Innova Biosciences

Guide to labeling your primary antibody

Innova Biosciences Guide

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Antibodies are used to detect and quantify antigens in techniques such as flow cytometry, ELISA, western blotting, immunohistochemistry and lateral flow. The antibody that binds to the antigen is called a 'primary an¬tibody' and it confers specificity to the assay. Most types of immunoassays also incorporate a 'label' whose purpose is to provide measurability. Assays are classed as 'direct' if the label is conjugated to the primary antibody or 'indirect' if the label is attached to another molecule, called a 'secondary reagent'. Labels include but are not limited to organic dyes, fluorescent proteins, colored particles and enzymes.

There are many methods of covalent antibody conjugation but the strategy usually is to attach the label to lysine residues. We discuss below the pros and cons of traditional approaches to conjugation and compare and contrast these methods with Lightning-Link®, a revolutionary technology that has transformed the way in which immunoreagents are made. Today, anyone with just 30 seconds of time can make his/her own primary antibody conjugate.

1. Why label your primary antibody?

All immunoassays can be greatly simplified by covalently attaching the label directly to the primary antibody. Some of the advantages of direct assays are:

(i) Avoidance of non-specific binding with labeled secondary antibodies.

(ii) Ability to multiplex with antibodies from the same spec¬ies.

(iii) Reduction in the number of incubation steps.

(iv) Reduction in the number of wash steps.

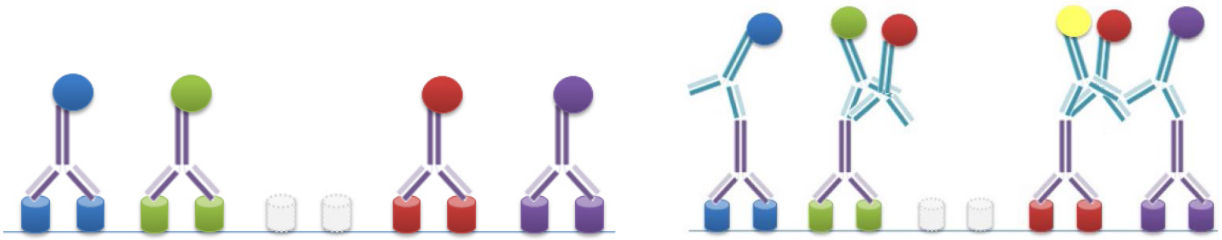
(v) Better data quality/less variability because of assay simplification.

In the indirect assay format the label is not physically attached to the primary antibody and all of the incubation and wash steps have to be repeated with the secondary reagent. Typically this is an anti-species antibody conjugated to a label which indirectly reports the presence or absence of antigen by binding to the primary antibody. We can state the disadvantages of the indirect assay format by writing the exact opposite of the five points listed above.

Multiplexed assays are particularly difficult to validate using the indirect assay approach. In Figure 1 (left panel), five directly labeled antibodies are being used to probe a cell surface which features four of the five antigens of interest. The matched colors of the labels and antigens illustrate schematically that the correct binding events have taken place. The more complex indirect approach (right panel) requires five primary antibodies, which must be from five different species, as well as five species-specific labeled secondary antibodies. If any of the secondary reagents 'crossover' onto the wrong primary antibody, which is a very common indirect assay problem, the results will be misleading.

In Figure 1 the apparent level and/or distribution of the red and green antigens will be wrong in the indirect assay, and the yellow antigen will be 'detected' when in reality it is not present at all.

Figure 1. The direct labeling approach is simpler and avoids problems such as cross-over.



2. Dissociation rates and amplification in indirect assays

One of the advantages of indirect assays is presumed amplification of the assay signal because more than one secondary antibody can bind to the primary antibody. Conceptually this is easy to understand and more than one secondary antibody may well bind. However, the dissociation rate of the primary antibody and its impact on the assay performance is seldom considered. The concentration of primary antibody in solution when the secondary reagent is added is zero. As the bound primary antibody does not have an infinitely high affinity, the system relaxes to a new equilibrium during the second part of the indirect assay and a steadily increasing proportion of the primary antibody returns 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 amplified is a diminishing amount of primary antibody. The same assay result, or an even better result, may be obtained more easily with a direct detection method. This is illustrated below in Figure 2 with immunoperoxidase staining of tonsil tissue.

