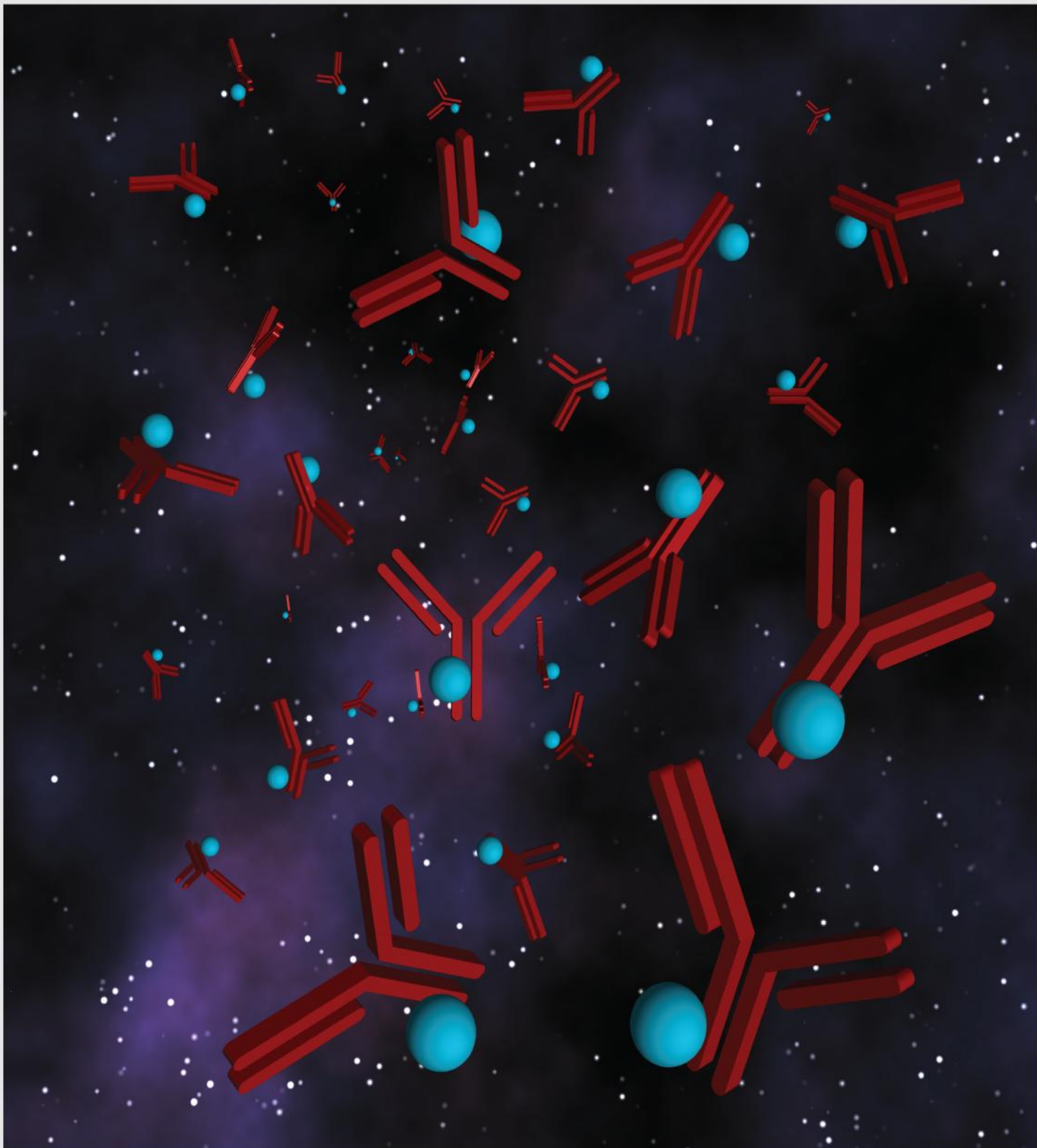


Guide to Antibody Labeling and Detection



Innova Biosciences
first for Antibody labeling

GUIDE TO ANTIBODY LABELING AND DETECTION

Anyone new to this field will find in the literature a bewildering number of antibody labeling protocols. It is difficult for the newcomer to know what the critical parameters are and what procedure should be applied in any given situation. With traditional labeling methods, a basic understanding of the principles of chemical modification is required because the antibody and/or label must be chemically ‘activated’ before the labeled antibody (or ‘conjugate’) can be formed.

