molecular probes®
by life technologies™

Alexa Fluor®, Pacific Blue™, and Pacific Orange™ Antibody Labeling Kits

Table 1 Contents and storage

Material	Amount	Storage*	Stability
Reactive dye (Component A)	5 vials	• 2–8°C • Protect from light • Do not freeze	When stored as directed, the kit components are stable for at least 3 months.
Sodium bicarbonate (MW = 84) (Component B)	~ 84 mg		
Purification resin (Component C)**	~ 10 mL		
Spin columns (Component D)	5 columns		
Collection tubes (Component E)	5 tubes		
* Individual kit components may be stored under the conditions listed on the component labels. The reactive dye (Component A) may be stored frozen at ≤–20°C or at 2–8°C. Do not freeze the purification resin (Component C).			
**30,000 MWCO (molecular weight cut-off) size–exclusion resin in phosphate-buffered saline (PBS), pH 7.2, and 2 mM sodium azide.			
Number of labelings: Each of the five vials of reactive dye provided in the kit is optimized for labeling ~100 µg of antibody. The kits are not suitable for labeling IgM.			

Introduction

The Alexa Fluor®, Pacific Blue™, and Pacific Orange™ Antibody Labeling Kits provide a convenient means to label small amounts of antibodies with the superior Alexa Fluor® dyes or the 405-nm excitable Pacific Blue™ and Pacific Orange™ dyes. These kits are optimized for labeling 100 µg of antibody per reaction. Comparably small amounts of other proteins (>40 kDa) can also be labeled. For labeling larger amounts of proteins (~1 mg), we recommend our Large-scale Protein Labeling Kits.

Each Antibody Labeling Kit contains everything you need to perform five separate labeling reactions and to purify the resulting conjugates. The reactive dye has either a succinimidyl ester (SE) or a tetrafluorophenyl (TFP) 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 an antibody; the kits are not suitable for labeling IgM antibodies.

Alexa Fluor® dyes produce protein conjugates that are typically brighter, more photostable, and less pH sensitive relative to the traditional dyes (AMCA, fluorescein/FITC, TRITC, etc.) and the Cy® dyes (Table 2). Unlike other dyes, the fluorescence of Alexa Fluor®-conjugates is insensitive to pH between pH 4 and 10. Also, various Alexa Fluor® dyes are resistant to quenching at high degrees of substitution and thus are brighter than similar proteins labeled with other dyes. The Alexa Fluor®-labeled antibodies can be used for multiple applications, including fluorescent microscopy, flow cytometry, Western blotting, ELISA, and indirect FISH. Pacific Blue™ and Pacific Orange™ are unique dyes designed for use with a violet laser (405 nm) for flow cytometry applications. Dye spectra and structures are provided in the Alexa Fluor®, Pacific Blue™, and Pacific Orange™ Dye Spectra and Structures User Guide (mp20189).

Table 2 Properties of Alexa Fluor®, Pacific Blue™, and Pacific Orange™ dyes

Cat. no.	Dye	Ex/Em maxima (nm)	Fluorescent color	Spectrally comparable* fluorophores	Commonly used filter sets
A20180	Alexa Fluor® 350	346/442	Blue	AMCA, eBFP, DAPI, Hoechst 33342, Hoechst 33258	DAPI
A20181	Alexa Fluor® 488	494/519	Green	Fluorescein/FITC, BODIPY® FL, DiO, Cy®2, Qdot® 525, GFP	FITC
A20182	Alexa Fluor® 532	530/554	Yellow	Rhodamine 6G, BODIPY® R6G, Qdot® 545, mBanana	R6G
A20183	Alexa Fluor® 546	554/570	Orange	Tetramethylrhodamine/TRITC, DiI, Cy®3, Qdot® 565, mOrange/OFP	TRITC
A20187	Alexa Fluor® 555	555/565	Orange	Tetramethylrhodamine/TRITC, R-PE, BODIPY® TMR, Cy®3, Qdot® 565, mOrange/OFP	TRITC
A20184	Alexa Fluor® 568	577/603	Orange/Red	Rhodamine Red, Qdot® 605, Cy®3.5, RFP, DsRed	RITC
A20185	Alexa Fluor® 594	590/617	Red	Texas Red®, BODIPY® TR, Qdot® 625, HcRed Tandem, mRaspberry	Texas Red®
A20186	Alexa Fluor® 647	650/668	Far Red**	APC, Qdot® 655, DDAO, DiD, DRAQ5™, TO-PRO®-3, Cy®5, IRDye® 650	Cy®5, APC
A20188	Alexa Fluor® 680	679/702	Far Red**	Qdot® 705, Cy®5.5, iRFP, IRDye® 680, IRDye® 700	Cy®5.5, LI-COR 700 channel
A20189	Alexa Fluor® 790	785/810	Far Red**	DiR, Qdot® 800, IRDye® 800	NIR, LI-COR 800 channel
P30013	Pacific Blue™	410/455	Blue	Hoechst 34580, TagBFP	Violet laser/ DAPI channel
P30014	Pacific Orange™	~400/551	Orange	Qdot® 545, Qdot® 565	Violet laser/PE channel

