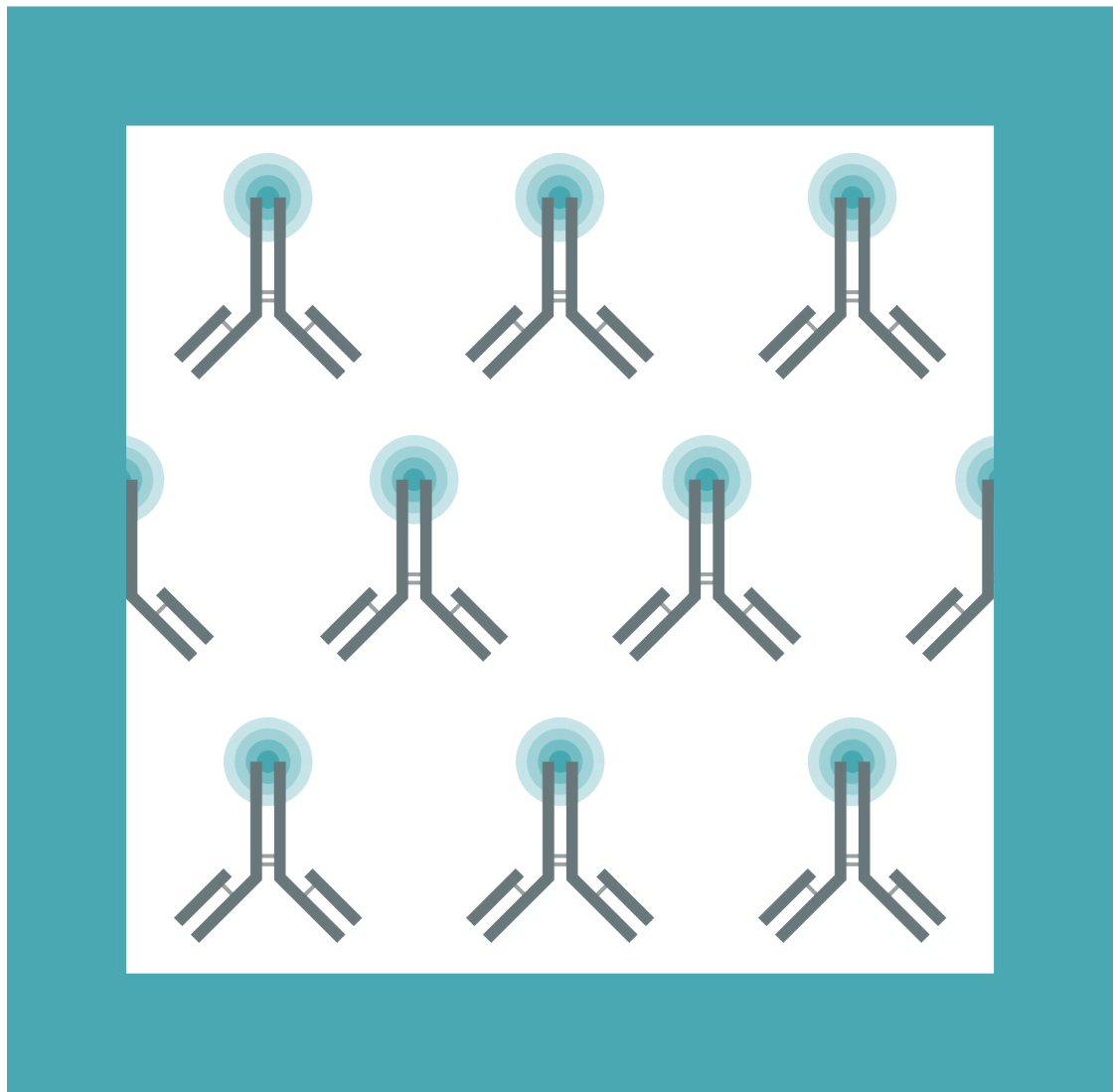


The complete guide to antibody conjugation

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Introduction

Whether it's a western blot analysis or profiling the tumor microenvironment using multiplex imaging, we rely on antibodies conjugated to various labels to visualize and understand protein structure, function, localization, and other protein-protein interactions.

