Alexa Fluor® 647 Monoclonal Antibody Labeling Kit

Catalog no. A20186

Table 1 Contents and storage

Material	Amount	Storage	Stability
Alexa Fluor® 647 reactive dye (Component A)	5 vials	• 2–6°C • Protect from light • Protect from moisture	When stored as directed, the kit components are stable for at least 3 months.
Sodium bicarbonate (MW = 84) (Component B)	~ 84 mg	• 2–6°C • Protect from moisture	
Purification resin (Component C)*	~ 10 mL	• 2–6°C • Do not freeze	
Spin columns (Component D)	5 columns	• 2–6°C	
Collection tubes (Component	5 tubes, 2 mL each		
*30,000 MW size–exclusion resin in phosphate-buffered saline (PBS), pH 7.2, plus 2 mM sodium azide.			
Approximate fluorescence excitation and emission maxima: Alexa Fluor® 647 reactive dye: 650/668 nm.			

Introduction

Alexa Fluor® 647 Monoclonal Antibody Labeling Kit provides a convenient means to label small amounts of monoclonal antibodies with the superior Alexa Fluor® 647 dye. Monoclonal antibodies are often available only in small quantities and this kit is optimized for labeling 100 µg of antibodies 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® 647 Protein Labeling Kit (Cat. no. A20173).

Alexa Fluor® 647 dye–labeled proteins, which have fluorescence excitation and emission maxima of approximately 650 nm and 668 nm, respectively (Figure 1, page 2). The wide spectral separation of this dye from commonly used red fluorophores such as Texas Red® and Alexa Fluor® 594 dyes makes it an ideal choice for use in multicolor applications. Moreover, the fluorescent signal from the Alexa Fluor® dye is pH insensitive between 4 and 10, and unlike Cy®5 dye, resistant to quenching at high degrees of substitution.

The Alexa Fluor® 647 Monoclonal Antibody Labeling Kit contains everything you need to perform five separate labeling reactions and to purify the resulting conjugates. The Alexa Fluor® 647 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 excitation and emission spectra of Alexa Fluor® 647 dye conjugated to goat anti-mouse IgG in pH 7.2 buffer

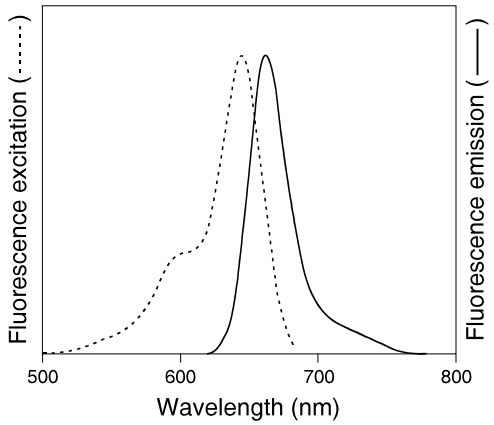
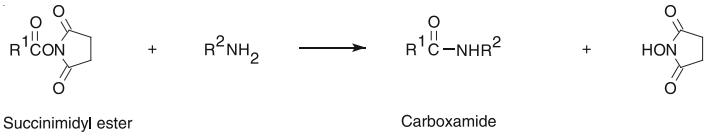


Figure 2 Reaction of a primary amine with a succinimidyl ester



Experimental Protocol

Protein Preparation

Each reaction is optimized for labeling 100 µg of monoclonal antibody. A similar mass of polyclonal antibody or other protein (>30 kDa) can also be labeled.

IMPORTANT: The purified protein must 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, the buffer needs to be replaced 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.

Note: Microdialysis apparatus for small volumes of proteins (e.g., 10–500 µL) can be obtained from Pierce Chemical Company (www.piercenet.com).

