Protein Labeling Kits (For Alexa Fluor™, Pacific Blue™, Fluorescein-EX, and Oregon Green™ 488)

Pub. No. MAN0019835 Rev. A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Protein Labeling Kits provide a convenient means to covalently label proteins with the superior Alexa Fluor™, Pacific Blue™, Fluorescein-EX, and Oregon Green™ 488 fluorescent dyes. These kits are optimized for labeling 1 mg of IgG per reaction and comparable amounts of other proteins (>20 kDa) can also be labeled. For labeling smaller amounts of antibodies (~100 µg), we recommend our antibody labeling kits.

Each Protein Labeling Kit contains everything you need to perform 3 separate labeling reactions and 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 3 vials of reactive dye provided in the kit is sufficient for labeling ~1 mg of an IgG antibody, although other proteins can also be labeled.

Alexa Fluor™ dyes produce protein conjugates that are typically brighter with better photostability and lower pH sensitivity relative to the traditional dyes (e.g., AMCA, fluorescein/FITC, TRITC, etc.) and the Cy™ dyes (Table 1). Unlike other dyes, the fluorescence of Alexa Fluor™ conjugates are 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. Alexa Fluor™-labeled antibodies can be used for multiple applications, including fluorescent microscopy, flow cytometry, western blotting, ELISA, and indirect FISH. Pacific Blue™ is a unique dye designed for use with a violet laser (405 nm) for flow cytometry applications. Dye spectra can be found at thermofisher.com

Table 1 Properties of Alexa Fluor™, Pacific Blue™, Fluorescein-EX, and Oregon Green™ 488 dyes.

Cat. No.	Dye	Ex/Em Maxima (nm)	Fluorescent Color	Spectrally Comparable Fluorophores[1]	Commonly Used Filter Sets
A10170	Alexa Fluor™ 350	346/442	Blue	AMCA, eBFP, DAPI, Hoechst 33342, Hoechst 33258	DAPI
A10235	Alexa Fluor™ 488	494/519	Green	Fluorescein/FITC, BODIPY™ FL, DiO, Cy2™, Qdot™ 525, GFP	FITC
A10236	Alexa Fluor™ 532	530/554	Yellow	Rhodamine 6G, BODIPY™ R6G, Qdot™ 545, mBanana	R6G
A10237	Alexa Fluor™ 546	554/570	Orange	Tetramethylrhodamine/TRITC, DiI, Cy3™, Qdot™ 565, mOrange/OFP	TRITC
A20174	Alexa Fluor™ 555	555/565	Orange	Tetramethylrhodamine/TRITC, R-PE, BODIPY™ TMR, Cy3™, Qdot™ 565, mOrange/OFP	TRITC
A10238	Alexa Fluor™ 568	577/603	Orange/Red	Rhodamine Red, Qdot™ 605, Cy3.5™, RFP, DsRed	RITC
A10239	Alexa Fluor™ 594	590/617	Red	Texas Red™, BODIPY™ TR, Qdot™ 625, HcRed Tandem, mRaspberry	Texas Red™
A20170	Alexa Fluor™ 633	632/647	Far Red[2]	APC, Qdot™ 655, BODIPY™ 630/650-X	Cy5™
A20173	Alexa Fluor™ 647	650/668	Far Red[2]	APC, Qdot™ 655, DDAO, DiD, DRAQ5™, TO-PRO™-3, Cy5™, IRDye™ 650	Cy5™, APC
A20171	Alexa Fluor™ 660	663/690	Far Red[2]	Cy5.5™	Cy5.5™
A20172	Alexa Fluor™ 680	679/702	Infrared	Qdot™ 705, Cy5.5™, iRFP, IRDye™ 680, IRDye™ 700	Cy5.5™, LICOR 700 channel
F10240	Fluorescein-EX	494/518	Green	Fluorescein/FITC, BODIPY™ FL DiO, Cy2™, Qdot™ 525, GFP	FITC
O10241	Oregon Green™ 488	496/524	Green	Fluorescein/FITC, BODIPY™ FL DiO, Cy2™, Qdot™ 525, GFP	FITC
P30012	Pacific Blue™	410/455	Blue	Hoechst 34580, TagBFP	Violet laser/DAPI channel

[1] 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™, Fluorescein-X, and Oregon Green™ 488 dyes.