Figure 2. IHC data demonstrating the direct and indirect labeling approaches.

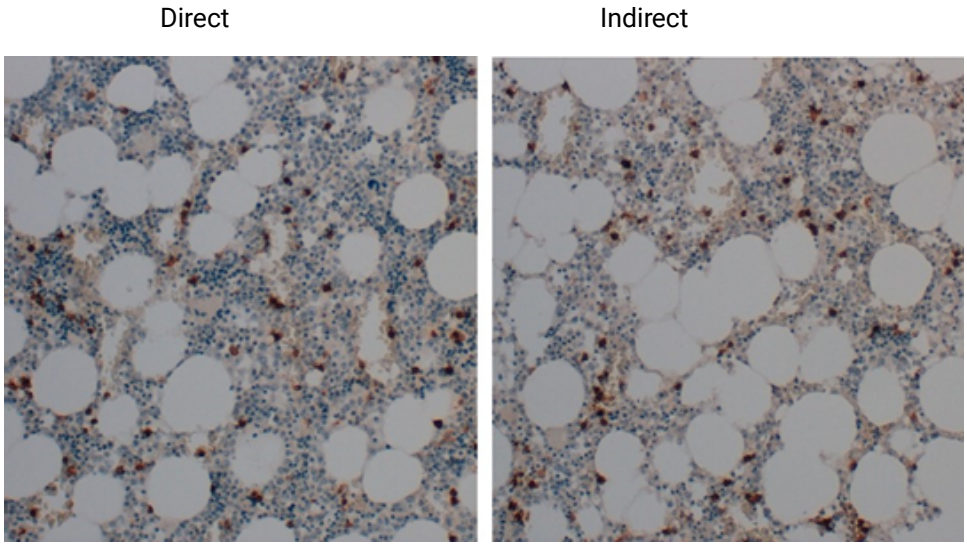


Figure 2 shows immunohistochemical staining of CD20 in formalin-fixed, paraffin-embedded sections of human tonsil. The left-hand image shows clone L26 directly conjugated to HRP. The right-hand image shows indirect labeling with clone L26 in combination with a high sensitivity poly-HRP detection system.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

3. Antibody Labeling Methods

Most (but not all) conjugation methods require knowledge of chemistry and experience in separation techniques e.g. fixed-column chromatography or spin columns in order to remove low-molecular-weight activation reagents from the label and/or antibody. Separation steps result in losses of materials, batch-to-batch variation of conjugates and complications in scaling up.

The main traditional chemical approaches to antibody labeling and modern methods such as Lightning-Link are described below together with the pros and cons of each approach. As labels are usually attached to the terminal amine group of lysine, primary amine-containing buffers and additives (e.g. Tris or glycine) should be avoided in conjugation work. Sodium azide, an anti-microbial agent, is also likely to be very problematic with most chemistries.

Considerations before you begin

(i) Antibody Concentration and Purity

The antibody will need to be reasonably pure (e.g. >90%, preferably >95%, without BSA) and ideally at a concentration of >0.5mg/ml. Many commercially available antibodies are suitable for immediate labeling, but crude samples such as hybridoma tissue culture supernatant (TCS), ascites fluid or serum must be purified to remove interfering proteins and/or small molecules. Note: TCS has relatively low concentrations of antibody. Antibodies are usually purified on protein A columns or antigen affinity columns (if available). Chemicals used in the purification of antibodies (e.g. Tris, glycine, citric acid) or that are added to antibodies (e.g. BSA, azide) can interfere in many conjugation chemistries.