This guide is a user friendly tool that allows you to learn the basics of common antibody labeling methods. It also describes [Lightning-Link](#) technology, which massively simplifies the production of labeled antibodies. The Lightning-Link approach requires no knowledge of chemistry and the hands-on time is just 30 seconds.

Antibodies and labels

Antibodies are widely used in immunoassays to detect and quantify antigens. The antibody that recognizes the antigen is referred to as the ‘primary’ antibody and confers specificity to the assay. A ‘label’ is also incorporated into the assay using one of two methods (see below - Indirect Vs. Direct Detection Methods) to provide measurability. Some commonly used immunoassay techniques are given in Table 1 along with examples of the types of labels that may be employed.

Table 1 Types of Immuno-experiments and associated labels

Immunoassay	Labels
Western Blotting	Enzymes (usually HRP, or alkaline phosphatase)
ELISA	Enzymes, Biotin/Streptavidin
Immunofluorescence	Fluorescent dyes
Immunohistochemistry	Enzymes, Biotin/Streptavidin
Flow Cytometry	Fluorescent proteins or dyes, Tandem dyes

Indirect Vs. Direct Detection Methods

The label in an immunoassay provides either ‘direct’ or ‘indirect’ detection of the antigen. With *direct* detection, the label is attached via a covalent bond to the primary antibody. Alternatively, using *indirect* detection, the label is covalently attached to a secondary antibody, which is allowed to bind to the primary antibody during the immunoassay.

In the case of indirect detection, the assay comprises two distinct parts: first, there is a period of incubation (usually one hour) with the *unlabeled* primary antibody, during which a small fraction of the antibody binds to the antigen (assuming of course that the antigen is present). Excess unbound primary antibody is then washed away and a labeled secondary reagent is added. After a period of incubation (again one hour), excess secondary reagent is washed away and the amount of label associated with the primary antibody (i.e. indirectly via the

secondary reagent) is quantified. The label usually results in the production of a colored substance or an increase in the amount of light emitted at a certain wavelength, if the antigen is present. In the absence of antigen there is no binding of the primary antibody and no binding of the secondary reagent, and thus no signal.

With direct detection, the prior covalent attachment of the label to the primary antibody means that only a single incubation step with the antigen is required and only a single 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 decrease assay variability and to improve data quality. Some of the often-stated pros and cons of direct/indirect detection methods are given in Table 2.

Table 2 Pros and Cons of Direct Vs Indirect Labeling

Method	Pros	Cons
Direct	<ul style="list-style-type: none"> • Quick methodology since only one antibody is used. • Non specific binding of secondary antibody is eliminated. 	<ul style="list-style-type: none"> • Immunoreactivity of the primary antibody may be reduced as a result of labeling. • Little signal amplification.
Indirect	<ul style="list-style-type: none"> • Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification. 	<ul style="list-style-type: none"> • Non specific binding may occur with the secondary antibody. • Extra incubation and wash steps are required in the procedure.

Despite the potential advantages of direct detection, many immunoassays today still employ the principle of indirect detection. Undoubtedly the main reason for this is that direct labeling of primary antibodies is relatively complicated and indeed, historically, antibody labeling has been carried out only by those with specialist knowledge of chemical-modification techniques.

What about the amplification afforded by the secondary reagent in indirect detection? Is the signal lower with the direct labeling approach? In many cases the ‘amplification’ of the indirect method may actually be illusory, as the primary antibody dissociates from the antigen during the incubation with the secondary reagent and during the following wash steps, thus what is actually being amplified is a diminishing amount of primary antibody. In many cases the same or even a better result can be obtained more easily with direct detection.

Some techniques commonly used in the labeling of antibodies and other biomolecules are outlined in the following section.

Antibody Labeling Methods

Antibodies like all proteins are composed of amino acids, and the side chain of lysine, which terminates in a primary amine (-NH₂), is almost always used to link labels covalently to antibody molecules. Despite the many variant labeling procedures in the literature, in reality

there are just four common approaches to labeling, which differ only in the way that the label is turned into a reactive derivative. Whatever approach is used the label almost without exception is covalently linked via lysine residues on the antibody molecule.

The four main chemical approaches for antibody labeling are summarized below:

1. NHS esters. In the case of fluorescent dye labels it is usual to purchase an activated form of the label with an inbuilt NHS ester (also called a 'succinimidyl ester'). The activated dye can be reacted under appropriate conditions with antibodies (all of which have multiple lysine groups). Excess reactive dye is removed by one of several possible methods (often column chromatography) before the labeled antibody can be used in an immunoassay.