This comprehensive guide covers the ins and outs of antibody conjugation, including the difference between direct and indirect assays, common labels used, different chemistries behind antibody labeling, and methods you can use to conjugate your primary antibody.

Antibody conjugation

Antibody conjugation, also known as antibody labeling, is the process of linking an antibody to a specific tag or label. Most immunoassays require antibodies either directly conjugated to a specific label or detected by a conjugated secondary antibody to provide a measurable signal.

When conjugated, a primary antibody allows for the direct detection of the target antigen of interest without needing a secondary antibody. Using direct detection has pros and cons, which are explored below.

Direct vs indirect assays

Assays are classed as direct if the label is conjugated directly onto the primary antibody. If the primary antibody is unconjugated, and the label is attached to another molecule, such as a secondary antibody, then the assay is classed as indirect (Figure 1).

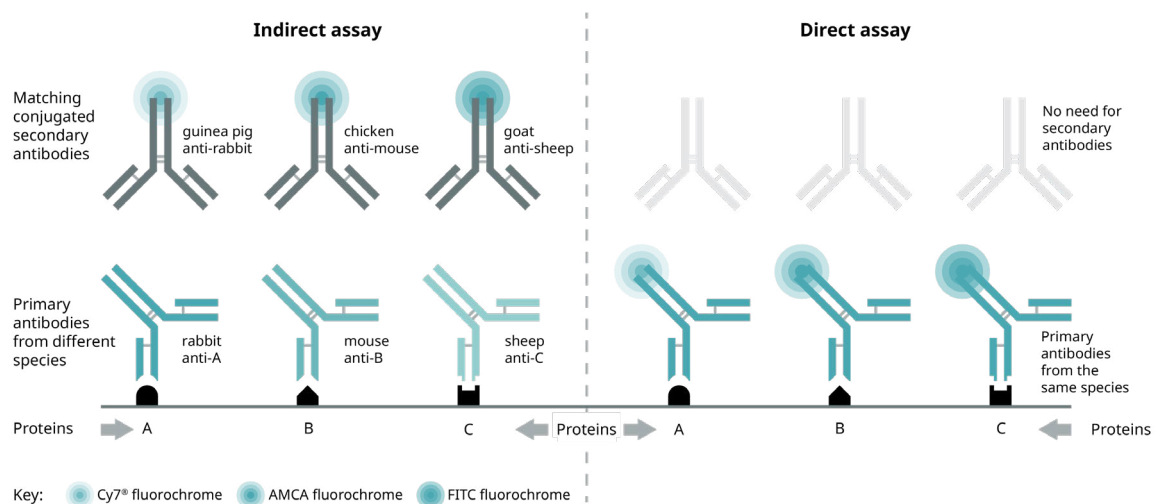


Figure 1. Indirect versus direct assays.

Choosing between direct and indirect methods

Direct and indirect methods have advantages and disadvantages you need to consider when choosing the most appropriate one for your experiment (see Table 1).

Table 1. Advantages and disadvantages of direct and indirect methods.

	Direct (conjugated primary antibody)	Indirect (primary antibody + conjugated secondary antibody)
Suitability	Suitable to detect high-medium expressed targets as there is no signal amplification generated by the secondary antibody.	Suitable for studying low-expressed proteins because of the signal amplification provided by the secondary antibody.
Time	Conjugated primary antibodies reduce the required number of steps, resulting in a shorter protocol. Only one round of incubation with the sample is necessary because the signal visualization comes from the conjugated primary antibody.	Using a secondary antibody results in a longer protocol due to the additional incubation steps required. In the first step, your sample must be incubated with the unconjugated primary antibody. In the next step, a secondary antibody conjugated to your label of interest will be incubated with the primary antibody to visualize your antigen.
Cost	Conjugated primary antibodies are usually more expensive than secondary antibodies.	Secondary antibodies are relatively inexpensive compared to primary antibodies and can be used to detect different primary antibodies.
Complexity	Fewer steps in the protocol simplify direct methods, meaning experiments are usually easier to design. Dye-antibody combination gives you more flexibility to build a multiplex assay.	You need to select the appropriate secondary antibody or multiple controls, which adds complexity. This is particularly relevant in multiplex experiments, which require several primary antibodies, each targeting different species, and several corresponding secondary antibodies, each conjugated to various dyes.
Species cross-reactivity	Species cross-reactivity is minimized in direct methods as the fluorophore is already conjugated to the primary antibody.	Secondary antibodies may cross-react with species other than the target. The use of pre-adsorbed secondary antibodies can prevent cross-reactivity.
Background	With direct methods, the background is reduced because the primary antibody is already conjugated and less likely to react with other antigens in your sample.	Because you're adding additional reagents to your experiment, the secondary antibodies may cross-react with other antigens in your sample, resulting in a higher background.