* The fluorophores listed have similar excitation and emission properties but may vary in brightness, photostability, water solubility, quantum yield, and pH response relative to the Alexa Fluor®, Pacific Blue™, and Pacific Orange™ dyes.

** Human vision is insensitive to light beyond ~650 nm, and therefore it is not possible to view the far-red–fluorescent dyes by looking through the eyepiece of a conventional fluorescence microscope.

Note: DiO, DiI, DiD, and DiR are the lipophilic, carbocyanine dyes.

Before You Begin

Preparing the Protein

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 (microdialysis apparatus for small volumes of proteins, e.g., 10–500 µL, can be obtained from ThermoFisher, Spectrum Laboratories, or other vendors). 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 (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 8–9, can be stored at 2–8°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**, above), dilute it to 1 mg/mL and then add 1/10th 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 8–9, is added to raise the pH of the reaction mixture, since succinimidyl esters and TFP esters react efficiently at alkaline pH.

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

The purification step removes the unbound dye from the dye-conjugated protein. In applications that utilize repeated wash steps after labeling with the dye-conjugated antibody, purification may not be necessary. For more infoamtion, see **Note [A]** (page 8).

2.1 Place a spin column (Figure 1) in a 13 × 100-mm glass tube.

Note: The enclosed spin column should have two frits inserted at the bottom (Figure 1 b). 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 a glass or plastic stir rod. If any resin from the column get past the frits and ends up in the collection tube with the conjugate, it will do no harm and removing it 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 by gravity.

2.3 Continue to add more of the suspension until the resin bed volume is ~1.5 mL.

2.4 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. Place the spin column in one of the provided collection tubes (Figures 1 c and d) 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:

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. Discard the buffer, but save the collection tube. The spin column is now ready for purifying the conjugated antibody.

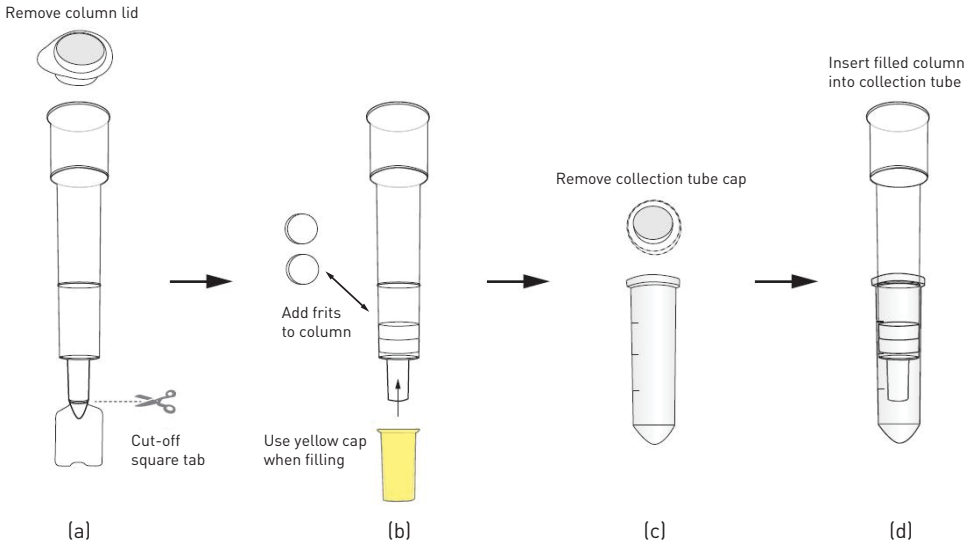
Note: A fixed angle rotor will suffice if a swinging bucket rotor is not available.

