Alexa Fluor® 546 Monoclonal Antibody Labeling Kit

Catalog no. A20183

Table 1 Contents and storage

Material	Amount	Storage	Stability
Alexa Fluor® 546 reactive dye (Component A)	5 vials	• ≤ –20°C • Dessicate • Protect from light	When stored properly, the kit components are stable for at least 3 months.
Sodium bicarbonate (Component B, MW = 84)	84 mg	• 2–6°C • Dessicate	
Purification resin (Component C, 30,000 MW size exclusion resin)	~10 mL in phosphate-buffered saline (PBS), pH 7.2, plus 2 mM sodium azide	• 2–6°C • Do not freeze	
Spin columns (Component D)	5 columns	• Room temperature	
Collection tubes (Component E)	5 tubes, 2 mL each		
Number of Labelings: Each vial of reactive dye contains the appropriate amount of dye to label approximately 100 µg of IgG (MW ~145,000).			
Approximate fluorescence excitation/emission maxima: 554/570 nm for Alexa Fluor® 546 conjugate.			

Introduction

Alexa Fluor® 546 Monoclonal Antibody Labeling Kit provides a convenient means to label small amounts of monoclonal antibodies with the Alexa Fluor® 546 dye. Monoclonal antibodies are often available only in small quantities and this kit is optimized for labeling 100 µg per reaction. Comparably small amounts of polyclonal antibodies or other proteins (>30 kDa) can also be labeled. For labeling larger amounts of proteins (~1 mg), we recommend our Alexa Fluor® 546 Protein Labeling Kit (Cat. no. A10237).

Alexa Fluor® 546 dye-labeled proteins have fluorescence excitation and emission maxima of approximately 554 nm and 570 nm, respectively (Figure 1, page 2), and are typically brighter than similar proteins labeled with the Cy®3 fluorophore.

The Alexa Fluor® 546 Monoclonal Antibody Labeling Kit contains everything you need to perform five separate labeling reactions and to purify the resulting conjugates. The Alexa Fluor® 546 reactive dye (Figure 2, page 2) has a succinimidyl ester moiety that reacts efficiently with primary amines of proteins to form stable dye–protein conjugates. Each of the five vials of reactive dye provided in the kit is optimized for labeling ~100 µg of a monoclonal antibody.

Figure 1 Normalized absorption and emission spectra of Alexa Fluor® 546 dye conjugated to goat anti-mouse IgG in pH 7.2 buffer

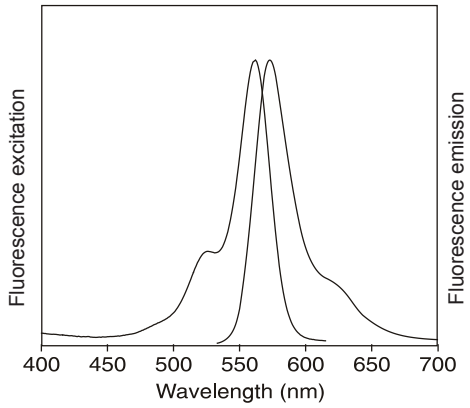
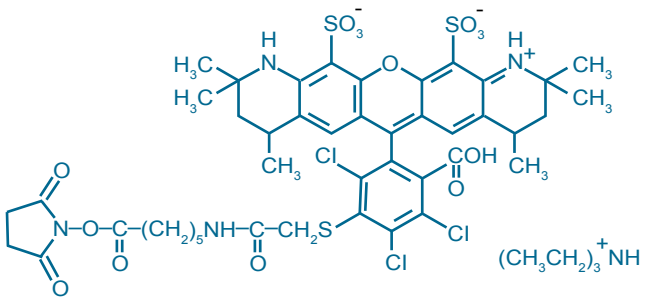


Figure 2 Alexa Fluor® 546 carboxylic acid, succinimidyl ester, triethylammonium salt (MW 1,159.60)



Before You Begin

Preparing the Protein

Each reaction is optimized to label 100 µg of monoclonal antibody. You can also label a similar mass of polyclonal antibody or other protein (≥40 kDa).

Important

For optimal labeling efficiency, the purified protein should be in a buffer free of ammonium ions or primary amines, as they will compete with the amine groups of the protein for the reactive dye. If the protein is in or has been lyophilized from an unsuitable buffer (*e.g.*, Tris or glycine) or purified with ammonium sulfate, replace the buffer with phosphate-buffered saline (PBS) by microdialysis. Impure antibodies or antibodies stabilized with bovine serum albumin (BSA) or gelatin will not label well. The presence of low concentrations of sodium azide (≤3 mM) or thimerosal (≤1 mM) will not interfere with the conjugation reaction.