Commonly used labels

Labels used to conjugate antibodies include fluorescent dyes, small molecules (eg, biotin), enzymes (horseradish peroxidase (HRP) or alkaline phosphatase (AP)), proteins (eg, R-PE, APC, streptavidin), latex or gold nanoparticles, and oligonucleotides. The choice of the label will depend on the experimental application, as shown in Table 2.

Table 2. Labels and their most common applications.

Experimental technique	Label(s)
Western blot	HRP, AP, fluorescent dyes
Immunofluorescence	Fluorescent dyes, including Alexa Fluor® dyes
Fluorescent immunohistochemistry (singleplex)	Fluorescent dyes
Fluorescent immunohistochemistry (multiplex)	Fluorescent dyes, oligonucleotides
Chromogenic immunohistochemistry	HRP, AP, Biotin
Flow cytometry	Fluorescent dyes, such as Alexa Fluor® dyes, and fluorescent proteins, including Phycoerythrin (PE), Allophycocyanin (APC), and tandem dyes
ELISA, ELISA-based applications	HRP, Biotin
Immuno-PCR, Proximity ligation assay (PLA), Single-cell proteomics	Oligonucleotides
Lateral flow assay (LFA)	Latex and gold nanoparticles, Fluorescent nanoparticles (Europium)
Mass cytometry (CyTOF®, Imaging Mass Cytometry™)	Metal ion tags

Antibody labeling chemistries

Suppose you concluded that a directly conjugated primary antibody is the best choice for your experiment. In this case, you can purchase a commercially available primary conjugated antibody or conjugate the primary antibody yourself, for example, using a commercial conjugation kit. The latest option can prove helpful if you cannot find the appropriate combination of antibody and label. In the next section, we describe common conjugation methods to help you decide which technique will enable you to complete your experiment.

Antibody labeling can be complex and typically requires a certain level of expertise. Conjugating an antibody using traditional labeling methods requires a basic understanding of the principles of chemical modification. Also, there are a variety of amino acid residues to which a label can be chemically attached. The most common are the primary amine groups on the lysines' side chain, sulfhydryl groups generated by cysteine reduction, or carbohydrate moieties.

Below we describe the most common methods to conjugate antibodies.

NHS ester method

Covalent conjugation via NHS ester groups is the most common antibody labeling method, which involves attaching the label of interest to the amine groups of lysine residues. The NHS ester method uses fluorescent dyes with a reactive N-Hydroxysuccinimide (NHS) group (Figure 2). After reacting with the antibody, any surplus reactive dye is removed through various separation processes. These separation processes usually take advantage of the label's small size compared to the antibody conjugate, as in gravity column chromatography. While these separation steps can result in conjugate losses, it allows removing of unbound dye, which can cause high background in your experiment.