(ii) Removal of interfering substances by dialysis

Dialysis can be used to remove all potentially interfering low-molecular-weight substances from antibodies. It is important to understand that the dialysis process is far 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 (indeed less may be needed) though the total time of the dialysis procedure will be greater. For example, the concentration of glycine (initially 50mM) in a 20ml sample of antibody is reduced to 200µM after dialysis against 1 x 5L of buffer. By contrast the concentration of glycine is only 0.4µM after sequential dialysis against 3 x 1L of buffer. The dialysis buffer should of course be compatible with the intended labeling reaction.

Main conjugation methods

A. NHS (succinimidyl) ester method

This approach is often used with fluorescent dyes which are widely available with a reactive NHS group. After reaction with the antibody any surplus reactive dye is removed by size separation using chromatography columns under gravity or spin columns in a microfuge. These separation steps result in losses of conjugate but 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 unstable and sensitive to moisture; when pots of NHS esters are opened and then stored in fridges/freezers the reactive groups decay.

All NHS esters must be dissolved in an organic solvent (e.g. DMSO) at a high concentration (to limit the amount of solvent added to the antibody). As solvents are never truly dry the NHS ester cannot be stored and must be used immediately after dissolution.

The NHS method of conjugation is often quite wasteful as the smallest amount of dye that can be weighed out accurately often greatly exceeds the actual quantity that is needed to label the antibody.
Dye-NHS ester reactions may be carried out at pH 7.2-8.0 in a primary amine-free buffer. A good choice is 50mM sodium phosphate buffer. The rate of reaction increases with increasing pH, but so does the rate of ester decomposition (hydrolysis).

Pros: Pre-activated dyes are widely available.

Cons: NHS esters are very unstable, applicable only to dye labels, and post-labeling purification is needed.

B. Two-tag method (heterobifunctional method)

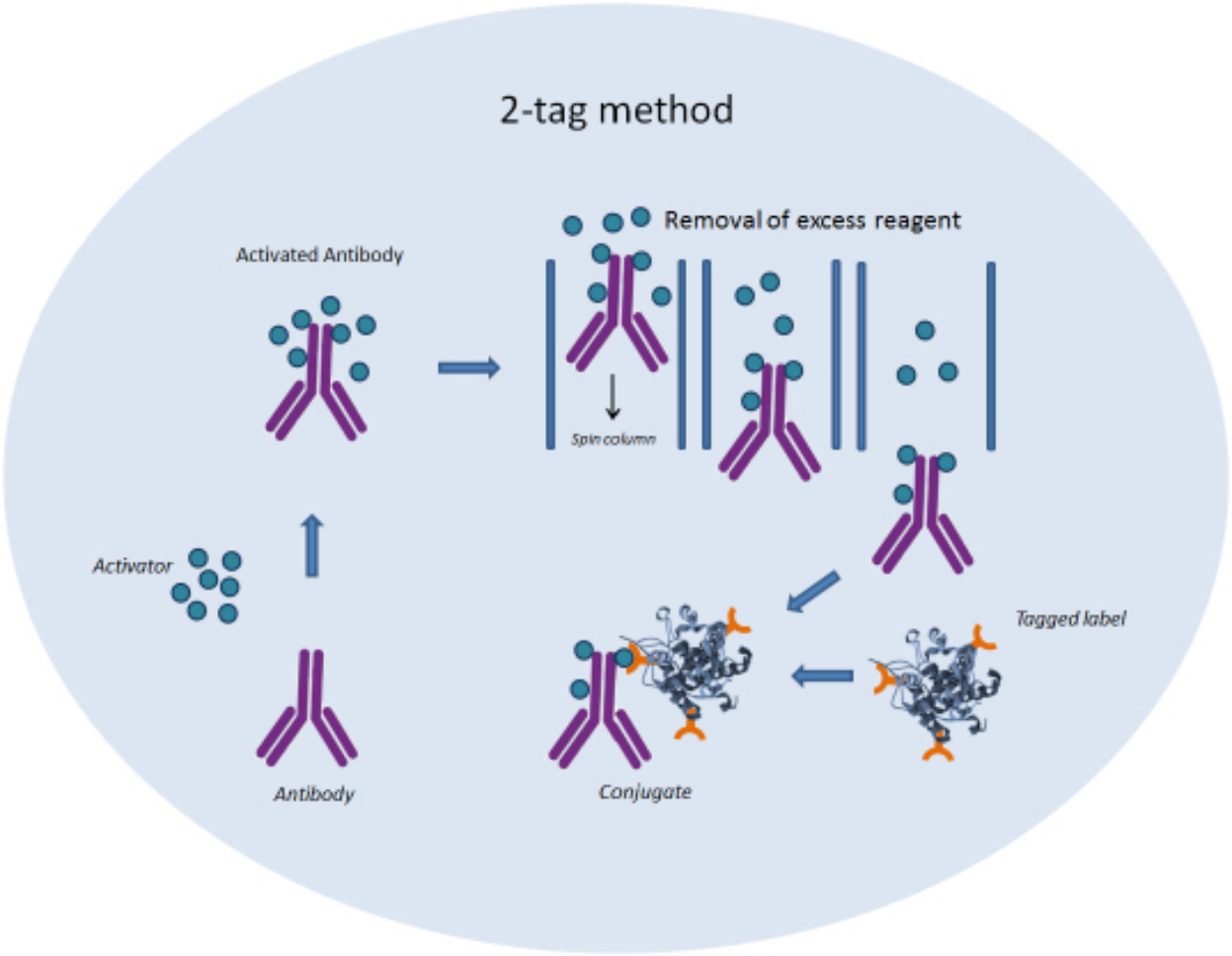
If the label is a protein molecule (e.g. alkaline phosphatase, HRP or phycoerythrin) both the antibody (Ab) and label (L) will have multiple lysine groups. Since molecules are usually attached via lysines there is a risk of forming Ab-Ab or L-L homodimers, or worse still high-molecular-weight aggregates.