Experimental Protocol

Labeling the Protein

1.1 Prepare a 1 M solution of sodium bicarbonate by adding 1 mL of deionized water to the provided vial of sodium bicarbonate (Component B). Vortex or pipet up and down until fully dissolved. You can store the bicarbonate solution, which will have a pH ~8.3, at 4°C for up to two weeks.

1.2 If the antibody to be labeled has a concentration of ≥1 mg/mL and is in an appropriate buffer (see **Preparing the Protein**, page 2), dilute it to 1 mg/mL and then add one-tenth volume of 1 M sodium bicarbonate buffer (prepared in step 1.1).

If the protein is a powder lyophilized from an appropriate buffer, prepare a 1 mg/mL solution of the antibody by adding an appropriate amount of 0.1 M sodium bicarbonate buffer to the protein. Prepare 0.1 M sodium bicarbonate buffer by diluting the 1 M solution 10-fold with deionized water.

Note: Bicarbonate, pH ~8.3, is added to raise the pH of the reaction mixture, since succinimidyl esters react efficiently at pH 7.5–8.5.

1.3 Transfer 100 µL of the protein solution (from step 1.2) to the vial of reactive dye. Cap the vial and gently invert it a few times to fully dissolve the dye. Violent agitation of the protein solution may result in protein denaturation.

Note: To visually confirm that the dye has fully dissolved, it may help to peel the label off the vial of reactive dye.

1.4 Incubate the solution for 1 hour at room temperature. Every 10–15 minutes, gently invert the vial several times to mix the two reactants and increase the labeling efficiency.

Note: During the incubation period, proceed to steps 2.1–2.4 to prepare a spin column for the purification of the labeled protein. This will take ~15 minutes.

Purifying the Labeled Protein

2.1 Place a spin column in a 13 × 100 mm glass tube or a plastic centrifuge tube.

Note: The enclosed spin column should have two frits inserted at the bottom. If the two frits are not present, one or both of them may be in the plastic bag. Insert the second or both frits into the column and push them down to the bottom of the column with the blunt end of a pen or pencil. If any gel filtration beads from the column get past the frits and end up in the collection tube with the conjugate, they will do no harm and removing them is optional.

2.2 Stir the purification resin (Component C), add 1.0 mL of the suspension into the column, and allow it to settle.

2.3 Continue to add more of the suspension until the bed volume is ~1.5 mL. Allow the column buffer to drain from the column by gravity. Initially, some pressure or a brief burst of centrifugation may be required to cause the first few drops of buffer to elute.

2.4 Place the spin column in one of the provided collection tubes, and centrifuge the column for 3 minutes at 1,100 × *g* using a swinging bucket rotor or a fixed angle rotor. To convert revolutions per minute (rpm) into relative centrifugal force (*g*-force), either consult the conversion chart provided by the centrifuge manufacturer or use the following equation:

Relative centrifugal force = (1.12×10^{-5}) $(\text{rpm})^2$ (radius)

where radius = radius in centimeters measured from the center of the centrifuge spindle to the bottom of the rotor bucket. Do not exceed 2,000 × *g*. Discard the buffer, but save the collection tube. The spin column is now ready for purifying the conjugated antibody.

2.5 Load the 100 µL reaction volume (from step 1.4, page 3) dropwise onto the center of the resin at the top of the spin column. Allow the solution to absorb into the gel bed.

2.6 Place the spin column into the empty collection tube and centrifuge for 5 minutes at 1,100 × *g*.

2.7 After centrifugation, the collection tube contains labeled protein in approximately 100 µL of PBS, pH 7.2, with 2 mM sodium azide; free dye remains in the column bed. Discard the spin column.

Determining the Degree of Labeling

3.1 Dilute a small amount of the purified conjugate (*i.e.*, labeled antibody) into PBS or other suitable buffer and measure the absorbance in a cuvette with a 1 cm pathlength at 280 nm (A_{280}) and 554 nm (A_{554}).

Note: The formulas given below is for measuring the absorbance in a cuvette with a 1 cm pathlength. If using a NanoDrop or a small volume cuvette, you must modify the molar extinction coefficients for the smaller pathlength. For example, if using a cuvette with a 1 mm pathlength, multiply the extinction coefficient by 10.