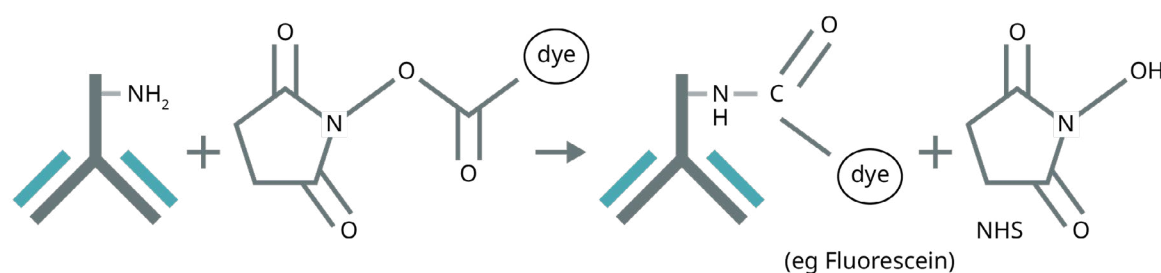


Figure 2. NHS ester reaction with an amine (pH 8.0).

Two-tag method (heterobifunctional method)

The two-tag method is generally used to conjugate protein molecules, for example, HRP. This is because both the antibody (Ab) and label (L) have multiple lysine groups, and there is a risk of forming Ab-Ab or L-L homodimers or high-molecular-weight aggregates.

The main advantage of the two-tag approach is that it provides a controlled reaction to form conjugated antibodies. However, pre-conjugation separation steps can lead to the loss of valuable antibody reagent (and label), uncertainty over the amount of recovered antibody, batch-to-batch variation, and lack of scalability.

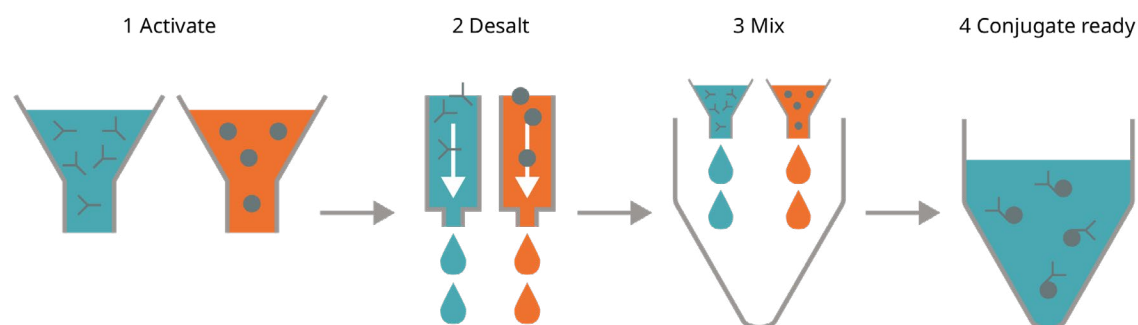


Figure 3. The two-tag approach to conjugation.

Site-specific conjugation

All labeling methods described above rely on specific aminoacidic residues in the chains of the antibody. However, the number and location of these amino acids can vary depending on the antibody chain sequences, and certain amino acids may belong to the antigen-binding site. Labels binding near the paratope can interfere with the antibody structure, charge, and affinity, and therefore its function.

Site-specific conjugation ensures that labeling happens far from the antigen-binding site, so labels do not create steric hindrance that could impact the antibody's binding affinity and avidity. Through enzymatic activation or modification of specific domains in the F_c region or antibody sequence engineering, it is possible to target specifically modified amino acids at pre-defined locations of the antibody chain to achieve a very high level of control and reproducibility over antibody labeling.

Cysteine-based conjugation

An alternative to primary amine modification is to target sulfhydryl groups, also called thiols. Pairs of cysteine sulfhydryl groups exist in proteins and antibodies as intra-chain disulfide bonds ($-S-S-$) and therefore require chemical modification (ie, reduction) to become available in their reactive form ($-SH$) for antibody labeling. Upon each disulfide bond reduction, two thiol groups are formed, which can be paired with thiol-reactive compounds, such as maleimides and pyridyl disulfides.

Targeting cysteine residues allows us to attach the label of interest away from the binding site region. However, not all antibodies will tolerate the chemical reduction, leading to the creation of unstable half antibodies that show lower avidity due to the presence of only one binding site.