A degree of control over the conjugation process may be exerted by using heterobifunctional reagents, linear molecules usually with a NHS ester group at one end and one of several possible functional groups (e.g. 'X' or 'Y') at the other end. The X and Y groups are often referred to as 'tags', 'linkers', or 'activators' and they will, if brought into contact with one another, react to form an X-Y link.

In a two-tag strategy of conjugation, some of the lysines on one molecule (e.g. the antibody) are reacted with the X-NHS ester to introduce the X tag (analogous to incorporating a fluorescent dye, see section A). Lysines on the other molecule (e.g. alkaline phosphatase) are reacted with the Y-NHS ester to introduce the Y group. The antibody and label are purified from excess heterobifunctional reagent by size separation and then mixed, at which point the X and Y tags on the protein molecules can react to form heterodimeric conjugates.

There are several types of X groups (e.g. X1, X2, X3) which are chemically very different from one another and each one must be paired with an appropriate Y group (e.g. Y1, Y2 or Y3). Thus labels with Y2 functionality cannot react with an antibody with X1 groups. Labels can sometimes be purchased with pre-attached 'Y' tags, but you always need to add the appropriate 'X' tag to the antibody yourself using the NHS ester method or pay someone else to do it. The two-tag approach is shown schematically in Figure 3.

Figure 3. The two-tag approach to conjugation.



Examples of the two-tag approach to conjugation include 'click chemistry', which is particularly important in the field of organic synthesis and has been applied in a limited way with antibodies. Hydrazone chemistry uses an aromatic aldehyde on one molecule which is paired with a hydrazide tag on the other. Maleimide and thiol tags represent another common pairing, and there are several other examples.

A major disadvantage of two-tag methods is the need to incorporate an 'X' tag into the antibody followed by a separation step. Pre-conjugation separation steps are always 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. The need for separation steps also means that one must begin the conjugation process with a significant amount of antibody.