2. Heterobifunctional reagents. If the label is a protein molecule (e.g. HRP, alkaline phosphatase, or phycoerythrin) the antibody labeling procedure is complicated by the fact that the antibody *and* label have multiple amines. In this situation it is usual to modify some of the lysines on one molecule (e.g. the antibody) to create a new reactive group (X) and lysines on the label to create another reactive group (Y). A 'heterobifunctional reagent' is used to introduce the Y groups, which subsequently react with X groups when the antibody and label are mixed, thus creating heterodimeric conjugates. There are many variations on this theme and you will find hundreds of examples in the literature on the use of heterobifunctional reagents to create labeled antibodies and other labeled biomolecules.

3. Carbodiimides. These reagents (EDC is one very common example) are used to create covalent links between amine- and carboxyl-containing molecules. Carbodiimides activate carboxyl groups, and the activated intermediate is then attacked by an amine (e.g. provided by a lysine residue on an antibody). Carbodiimides are commonly used to conjugate antibodies to carboxylated particles (e.g. latex particles, magnetic beads), and to other carboxylated surfaces, such as microwell plates or chip surfaces. Carbodiimides are rarely used to attach dyes or protein labels to antibodies, although they are important in the production of NHS-activated dyes (see above).

4. Sodium periodate. This chemical cannot be employed with the vast majority of labels but is quite an important reagent in that it is applicable to HRP, the most popular diagnostic enzyme. Periodate activates carbohydrate chains on the HRP molecule to create aldehyde groups, which are capable of reacting with lysines on antibody molecules. Since HRP itself has very few lysines it is relatively easy to create antibody-HRP conjugates without significant HRP polymerization.

You may find more detailed instruction on these four approaches from any specialist book on antibody labeling.

Buffers and Additives – Key Considerations for Antibody Labeling

It is important to remember that your antibody will contain substances other than antibody; minimally a buffer and/or salts, and possibly other proteins and additives. Compatibility of the mixture with labeling methods may not have been a key consideration when the antibody was initially purified and formulated; occasionally it will be necessary to re-purify the antibody prior to carrying out the labeling reaction. Purification may involve the removal of stabilizing proteins (e.g. BSA) or the removal of low molecular weight substances, such as sodium azide, tris buffer or glycine. The different labeling methods may be negatively

impacted to different extents by the various additives but, as mentioned earlier, the majority of labeling methods exploit lysine residues on antibodies, thus substances with primary amines should generally be avoided in antibody labeling procedures. For example, glycine is often used to elute antibodies from antigen affinity columns and antibodies that have been purified in this way should be dialysed before use in labeling reactions. One should be particularly aware with dialysis that the removal of unwanted low molecular weight substances is more efficient if the buffer is changed two or three times. This does not mean that you have to make three times as much buffer, quite the contrary, though the total time required for the dialysis procedure will be greater. For example, calculation shows that dialysis against 3 x 1L volumes is far more effective than dialysis against a single 5L volume. PBS, MES or bicarbonate are often recommended as suitable buffers in which to carry out conjugation reactions; the best buffer is determined from the pH requirements of the labeling reaction.

Antibody Concentration and Purity

For most labeling reactions, the antibody will need to be reasonably pure (e.g. >90%, preferably >95%) and at a concentration of >0.5mg/ml. Many commercially available antibodies are provided in a form suitable for labeling, but you should be aware that this is not always so. For example, antibodies may be sold in the form of 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. Purification (e.g. on protein A columns) is more or less obligatory if TCS is your starting point. Ascites fluids and crude serum have higher concentrations of antibody than TCS, but again these materials are impure and contain high concentrations of amino acids; further purification of the antibody will generally be required.

At this point it is probably useful to provide a summary of the above: you now know about (i) the key differences between direct and indirect detection; (ii) the four main approaches to antibody labeling; (iii) the importance of buffer formulations and antibody concentration

In reality, today, because of advances in antibody labeling technology, anyone who can use a hand-held pipette can make labeled antibodies with ease. The [Lightning-Link](#) one-step antibody labeling method is discussed below.