Glycan-based conjugation

Glycans are carbohydrate-based polymers made by all living organisms. This site-specific method targets carbohydrate residues on the Fc region of all IgG antibodies. Hence, this method allows us to attach the desired label far away from the antigen binding site.

The original chemical method was relatively complex and harsh, depending upon cis-diol oxidation to create aldehydes groups that react with hydrazides. Innovative and gentler approaches involve using enzymes to specifically attach an azido moiety to the N-linked glycans on the heavy chains of an IgG antibody; once azido-modified, the label of interest can be attached to the antibody via “click” chemistry.

Conjugation kits

The development of conjugation kits has simplified the traditionally complex conjugation processes, offering an excellent option for those without access to the expertise required to execute and optimize the complex methods described above. Most importantly, this option can prove helpful if you can't find the appropriate combination of antibody and label readily available as an "off the shelf" product.

Conjugation kits, such as Lightning-Link[®], use a simple, three-step process whereby the antibody to be labeled is added into a vial of a lyophilized mixture containing the desired label. The conjugated antibody is then ready to use without the need for post-conjugation purification steps, removing the risk of antibody loss. Figure 4 illustrates the Lightning-Link[®] conjugation kit process.

The advantages of this method include reproducibility and scalability, with conjugation kits that work with 10µg–100mg of antibody. Also, the kits' ability to form antibody-label conjugates at a neutral pH in just three steps reduces the amount of hands-on bench time. Furthermore, the range of more than 45 different labels, as in Lightning-Link[®] kits, allows flexibility in the choice of application and multiplex panel development.

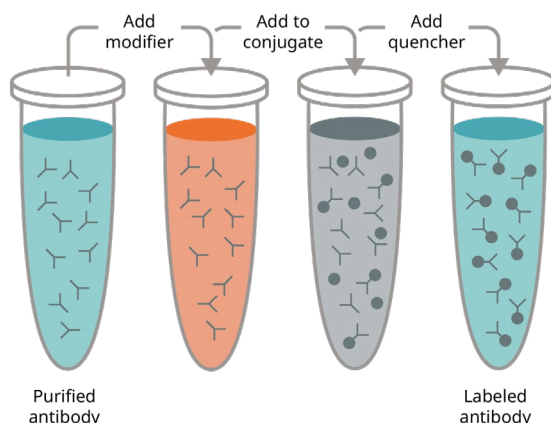


Figure 4. The Lightning-Link[®] kit labeling method.

You can read more about Lightning-Link[®] kits [here](#).

Four key considerations to generate high-quality conjugates

The conjugation efficiency of most conjugation chemistries, especially NHS-ester-based methods, relies on several factors, including your application, antibody purity and concentration, and buffer constituents.

To generate a high-quality conjugated antibody, follow these four steps:

- 1. Select the best label for your application:** The choice of the label will largely depend on the final application. Table 2 lists labels and their most common applications.
- 2. Make sure your antibody is purified:** Only purified antibodies are suitable for conjugation (ie, not crude serum or tissue culture supernatant).
- 3. Check the concentration of your antibody:** the antibody should be at least 1mg/mL.
- 4. Confirm the antibody is in a suitable buffer:** certain buffer constituents can be incompatible with the labeling reaction (see Table 4).

To achieve the best results with antibody conjugation, we recommend choosing an antibody in a buffer formulation free from glycerol, BSA and sodium azide. These additives and other components that can interfere with the conjugation reaction will hinder the antibody's labeling efficiency and result in poor-quality conjugated antibodies.

Abcam offers carrier-free antibodies specifically designed for antibody labeling. Carrier-free antibodies are conjugation-ready because they are provided in the most suitable buffer formulation and concentration. Choosing a carrier-free antibody will save you time and money due to unnecessary antibody loss post purification, concentration or buffer exchange.

If your antibody does not meet the requirements specified above, you can easily purify or concentrate your antibody using our range of [purification and concentration kits](#).

Table 4. Buffer formulation components that can interfere with conjugation reaction and purification kits.