Pros: Controlled reaction to form heterodimers.

Cons: NHS ester derivatives are needed to add tags, separation steps, batch-to-batch variability, and high antibody consumption.

C. Carbodiimide method

Carbodiimide reagents are used to convert carboxyl groups into a highly reactive intermediate, an o-acylisourea, which can react with amines (e.g. lysines). Carbodiimides leave no trace in the final conjugate and are therefore sometimes called 'zero-length crosslinkers'. Hundreds of carbodiimides are known but as most of them are insoluble in water they are used mainly in synthetic organic chemistry. One such application of carbodiimides is the production of NHS-activated dyes and NHS-activated tags and linkers, which we discussed earlier.

The water-soluble carbodiimide most often used in biology is EDC (EDAC). Note: EDC is never used to attach protein labels to antibodies because both the antibody and label have amine and carboxyl groups. EDC is normally used to attach antibodies to carboxylated particles (e.g. latex, gold or magnetic). Sometimes N-hydroxysuccinimide (NHS) is added with EDC so that the reaction with amines proceeds via the relatively stable NHS ester. After activation of the carboxyl groups on the particles any excess reactants are washed away (e.g. by spinning down the particles) and the antibody is then added. Carbodiimides also find use in the covalent immobilisation of antibodies on surfaces, such as microwell plates or chip surfaces.

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 a particularly effective buffer at pH 4.7. A reaction pH of around pH 5.5-6.0 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 (> pH 7). Phosphate buffer is also commonly recommended for EDC reactions; it is acceptable but not the best choice, as phosphate reduces reaction efficiency.

Pros: Relatively simple.
Cons: Risk of excessive crosslinking with biomolecules, and EDC is moisture sensitive.

D. Periodate method

Sodium periodate is a powerful oxidizer and can be used to activate the glycoprotein horseradish peroxidase (HRP), the most popular diagnostic enzyme. Periodate reacts with the carbohydrate portion of the molecule to create aldehyde groups, which can react with lysine residues in antibodies. HRP itself has relatively few lysines which is helpful in limiting self-polymerization of the label. A major advantage of the periodate approach is that antibodies in their native form can be linked directly to periodate-activated HRP i.e. no tags have to be added to the antibody.

Conjugations with aldehyde-HRP are carried out at pH 9.5 in an amine-free buffer (e.g. 100mM sodium carbonate). The resulting bonds formed between lysines and aldehydes are reversible unless stabilized by treatment with sodium cyanoborohydride.

Pros: Simple, no tags on the antibody are needed.
Cons: Not widely applicable, high pH, and a cyanide compound is needed for stabilization.

E. Isothiocyanate method

This method is worthy of a mention because of the historical significance of FITC (fluorescein isothiocyanate), which was (and still is) used to make fluorescein derivatives of antibodies and proteins. Isothiocyanate analogues of other dyes are also commercially available (e.g. TRITC), but NHS esters are 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). As with NHS esters, excess dye reagent has to be removed by chromatography before the conjugate can be used. FITC needs to be dissolved in an organic solvent (e.g. DMSO, DMF).

Isothiocyanate reactions should be carried out at pH 9 or greater, otherwise the reaction with lysines will be very slow. Sodium bicarbonate/sodium carbonate buffer (100mM) is most commonly used.

Pros: Isothiocyanate derivatives of dyes are more stable than corresponding NHS esters