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

Figure 1 Spin column – adding frits to spin column and assembly with collection tube.



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 the absorbance maximum (λ_{max}) for the respective dye (A_{dye}). See Table 3 for values. If using a NanoDrop® or a cuvette that may provide a shorter or longer pathlength, see **Note [B]** (page 8) to modify the calculation.

3.2 Calculate the concentration of protein in the sample:

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

where 203,000 is the molar extinction coefficient (ε) in $cm^{-1}\ M^{-1}$ of a typical IgG at 280 nm and it is suitable for IgA, IgD, and IgE as well. The value CF_{280} is a correction factor for the fluorophore's contribution to the absorbance at 280 nm.

3.3 Calculate the degree of labeling:

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

where ε_{dye} (in $cm^{-1}M^{-1}$) is the approximate molar extinction coefficient of the specific dye. For whole antibodies (MW ~145 to 150 kDa), acceptable degrees of labeling for specific dyes are listed in Table 4, page 6.

Table 3 Properties of Alexa Fluor®, Pacific Blue™, and Pacific Orange™ dyes

Cat. no.	Dye	Molecular Weight	λ_{max}*	Em*	ε_{dye}**	CF_{280}†
A20180	Alexa Fluor® 350	410	346	442	19,000	0.19
A20181	Alexa Fluor® 488	885	494	519	71,000	0.11
A20182	Alexa Fluor® 532	724	530	554	81,000	0.09
A20183	Alexa Fluor® 546	~1160	554	570	104,000	0.12
A20187	Alexa Fluor® 555	~1260	555	565	150,000	0.08
A20184	Alexa Fluor® 568	792	577	603	91,300	0.46
A20185	Alexa Fluor® 594	820	590	617	73,000	0.56
A20186	Alexa Fluor® 647‡	~1300	650	668	239,000	0.03
A20188	Alexa Fluor® 680‡	~1200	679	702	184,000	0.05
A20189	Alexa Fluor® 790‡	~1750	785	810	260,000	0.08
P30013	Pacific Blue™	339	409	455	30,000	0.20
P30014	Pacific Orange™	~750	397	551	24,500	0.60

* Fluorescence absorbance and emission maxima, in nm, conjugated to an IgG antibody.
** Extinction coefficient at λ_{max} in $cm^{-1}\ M^{-1}$.
† Correction factor for absorption readings (A_{280}) at 280 nm; e.g., $A_{280,\ actual} = A_{280,\ observed} - (CF_{280} \times \lambda_{max})$.
‡ Human vision is insensitive to light beyond ~650 nm, and therefore it is not possible to view the far-red–fluorescent dyes by looking through the eyepiece of a conventional fluorescence microscope.

Table 4 Acceptable degrees of labeling (DOL) for a whole IgG

Cat. no.	Dye	DOL*
A20180	Alexa Fluor® 350	3–8
A20181	Alexa Fluor® 488	4–9
A20182	Alexa Fluor® 532	3–8
A20183	Alexa Fluor® 546	2–7
A20187	Alexa Fluor® 555	4–7
A20184	Alexa Fluor® 568	3–6
A20185	Alexa Fluor® 594	3–6
A20186	Alexa Fluor® 647	3–7
A20188	Alexa Fluor® 680	3–7
A20189	Alexa Fluor® 790	1–4
P30013	Pacific Blue™	4–7
P30014	Pacific Orange™	12–20

* Moles of dye per mole of protein.

Example Calculations

The example below shows the calculations of the protein concentration and degree of labeling (DOL) for an Alexa Fluor® 488-conjugated antibody.