Unconjugated antibody properties	Compatibility	Notes
Purified antibody	Yes	–
Antibody in ascites fluid, serum, hybridoma or tissue culture media.	No	Serum Antibody Purification Kit (Protein A) (ab109209) and Serum Antibody Purification Kit (Protein G) (ab128751) are suitable for purifying IgG fractions from serum and ascites fluid.
Antibody concentration	> 1 mg/mL	The Antibody Concentration and Clean-Up Kit (ab102778) can be used to concentrate your antibody. Use the BCA Protein Assay Kit II (ab287853) to check your antibody concentration after you have removed any buffer components that could interfere with the concentration measurement.
pH	6.5 – 8.5	–
Amine free buffer (eg, MES, MOPS, HEPES, PBS), NaCl, Borate buffer, Chelating agents (eg, EDTA), Sugars.	Yes	–
BSA	Only at < 0.1%, w/v	Avoid if intending to use conjugated antibodies for IHC; BSA may cause background staining. The BSA Removal Kit (ab173231) could be used.

Sodium azide	Only at < 0.1%, w/v	Remove any sodium azide if labeling with HRP, as it is an irreversible inhibitor of HRP activity. The Antibody Concentration and Clean-Up Kit (ab102778) can be used for this.
Glycerol	≤ 50 %, v/v	Use only at concentrations up to 50%.
Gelatin	Only at < 0.1%	Use only at concentrations <0.1%. Avoid if intending to use conjugated antibodies for IHC; Gelatin may cause background staining.
Tris	< 50 mM	The Antibody Concentration and Clean-Up Kit (ab102778) can be used to remove any excess Tris from the buffer.
Glycine	No	The Antibody Concentration and Clean-Up Kit (ab102778) can be used to remove Glycine from the buffer.
Thiomersal / Thimerosal, Merthiolate, Proclin 300, Nucleophilic components (Primary amines, eg, amino acids or ethanolamine and thiols, eg, mercaptoethanol or DTT).	No	–

Please note that, individually, the concentrations shown should not affect the conjugation reaction. However, in combination with additional compounds that are not recommended above a certain concentration, the reaction may be affected.

Confirming successful antibody conjugation

Before proceeding to the next step of your experiment, it is crucial to confirm successful antibody conjugation. Measuring the degree of labeling using a lateral flow assay can confirm successful antibody conjugation.

Our range of [conjugation check kits](#) allows you to confirm the successful labeling of an antibody conjugate. These kits are suitable for the qualitative verification of IgG antibodies and are compatible with any other conjugation technology that uses colored labels. Alternatively, you can test conjugation success using a preliminary experiment in the application of interest.

Lightning-Link® Antibody Labeling Kits

Our full range of fluorescent labels

Fluorescent label	Maximum absorbance (nm)	Excitation colour	Suggested excitation laser (nm)	Maximum emission (nm)	Emission colour
AMCA**	352		355	452	
DyLight® 350**	354		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	
Alexa Fluor® 555**	552		532	570	
DyLight® 550**	556		561	584	
Atto 565**	570		561	598	
Alexa Fluor® 568**	580		532	604	
Alexa Fluor® 594**	592		532	620	
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	
Alexa Fluor® 647**	652		640	672	
DyLight® 650**	656		633, 635, 640	686	
Cyanine Dye 5.5**	680		640*	705	
DyLight® 680**	686		640*	716	
Alexa Fluor® 700**	695		640*	720	
DyLight® 755**	756		640*	794	
DyLight® 800	776		640*	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 Maximum Absorbance, so performance will be compromised if this dye is used with the suggested laser line. ** Lightning-Link® Fast kit. *** Both Fast and standard Lightning-Link® kits.

Visit abcam.com/kits/antibody-conjugation-kits to see our full range including nanoparticles such as Gold, Latex, Europium and Magnetic beads and oligonucleotide conjugation kits

For more information, please visit abcam.com/kits/antibody-conjugation-kits

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Additional resources

Antibody conjugation

- [Antibody labeling kits: resources and products](#)
- [Conjugation kit application note](#)
- [Conjugation kits FAQs](#)
- [Lightning-Link® kit protocol video](#)
- [Antibody conjugation solutions](#)

General

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- [Protocols](#)