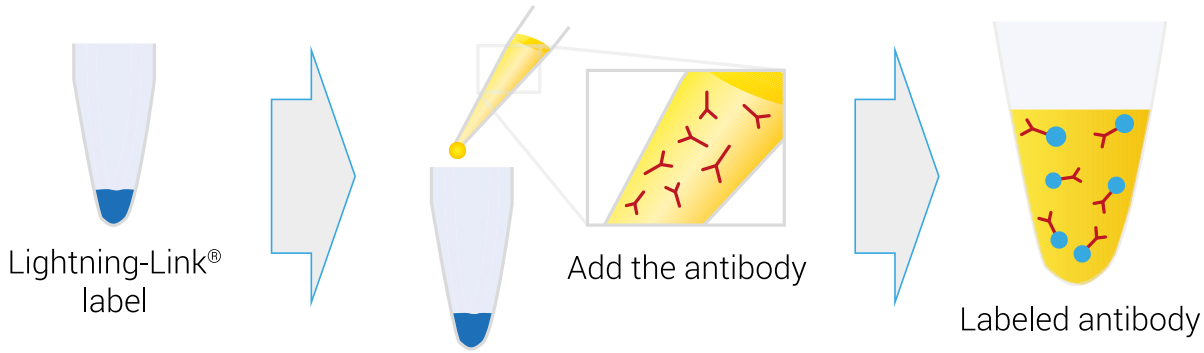
Cons: Not very reactive, high pH needed, limited to a relatively small number of dyes.

F. Lightning-Link® method

Lightning-Link technology is a sophisticated approach to conjugation that eliminates all separation steps, thus circumventing many difficulties associated with traditional procedures: loss of material during separation steps, batch-to-batch variation and difficulties in scaling up.

The Lightning-Link process is summarized in Figure 4. The antibody to be labeled is added to a vial of lyophilized mixture containing the particular label of interest; over 40 labels are available in this format including dyes, proteins and enzymes. Dissolution of the vial contents activates the chemicals that mediate the antibody labeling reaction. The byproducts of the reaction are completely benign and antibody recovery is 100%. Furthermore the hands-on time is just thirty seconds, and this is true whatever the scale of reaction (10µg-100mg range).

Figure 4. Lightning-Link antibody labeling process



Although the antibody labeling procedure from a user's perspective is just one-step, the technology behind this approach is very sophisticated and involves an orchestrated set of reactions which leads to the controlled formation of antibody-label conjugates at neutral pH. Lightning-Link conjugates exhibit performance characteristics that are identical to or better than those prepared with laborious multistep procedures.

For any new antibody, or in situations where the antibody is extremely valuable and in limited supply, trial conjugates can be made at just 10µg scale (because there are no separation steps). The simplicity of the approach also means that it is easy to scale up the amount of antibody to be labeled without any change in conjugate performance.

Pros: 30 seconds hands-on time, one chemistry for all dye or protein labels, no organic solvents, no separation steps, trial conjugations at 10µg scale, fully scalable.

Cons: Fixed amount of antibody must be used (custom vial sizes possible).

G. One-step particle conjugation methods

Covalent conjugation of antibodies to nanoparticles and microparticles is quite challenging and unwanted aggregation is a major technical issue. Particles are often carboxylated and activation with carbodiimide, which is clearly an obligatory step, is itself a major cause of aggregation. Most protocols recommend the use of sonicators to break up aggregates, but solutions that address the root cause of the problem are now emerging (see below).

(i) InnovaCoat® GOLD (10nm, 20nm, 40nm, 60nm and 80nm sizes).

Operationally the conjugation of InnovaCoat GOLD particles is identical to that of Lightning-Link reactions (see Figure 4). While the chemistry is different, conjugation is still via lysines on the antibody. A unique protective coat around the particle dramatically increases colloidal stability and allows the particles to be freeze-dried with all of the necessary conjugation chemicals. Reconstitution of the dry mixture with the antibody to be labelled triggers coupling of the antibody to the surface coat. Passive (non-covalent) absorption procedures using uncoated particles are also used to make gold conjugates, but are not discussed here.

Pros: Simple, high colloidal stability, fully scalable, and other functionalities are available.

Cons: Fixed amount of antibody must be used (custom vial sizes possible).

(ii) Innova LATEX (400nm, red, black and blue)

While latex and gold are very different materials, the particles share applications (e.g. lateral flow) and present similar technical challenges (e.g. aggregation). As latex particles are usually provided with a carboxyl surface, carbodiimide-mediated conjugation is commonly used. However, using techniques similar to those described above for InnovaCoat GOLD particles it is now possible to carry out one-step conjugations with freeze-dried latex particles.