[2] 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.

Contents and storage

Material	Amount	Storage[1]	Stability
Reactive Dye (Component A)	3 vials (each containing a magnetic stir bar)	• Store at 2–6°C protected from light. • Do not freeze.	When stored properly, kit components are stable for at least 3 months.
Sodium bicarbonate (MW=84) (Component B)	84 mg		
Purification columns (Component C)[2]	3 each		
Collection tubes	6 tubes		
Number of labelings: Each vial of reactive dye contains the appropriate amount of dye to label approximately 1 mg of IgG (MW ~145,000) as 0.5 mL of IgG solution at 2 mg/mL.			

[1] The kit can be stored under the conditions listed. For optimal storage conditions of individual components, refer to the labels on the vials or bags. Note that the reactive dye (Component A) may be stored frozen at ≤–20°C or at 2–8°C. Do not freeze the purification columns (Component C).

[2] The resin in each column is supplied in a 0.1 M NaCl/0.05% sodium azide solution.

Equipment required but not supplied

- Benchtop centrifuge capable of 1,000 × *g*

Labeling protocol

Prepare the proteins

- For optimal labeling efficiency, the purified protein must be in a buffer free of ammonium ions or primary amines.
- If the protein is in an unsuitable buffer (e.g., Tris or glycine), the buffer should be replaced with phosphate-buffered saline (PBS) by dialysis or another method. Impure proteins (e.g., antibodies in crude serum) 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.
- This kit can be used to label virtually any protein, although the following protocol has been optimized for labeling IgG antibodies. Each vial of reactive dye contains the appropriate amount of dye to label approximately 1 mg of IgG (MW ~145,000) as 0.5 mL of IgG solution at 2 mg/mL.

For tips on optimizing the procedure for other proteins or for antibody solutions at lower concentrations, see “Optimize the kit for use with other proteins and/or concentrations” on page 4 or “Optimization and troubleshooting” on page 4.

Label the protein

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 4°C for up to 2 weeks.
2. If the protein concentration is greater than 2 mg/mL, the protein should be diluted to 2 mg/mL in a suitable buffer (e.g., PBS or 0.1 M sodium bicarbonate.
3. To 0.5 mL of the 2 mg/mL protein solution, add 50 µL of 1 M bicarbonate prepared in step 1.
   Note: Bicarbonate, pH~8.3, is added to raise the pH of the reaction mixture, since TFP and succinimidyl esters react efficiently at alkaline pH.
4. Allow a vial of reactive dye to warm to room temperature. Transfer the protein solution from step 3 to the vial of reactive dye. This vial contains a magnetic stir bar. Cap the vial and invert a few times to fully dissolve the dye. Stir the reaction mixture for 1 hour at room temperature.

Purify the labeled proteins

Thermo Scientific™ Zeba™ Dye and Biotin Removal Spin Columns in this kit contain a ready-to-use resin that is uniquely designed for rapid removal of non-conjugated fluorescent dyes with exceptional protein recovery. The purification resin is designed to separate free dye from proteins with MW >20 kDa. For smaller proteins, gel filtration media of a suitable molecular weight cutoff should be selected. Labeled peptides may be separated from free dye by TLC or HPLC. Removal of free dye after a labeling reaction is essential for the accurate determination of dye to protein ratios. For optimal protein recovery and dye removal, ensure that the appropriate amount of sample and buffer conditions are used.

IMPORTANT! Protein conjugates that are between 20-50 kDa require a more alkaline buffer system to elute and will retain on the column if the buffer system is not changed. See procedure below for purifying 20-50 kDa conjugates.

Procedural guidelines

- Do not reuse the purification resin.
- Limit DMF and other organic solvents to ≤10% of solvent volume loaded onto the column.
- If labeling a 20-50 kDa protein, refer to “Purify 20-50 kDa conjugates” on page 2 to ensure conjugate recovery.

