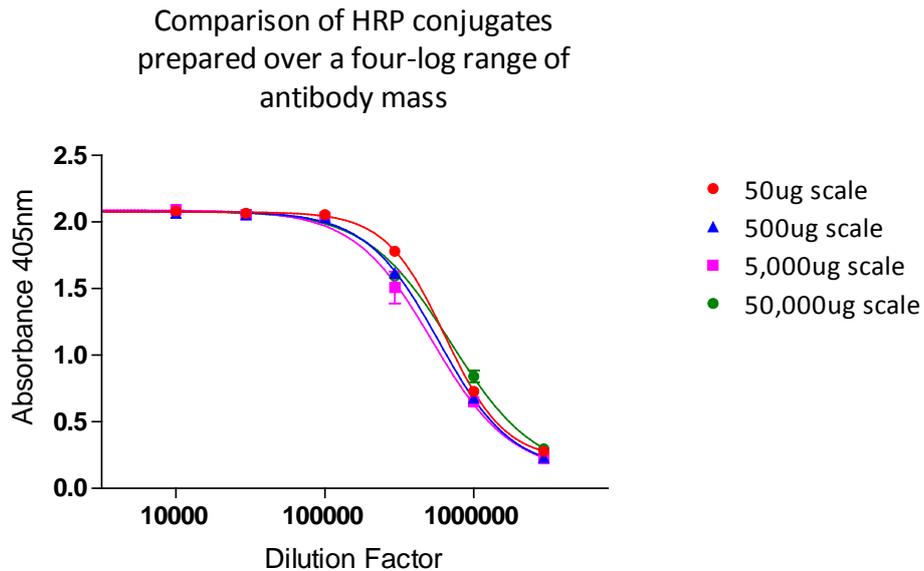


Figure 5: Goat Anti-rabbit IgG antibody was conjugated to Lightning-Link® horseradish peroxidase at four scales. Conjugates were serially diluted and tested on the same rabbit IgG-coated plate. As can be seen there is no significant difference in ELISA performance.

Antibodies (either primary or secondary) labeled with the exceptionally mild Lightning-Link® technique exhibit levels of performance that are better than those prepared with traditional procedures, as illustrated in Figure 6 below.

Figure 6. ELISA of conjugates prepared with a traditional method versus Lightning-Link®

Alkaline Phosphatase anti-Human IgG (Fc) Conjugates Analysed By ELISA

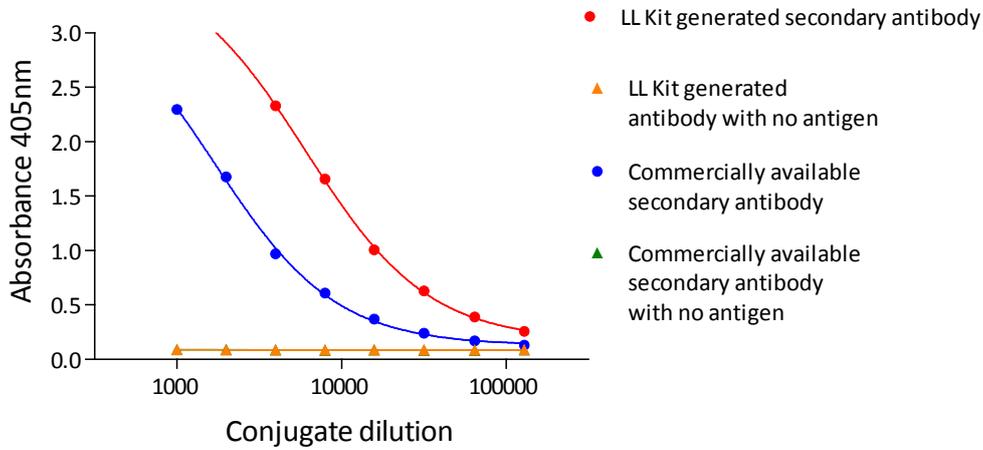


Figure 6: Anti-human IgG monoclonal antibody was purchased pre-conjugated to alkaline phosphatase from a commercial supplier. The same antibody was purchased in un-conjugated form and then conjugated to alkaline phosphatase using a Lightning-Link® (LL) kit. The Lightning-Link® conjugate demonstrates enhanced titre and sensitivity.

Summary

The use of labeled primary antibodies in immunoassays leads to improved assay performance and elimination of tedious wash steps. The main antibody conjugation techniques have been described, which we hope will give you the confidence to make your own labeled primary antibodies. A quick reference table of the different approaches to conjugation is provided in the Appendix. At its simplest, with Lightning-Link®, the act of conjugating an antibody is reduced to a simple pipetting operation taking less than 30 seconds. Since all antibodies are compatible with Lightning-Link® in their *native* form, the operation of adding tags or linkers to antibodies is now redundant. It is fair to say that antibody labeling has never been easier.

Appendix: Key features of the main antibody conjugation technologies.

Chemistry	Avoids tagging & pre-conjugation separation steps?	Avoids post-conjugation separation steps?	Works with enzymes and fluorescent labels?	Works with dyes & small molecules?	Ease of scaling up	Hands-on time	10ug scale possible?	Typical antibody yield	Other comments
NHS ester	Yes	No	No	Yes	Moderate	> 15 min	No	50-80%	NHS ester unstable, variable efficiency
Isothiocyanate	Yes	No	No	Yes	Moderate	> 15 min	No	50-80%	Not very reactive, high pH needed
Carbodiimide	Yes	No	No	Yes/No ³	Moderate	> 15 min	No	50-80%	Never used to attach protein labels
Two-tag methods	No	Yes	Yes	Yes	Difficult	> 60 min	No	20-50%	Inherently variable because of complexity
Periodate	Yes	No ¹	Yes/No ²	No	Moderate	> 15 min	Yes/No ¹	70-80%	Requires hazardous chemicals
Lightning-Link	Yes	Yes	Yes	Yes	Easy	30 seconds	Yes	100%	One step, no losses, over fifty labels

1. Possibly (if sodium cyanoborohydride is not removed).

2. Limited range of application, only for use with glycoprotein labels, and mainly with HRP.

3. Commonly used in reactions involving small molecules, but almost never in reactions of small molecules with antibodies.