Pros: Easy, high colloidal stability during conjugation, fully scalable, three colors available.

Cons: Limited number of particle types have been converted to this new format.

Appendix: Summary of covalent technologies used to attach labels to antibodies

	Lightning-Link	Gold & Latex (Innova)	NHS ester	Isothio-cyanate	Carbodi-imide	Two-tag	Periodate
Avoids tagging of antibody	Yes	Yes	Yes	Yes	Yes	No	Yes
Avoids post-conjugation separations	Yes	Yes[1]	No	No	No	Yes	Yes[2]
Used to attach enzymes	Yes	n/a	No	No	No	Yes	Yes[3]
Used to attach dyes or small molecules	Yes	n/a	Yes	Yes	No	No	No
Scalability	Easy	Easy	Hard	Hard	Easy	Very difficult	Hard[4]
Hands-on time 30 seconds	30 seconds	1-3 min	>15 min	>15min	>15min	>60 min	>15 min
10µg scale possible?	Yes	Yes (also 1µg)	No	No	No	No	Yes[2]
Typical antibody yield	100%	100%[1]	50-80%	50-80%	50-80%	20-50%	70-80%
Other comments	One step, no losses	One step, no losses	NHS esters unstable	High pH needed	Used with particle labels	Complex multi-step process	Chemical hazards

1. If particles are buffer-exchanged some small losses of particles would be expected.
2. Only if sodium cyanoborohydride is not removed.
3. Out of the commonly used labels only applicable to HRP.
4. Easier if the label is purchased pre-activated by periodate.

Lightning-Link® Fluorescent dyes

The below table contains the specifications of all our Lightning-Link® fluorescent dyes:

Fluorescent Label	Maximal Absorbance (nm)	Excitation colour	Suggested Excitation Laser Line (nm)	Maximal Emission (nm)	Emission Color
AMCA	352	N/A	355	452	
DyLight® 350	354	N/A	355	432	
Atto 390	388		405	468	
DyLight® 405	402		405	428	
PerCP	484		488	678	
PerCP/Cy5.5	484		488	692	
DyLight® 488	496		488	524	
Alexa Fluor® 488	496		488	524	
Fluorescein	498		488	532	
R-Phycoerythrin	498, 544, 566†		488, 532, 561	580	
PE/Texas Red®	498, 544, 566†		488, 532, 561	618	
PE/Atto594	498, 544, 566†		488, 532, 561	632	
PE/Cy5	498, 544, 566†		488, 532, 561	672	
PE/Cy5.5	498, 544, 566†		488, 532, 561	700	
PE/Cy7	498, 544, 566†		488, 532, 561	782	
Atto488	504		488	530	
B-Phycoerythrin	546		561	580	
Cyanine Dye 3	552		561	576	
Rhodamine	555		561	588	
DyLight® 550	556		561	584	
Atto 565	570		561	598	
DyLight® 594	594		561*	629	
Texas Red®	596		561*	616	
DyLight® 633	628		633, 635, 640	660	
Atto 633	634		633, 635, 640	660	
FluoProbes647H	650		633, 635, 640	684	
Cyanine Dye 5	652		633, 635, 640	678	
Allophycocyanin	652		633, 635, 640	666	
APC/Cy5.5	652		633, 635, 640	700	
APC/Cy7	652		633, 635, 640	790	
DyLight® 650	656		633, 635, 640	686	
Cyanine Dye 5.5	680		640*	705	
DyLight® 680	686		640*	716	
Atto700	704		640*	724	
DyLight® 755	756		750	794	
DyLight® 800	776		750	798	

† (R-)PE has three maxima, and all can be used. The optimal will depend on the application

* This Laser Line is some distance from the Maximal Absorbance, so performance will be compromised if this dye is used with the suggested Laser Line.

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