Prepare the spin column

1. Twist to remove the bottom plug of the column, then loosen the cap. Do not remove the cap.
2. Place the column in a collection tube, then centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes to remove the storage buffer. Discard the flowthrough.
3. If using a fixed angle rotor, place a mark on the side of the column facing away from the rotor center. For all subsequent centrifugation steps, place the column in the centrifuge with the mark facing away from the rotor center.

   IMPORTANT! Improper orientation of the column during centrifugation can result in reduced small molecule removal.
4. If desired, the resin storage buffer can be exchanged using a buffer of choice. To exchange, add 2 mL of equilibration buffer to the column, then centrifuge at 1,000 × *g* for 2 minutes. Discard the flowthrough.

Purify 20-50 kDa conjugates

If purifying a 20–50 kDa protein, a buffer exchange is required to ensure conjugate recovery.

1. Following storage buffer removal, apply 500 µL of 0.2 M, pH 9.4 bicarbonate buffer to the column (Cat. No. 28382).
2. Centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes.
3. For optimal conjugate recovery, repeat steps 1 and 2 two more times for a total of 3 column washes to ensure equilibration.

Process the sample

1. Place the prepared column into a new collection tube, then remove the cap.
2. Slowly apply the reaction mixture (~0.5 mL) to the center of the settled resin.
3. Centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes to collect the sample. The sample will be in the collection tube and the column can now be discarded.
4. *(Optional)* The column may be washed with an additional ~0.5 mL of suitable buffer (e.g., PBS) to maximize the recovered sample, applied as in steps 2 and 3. Note that this extra wash step will dilute the recovered conjugate and may be omitted if higher concentration is desired.

Determine the Degree of Labeling *(Optional)*

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 path length at 280 nm (A_{280}) and the absorbance maximum (λ_{max}) for the respective dye (A_{dye}). If using a NanoDrop™ or cuvette that may provide a shorter or longer path length, see “Note B” on page 4 to modify the calculation.

2. Calculate the concentration of protein in the sample:

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

Where 203,000 is the molar extinction coefficient (ε) in $cm^{-1}M^{-1}$ of a typical IgG at 280 nm suitable for IgA, IgD, and IgE. The value CF_{280} is a correction factor for the fluorophore's contribution to the absorbance at 280 nm (see Table 2).

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 (see Table 2). For whole antibodies (MW ~145 to 150 kDa), acceptable degrees of labeling for specific dyes are listed in the tables below.

Table 2 Properties of Alexa Fluor™, Oregon Green™ 488, Pacific Blue™, and Fluorescein-EX dyes.

Cat. No.	Dye	Molecular Weight	λ_{max}[1]	Em[1]	ε_{dye}[2]	CF_{280}[3]
A10170	Alexa Fluor™ 350	410	346	442	19,000	0.19
A10235	Alexa Fluor™ 488	885	494	519	71,000	0.11
A10236	Alexa Fluor™ 532	724	530	554	81,000	0.09
A10237	Alexa Fluor™ 546	~1160	554	570	104,000	0.12
A20174	Alexa Fluor™ 555	~1260	555	565	150,000	0.08
A10238	Alexa Fluor™ 568	792	577	603	91,300	0.46
A10239	Alexa Fluor™ 594	820	590	617	73,000	0.56
A20170	Alexa Fluor™ 633[4]	~1200	632	647	100,000	0.55
A20173	Alexa Fluor™ 647[4]	~1300	650	668	239,000	0.03
A20171	Alexa Fluor™ 660[4]	~1100	663	690	132,000	0.1
A20172	Alexa Fluor™ 680[4]	~1150	679	702	184,000	0.05
F10240	Fluorescein-EX	591	494	518	68,000	0.2
O10241	Oregon Green™ 488	509	496	524	70,000	0.12
P30012	Pacific Blue™	339	410	455	30,000	0.20

[1] Fluorescence absorbance and emission maxima (nm) conjugated to an IgG antibody.

[2] Extinction coefficient at λ_{max} in $cm^{-1}M^{-1}$.

[3] Correction factor for absorption readings (A_{280}) at 280 nm (e.g., $A_{280, actual} = A_{280, observed} - (CF_{280} \times \lambda_{max})$.

[4] 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 3 Acceptable degrees of labeling (DOL) for a whole IgG.