4.1 Calculate the concentration of protein in the sample:

$$\text{Protein concentration (M)} = \frac{[A_{280} - (A_{494} \times 0.11)] \times \text{dilution factor}}{203{,}000}$$

where 203,000 is the molar extinction coefficient (ε) in $cm^{-1}\ M^{-1}$ of a typical IgG at 280 nm. The value 0.11 is the correction factor for the contribution of Alexa Fluor® 488 to the absorbance at 280 nm.

4.2 Calculate the degree of labeling (DOL):

$$\text{Moles dye per mole protein} = \frac{A_{494} \times \text{dilution factor}}{71{,}000 \times \text{protein concentration (M)}}$$

where 71,000 is the approximate molar extinction coefficient (ε_{dye}) of the Alexa Fluor® 488 dye at 494 nm.

Storing the Conjugates

Store the labeled protein at 2–8°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 2–8°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 the recommended DOL (moles of fluorophore per mole of 145,000–150,000 MW protein; Table 4), 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 3) is designed to raise the pH of the reaction mixture to ~8, because TFP- and 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 2–8°C after an initial incubation of one hour at room temperature.

Over-labeling

If calculations indicate that the protein conjugate is labeled with significantly higher DOL than recommended (moles of fluorophore per mole of 145,000–150,000 MW protein; Table 4), 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 5). 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.

Notes

Note [A] We have a tested a shortened workflow to determine whether the final column purification step (steps 2.1 to 2.7) was absolutely necessary to produce labeled protein conjugates suitable for immunocytochemical staining. We prepared labeled secondary antibodies using three of our most popular protein labeling kits—the Alexa Fluor® Large-scale Protein Labeling Kit, Alexa Fluor® Antibody Labeling Kit, and SAIVI™ Alexa Fluor® Antibody Labeling Kit. For each kit, we followed either: 1) the standard protocol including the final column purification step to remove free dye, or 2) a simplified protocol in which the final purification step was omitted. All three Molecular Probes® labeling kits produced fluorescent conjugates that effectively stained cells, even without the column purification step. As expected, the standard protocol with column purification produced slightly higher signal:noise ratios; however, we encourage researchers to consider whether the column purification step would significantly alter the outcome of their experiments. Furthermore, we found that the addition of Image-iT® FX signal enhancer (available separately, Cat. no. I36933) to the cells prior to staining with the labeled protein conjugate reduced the slight background fluorescence due to the presence of free dye, producing results that were nearly indistinguishable from those obtained with a column-purified conjugate.

Tip: After the labeling reaction, any unbound reactive dye may be quenched upon the addition of a small amount of a concentrated Tris or glycine buffer, or other small molecule with a primary amine.

More importantly, we found that, even without the column purification step, the Molecular Probes® labeling kits produced fluorescent conjugates that were far superior to those of the other one-step labeling kits tested, in terms of signal strength and background fluorescence. Thus, with this new simplified workflow, Molecular Probes® labeling kits provide one-step labeling convenience with high yields and bright results.

Note [B] Published extinction coefficients for protein and dyes are with the pathlength of 1 cm (cm^{-1} M^{-1}) unless specified otherwise. If using a NanoDrop®, the nominal pathlength is 1 mm. For the DOL calculation, multiply the ε of the protein and ε_{dye} by 10. If using a cuvette of a pathlength smaller than 1 cm, multiply the extinction coefficient by the ratio of the cuvette pathlength per 1 cm (10 mm). For example, if using a cuvette with a 2 mm pathlength, (10 mm / 2 mm) = 5. Multiply the extinction coefficient by 5.

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

Cat. no.	Product Name	Unit Size
A20180	Alexa Fluor® 350 Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
A20181	Alexa Fluor® 488 Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
A20182	Alexa Fluor® 532 Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
A20183	Alexa Fluor® 546 Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
A20187	Alexa Fluor® 555 Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
A20184	Alexa Fluor® 568 Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
A20185	Alexa Fluor® 594 Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
A20186	Alexa Fluor® 647 Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
A20188	Alexa Fluor® 680 Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
A20189	Alexa Fluor® 790 Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
P30013	Pacific Blue™ Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit
P30014	Pacific Orange™ Antibody Labeling Kit (5 reactions. 100 µg each)	1 kit

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Additional international offices are listed at
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These high-quality reagents and materials must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Read the Safety Data Sheet provided for each product; other regulatory considerations may apply.

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