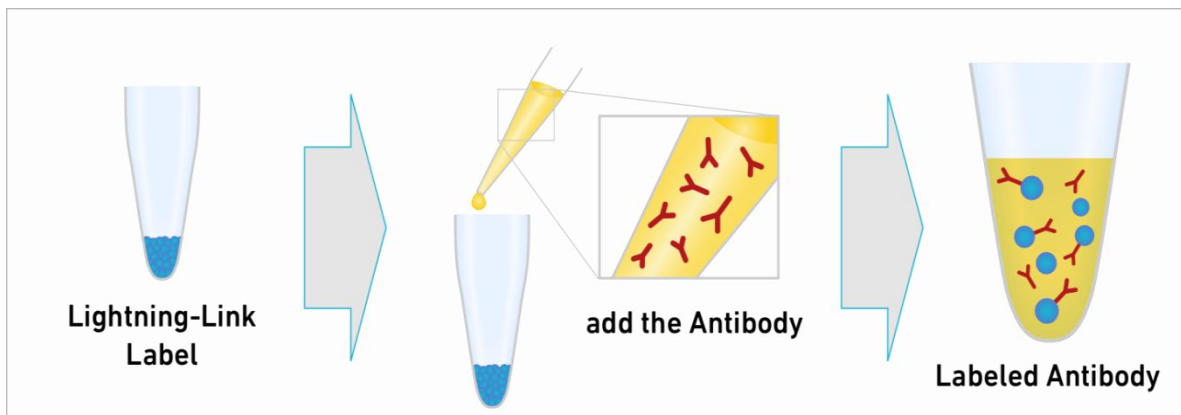
Lightning-Link – The World’s easiest Antibody Labeling Kits

The simplification of the antibody labeling process (most notably the elimination of column separation steps) circumvents many issues that have beset traditional procedures for years - loss of material, sample dilution during column chromatography, batch-to-batch variation and difficulties in scaling up.

The Lightning-Link process is summarized in *Figure 1*. You simply pipette the antibody to be labeled into a vial of lyophilized mixture containing the label of interest. Dissolution of the vial contents activates the chemicals that mediate the antibody labeling reaction. As there are no purification or separation steps (byproducts of the reaction are completely benign), antibody recovery is close to 100%. Furthermore the labeling reaction can be set up using this

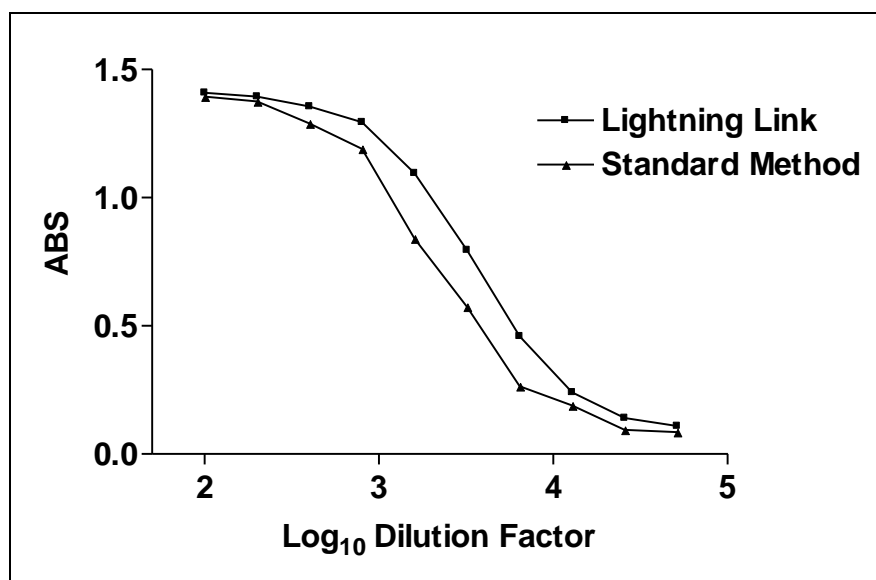
simple method in less than thirty seconds. You can see a timed demonstration ([Lightning-Link video](#)) of the antibody labeling process.

Figure 1 Lightning-Link antibody labeling process



Although the antibody labeling procedure is very simple, the chemical approach is sophisticated – allowing the formation only of antibody-label conjugates in a gentle and controlled process. Antibodies labeled with Lightning-Link exhibit performance characteristics that are identical with, or better than, those prepared with laborious multistep traditional procedures – please see figure 2 below.