Conjugation Reaction

1.1 Prepare a 1 M solution of sodium bicarbonate by adding 1 mL of deionized water (dH_2O) to the provided vial of sodium bicarbonate (Component B). Vortex or pipet up and down until fully dissolved. The bicarbonate solution, which will have a pH ~8.3, can be stored 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 **Protein Preparation**, above), 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 dH_2O.

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 can 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 in order to mix the two reactants and increase the labeling efficiency.

Note: During the incubation period, proceed to steps 2.1–2.4, below, 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.

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), then 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 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 1100 × *g* using a swinging bucket 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:

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

where radius = radius in centimeters measured from the center of the centrifuge spindle to the bottom of the rotor bucket. Discard the buffer, but save the collection tube. The spin column is now ready for purifying the conjugated antibody.

Note: A fixed angle rotor will suffice if a swinging bucket rotor is not available.

2.5 Load the 100 µL reaction volume (from step 1.4, above) dropwise onto the center 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 1100 × g.

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

Determining the Degree of Labeling

3.1 Dilute a small amount of the purified conjugate into PBS or other suitable buffer and measure the absorbance in a cuvette with a 1-cm pathlength at 280 nm (A_{280}) and 650 nm (A_{650}).

3.2 Calculate the concentration of protein in the sample:

$$\text{Protein concentration (M)} = \frac{[A_{280} - (A_{650} \times 0.03)] \times \text{dilution factor}}{203{,}000}$$

where 203,000 is the molar extinction coefficient (ε) in $cm^{-1}M^{-1}$ of a typical IgG at 280 nm, and 0.03 is a correction factor for the fluorophore's contribution to the absorbance at 280 nm.

3.3 Calculate the degree of labeling:

$$\text{Moles dye per mole protein} = \frac{A_{650} \times \text{dilution factor}}{239{,}000 \times \text{protein concentration (M)}}$$

where 239,000 $cm^{-1}M^{-1}$ is the approximate molar extinction coefficient of the Alexa Fluor® 647 dye at 650 nm. For IgGs, we find that labeling with 3–7 moles of Alexa Fluor® 647 dye per mole of antibody is optimal.

Storing the Conjugates

Store the labeled protein at 4°C, protected from light. If the final concentration of purified protein conjugate is less than 1 mg/mL, add BSA or other stabilizing protein at 1–10 mg/mL. In the presence of 2 mM sodium azide, the conjugate should be stable at 4°C for several months. For longer storage, divide the conjugate into small aliquots and freeze at –20°C. **Avoid repeated freezing and thawing. Protect from light.**

Troubleshooting

Under-labeling

If calculations indicate that the protein is labeled with significantly less than three moles of fluorophore per mole of 145,000 MW protein, your protein is probably under-labeled. A number of conditions can cause a protein to label inefficiently.

- Trace amounts of primary amine–containing components in the buffer 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 2) is designed to raise the pH of the reaction mixture to ~8, because 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. Either more bicarbonate can be added or the buffer can be exchanged 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 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 eight 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 fluorescence quenching of the conjugate. To reduce the amount of labeling, 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 spin columns, it is possible that trace amounts of free dye will remain in the conjugate solution after purification. 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**, page 4). Remaining traces of free dye can be removed 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. no.	Product Name	Unit Size
A20186	Alexa Fluor® 647 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit

Contact Information

Corporate Headquarters
5791 Van Allen Way
Carlsbad, CA 92008
USA
Phone: +1 760 603 7200
Fax: +1 760 602 6500
Email: techsupport@lifetech.com

European Headquarters
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF
UK
Phone: +44 141 814 6100
Toll-Free Phone: 0800 269 210
Toll-Free Tech: 0800 838 380
Fax: +44 141 814 6260
Tech Fax: +44 141 814 6117
Email: euroinfo@invitrogen.com
Email Tech: eurotech@invitrogen.com

Japanese Headquarters
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Japan
Phone: +81 3 5730 6509
Fax: +81 3 5730 6519
Email: jpinfo@invitrogen.com

Additional international offices are listed at www.lifetechnologies.com

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