3.2 Calculate the concentration of protein in the sample:

$$\text{protein concentration (M)} = \frac{[A_{280} - (A_{554} \times 0.12)] \times \text{dilution factor}}{203{,}000}$$

where 203,000 is the molar extinction coefficient of a typical IgG in $\text{cm}^{-1}\,\text{M}^{-1}$, and 0.12 is a correction factor to account for absorption of the dye at 280 nm.

Note: Non-IgG proteins will likely have significantly different molar extinction coefficients.

3.3 Calculate the degree of labeling:

$$\text{moles dye per mole protein} = \frac{A_{554} \times \text{dilution factor}}{104{,}000 \times \text{protein concentration (M)}}$$

where 104,000 is the approximate molar extinction coefficient, in $\text{cm}^{-1}\text{M}^{-1}$, of the Alexa Fluor® 546 dye at 554 nm. For IgGs, we find that labeling with 2–7 moles of Alexa Fluor® 546 dye per mole of antibody is optimal.

Storing and Handling the Conjugates

Store the labeled antibody at 2–6°C, **protected from light**. If the final concentration of purified antibody conjugate is less than 1 mg/mL, add bovine serum albumin (BSA) or other stabilizing protein to 1–10 mg/mL.

In the presence of 2 mM sodium azide, the conjugate is stable at 4°C for at least three months. For long-term storage, divide the solution into small aliquots and freeze at ≤–20°C. **Avoid repeated freezing and thawing. Protect from light.**

For other proteins, store the dye-conjugated protein under optimal conditions for that molecule; stability of your conjugate will be similar to that observed with the unconjugated protein. **Protect from light.**

Troubleshooting

Under-Labeling

If calculations indicate that the protein is labeled with significantly less than two moles of fluorophore per mole of IgG antibody, your protein could possibly be under-labeled. A number of conditions can cause a protein to label inefficiently:

- Trace amounts of primary amine-containing components in the buffer will react with the dye and decrease the efficiency of protein labeling. If your protein has been in amine-containing buffers (*e.g.*, Tris or glycine), dialyze *extensively* versus PBS before labeling.
- Dilute solutions of protein (<1 mg/mL) will not label efficiently.
- The addition of sodium bicarbonate (step 1.2, page 3) is designed to raise the pH of the reaction mixture to ~8, as succinimidyl esters react most efficiently with primary amines at slightly alkaline pH. If the protein solution is strongly buffered at a lower pH, the addition of bicarbonate will not raise the pH to the optimal level. To raise the pH to the optimal level, you can add more bicarbonate, or exchange the buffer with PBS, which is only weakly buffered, or with 0.1 M sodium bicarbonate, pH 8.3, by dialysis or other method prior to starting the reaction.
- Because proteins, including different antibodies, react with fluorophores at different rates and retain biological activity at different degrees of dye labeling, the standard protocol may not always result in optimal labeling. To increase the amount of labeling, you can relabel the same protein sample, or you can label a new protein sample using either less protein or more reactive dye per reaction. To increase the amount of dye in the reaction, you can combine the contents of two vials of reactive dye together. Some researchers obtain better labeling with overnight incubations at 4°C after an initial incubation of one hour at room temperature.

Over-Labeling

If calculations indicate that the protein conjugate is labeled with significantly more than seven moles of fluorophore per mole of IgG antibody, your protein is probably over-labeled. Although conjugates with a high number of attached dye molecules may be acceptable for use, over-labeling can cause aggregation of the protein conjugate and can also reduce the antibody's specificity for its antigen—both of which can lead to nonspecific staining. Over-labeling can also cause quenching of the attached dyes, which will decrease the fluorescence of the conjugate. To reduce the amount of labeling next time, you can add more protein to your reaction to decrease the molar ratio of dye to protein or allow the reaction to proceed for a shorter time.

Inefficient Removal of Free Dye

Although we have had good success in removing free dye from protein conjugates with the provided columns, it is possible that trace amounts of free dye will remain in the conjugate solution after purification, particularly if a low molecular weight protein is labeled. The presence of free dye, which can be determined by thin layer chromatography, will result in erroneously high calculated values for the degree of labeling (see **Determining the Degree of Labeling**). You can remove the remaining traces of free dye by applying the conjugate to another column or by extensive dialysis.

Protein or Protein Conjugate Remains on the Spin Column

If the protein did not elute during centrifugation, do not add additional buffer to the column. Instead, re-centrifuge one or more times in order to elute the protein.

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat #	Product Name	Unit Size
A20183	Alexa Fluor® 546 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
Related products		
A10237	Alexa Fluor® 546 Protein Labeling Kit *3 labelings*	1 kit

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