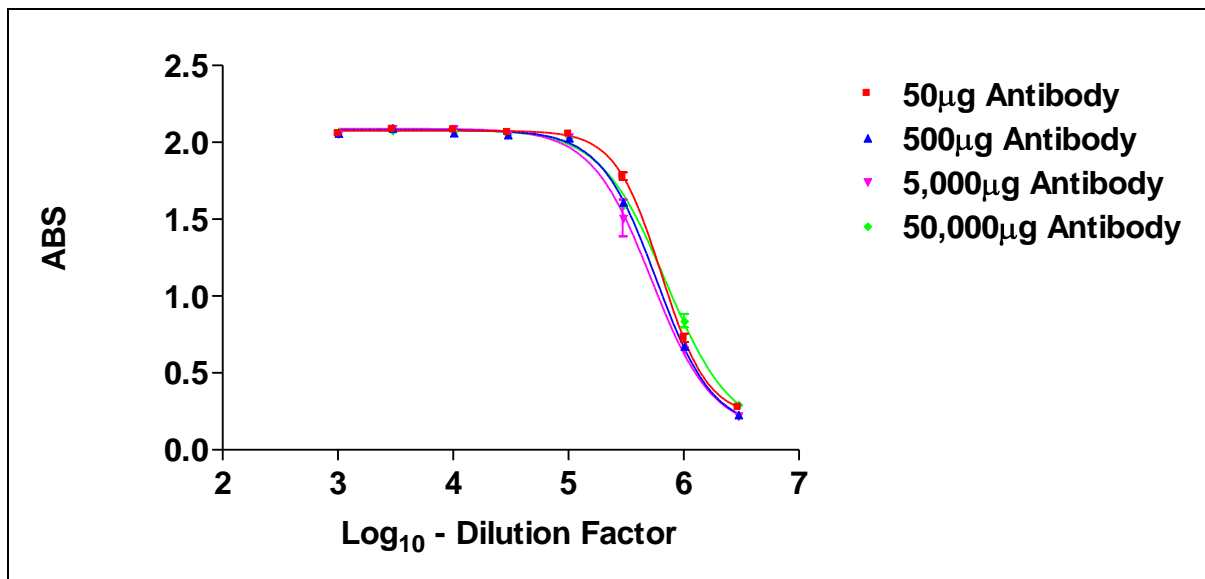
Figure 2 ELISA of conjugates prepared with a standard method Vs Lightning-Link



The simplicity of the Lightning-Link approach means that it is very easy to scale up (or scale down) the amount of antibody to be labeled. Whether you are labeling small or bulk amounts of antibody, the hands on time remains the same – just 30 seconds. Because there are no new technical issues created by scaling up, the performance of trial conjugate prepared at small scale is essentially identical to that prepared at bulk scale (see Figure 3 below). For any new antibody, or in situations where the antibody is extremely valuable and in limited supply,

Lightning-Link kits allow labeling reactions to be carried out with as little as 10µg of antibody. Other regular pack sizes include 100ug, 1mg and 5mg.

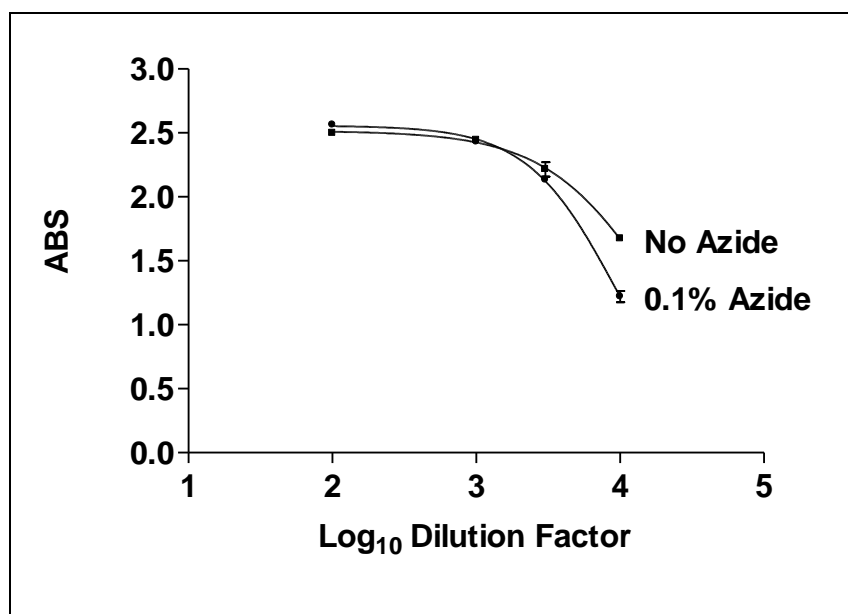
Figure 3 ELISA of antibodies labeled at various scales with Lightning-Link



Although the hands-on aspect of doing antibody labeling reactions is now incredibly simple; as mentioned above it is still necessary to think about what else is present in your preparation of antibody before undertaking a labeling reaction. However, Lightning-Link is remarkably tolerant of many additives found in antibody preparations.