Cat. No.	Dye	DOL[1]
A10170	Alexa Fluor™ 350	3–8
A10235	Alexa Fluor™ 488	4–9
A10236	Alexa Fluor™ 532	3–8
A10237	Alexa Fluor™ 546	2–7
A20174	Alexa Fluor™ 555	4–7
A10238	Alexa Fluor™ 568	3–6
A10239	Alexa Fluor™ 594	3–6
A20170	Alexa Fluor™ 633	1.5–4
A20173	Alexa Fluor™ 647	3–7
A20171	Alexa Fluor™ 660	3–7
A20172	Alexa Fluor™ 680	3–7
F10240	Fluorescein-EX	4–8
O10241	Oregon Green™ 488	4–8
P30012	Pacific Blue™	4–7

[1] Moles of dye per mole of protein.

Example calculations

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

1. Calculate the concentration of protein in the sample:

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

Where 203,000 is the molar extinction coefficient (ε) in cm-1M-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.

2. Calculate the degree of labeling:

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

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

Store and handle 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.
- For optimal use, centrifuge solutions of conjugates in a microcentrifuge before use; only the supernatant should then be used in the experiment. This step will remove any aggregates that may have formed during storage.

Optimize the kit for use with other proteins and/or concentrations

- Proteins at less than 2 mg/mL
  - Proteins at concentrations less than 2 mg/mL will not label as efficiently. If the protein cannot be concentrated to ~2 mg/mL, you may wish to use less than 1 mg protein per reaction to increase the molar ratio of dye to protein. In addition, using a dilute protein solution, especially at <1 mg/mL will make it more difficult to efficiently remove the unconjugated dye from the dye-labeled protein with acceptable yields, since the provided purification columns are designed to purify conjugates from a total volume of less than 1 mL. For reaction volumes greater than 1 mL, you can divide the solution of the conjugate and apply it to multiple purification columns or, to avoid further dilution of the conjugate, you can remove free dye by extensive dialysis.
- Proteins with molecular weight (MW) other than ~145,000
  - Typically, lower MW proteins require fewer dye molecules and higher MW proteins require more dye molecules per protein for optimal labeling. For this reason, we recommend initially performing the reaction with 0.5 mL of 2 mg/mL protein solution, as described for IgGs. The labeling conditions can then be optimized based on the initial results, if desired.

Optimization and 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 3), 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 2) 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 1 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, 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

Despite removing most free dye from protein conjugates using 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 (“Determine the Degree of Labeling (Optional)” on page 2). 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, the conjugate may be eluted using a high salt concentration buffer (e.g., PBS, 0.3 M NaCl) or with 0.2 M, pH 9.4 bicarbonate. Incubate the column with end over end mixing for 1 minute, then elute the conjugate by spinning at 1,000 × *g* for 2 minutes. We recommend re-purifying the conjugate using size-exclusion resin, dialysis, or spin filtration.

Notes

Note A

We have tested a shortened workflow to determine whether the final column purification step (“Prepare the spin column” on page 2) was absolutely necessary to produce labeled protein conjugates suitable for immunocytochemical staining. We prepared labeled secondary antibodies using 3 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 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.

Note: 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 fluoroscent 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, these 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 path length is 1 mm. For the DOL calculation, multiply the ε of the protein and ε_{dye} by 10. If using a cuvette of a path length smaller than 1 cm, multiply the extinction coefficient by the ratio of the cuvette path length per 1 cm (10 mm). For example, if using a cuvette with a 2 mm path length, (10 mm/2 mm = 5). Multiply the extinction coefficient by 5.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

Life Technologies Corporation | 29851 Willow Creek | Eugene, OR 97402
For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

The information in this guide is subject to change without notice.

DISCLAIMER: TO THE EXTENT ALLOWED BY LAW, THERMO FISHER SCIENTIFIC INC. AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Revision history: Pub. No. MAN0019835

Revision	Date	Description
A.0	21 January 2021	New manual.

Important Licensing Information: These products may be covered by one or more Limited Use Label Licenses. By use of these products, you accept the terms and conditions of all applicable Limited Use Label Licenses.