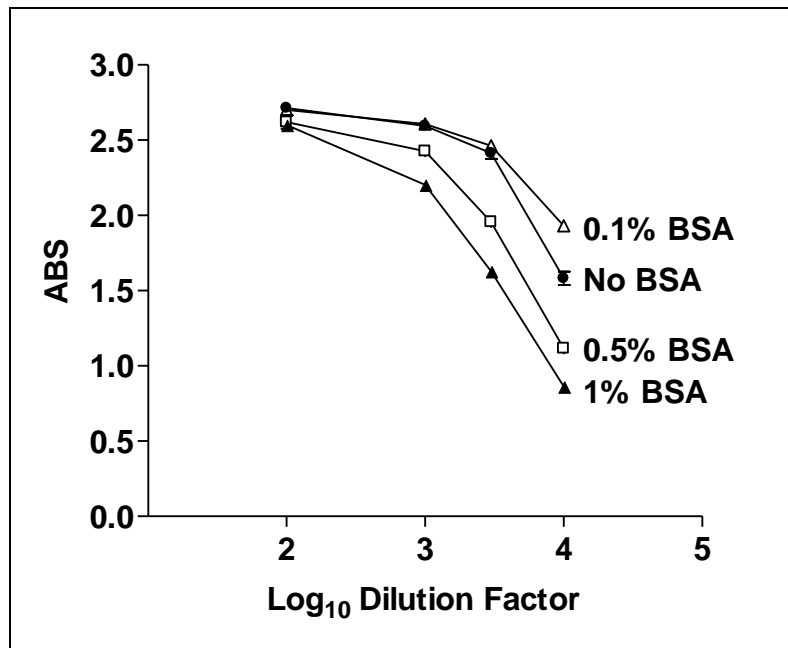
Sodium azide, which is widely employed as an antimicrobial agent in commercially available antibodies, typically at levels of 0.02% or 0.1%, causes serious interference with some labeling methods. Fortunately, the [Lightning-Link](#) approach is minimally impacted by sodium azide (figure 4) at concentrations of 0.1% or less.

Figure 4 – ELISA using antibodies labeled in the presence/absence of sodium azide



Another common additive in commercially available antibodies is BSA, which is used as a stabilizer. While one would anticipate serious interference from a molecule such as BSA in labeling reactions, BSA has only a very modest impact on Lightning-Link. Indeed, the possible benefit of removing BSA from very small amounts of antibody is likely to be offset by losses of antibody during any attempted purification. For example, with 1% BSA, which equates to a 20-fold molar excess over 1mg/ml antibody, the normal ELISA absorbance could be achieved simply by diluting the antibody labeled in the presence of BSA by 1/3000, instead of 1/10,000 for the BSA-free reaction (see figure 5).

Figure 5 – ELISA of labeled antibodies made in presence of BSA



It was discussed earlier that antibodies are sometimes supplied as TCS or ascites fluid. Innova also provides a range of antibody purification and clean up products so that every antibody preparation can be made compatible with the world's easiest [antibody labeling](#) technology:

[AbSelect™](#) Antibody Purification System readily removes unwanted substances such as proteins and amino acids that interfere with labeling reactions. It can be used to purify antibodies from crude samples such as ascites fluid or serum.

[Antibody Concentration and Cleanup](#) Kit allows for the quick and easy concentration of antibodies. The kit can also be used to reduce the concentration of any undesirable low molecular weight additives.

Go Direct!

With Lightning-Link technology you are now able to label any antibody with any one of over 45 labels to generate novel antibody research tools for your pioneering research. Direct

labeling of antibodies greatly simplifies immuno-experiments; without the problems of crossover and/or non specific binding from secondary antibodies it is far easier to obtain high quality data. A reduction in the number of tedious incubation and wash steps also saves time and money. Moreover you can now label as little as 10ug of antibody and scale up with ease.

To see the full range of [Lightning-Link](#) kits see our [product listing](#) at innovabiosciences.com.

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