Pacific Orange™ Monoclonal Antibody Labeling Kit

Table 1. Contents and Storage Information.

Materials	Amount	Concentration	Storage Upon Receipt	Stability
Pacific Orange™ reactive dye (component A)	5 vials	NA	• 2–6°C • Protect from light • Protect from moisture	When stored as directed, kit components are stable for at least 3 months.
Sodium bicarbonate (component B)	84 mg (MW = 84)	NA	• 2–6°C • Protect from moisture	
Purification resin (component C)	~10 mL of 30,000 MW size-exclusion resin in phosphate-buffered saline (PBS), 2 mM sodium azide, pH 7.2	NA	• 2–6°C • DO NOT FREEZE	
Spin columns (component D)	5	NA	2–6°C	
Collection tubes (component E)	5 tubes (2 mL each)	NA	2–6°C	

NA = Not Applicable.

Number of Reactions: each vial of Pacific Orange™ reactive dye contains enough dye to label ~100 µg of a monoclonal antibody.

Spectral Data: Pacific Orange™ dye ~400/551 nm. See Figure 1.

Introduction

Molecular Probes' Pacific Orange™ Monoclonal Antibody Labeling Kit provides a convenient means to label small amounts of monoclonal antibodies with the violet-excitable Pacific Orange™ 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 Pacific Orange™ Protein Labeling Kit (P30016).

Pacific Orange™ dye–labeled proteins have fluorescence excitation and emission maxima of approximately 400 nm and 551 nm, respectively (Figure 1).

The Pacific Orange™ Monoclonal Antibody Labeling Kit contains everything you need to perform five separate labeling reactions as well as to purify the resulting conjugates. The Pacific Orange™ reactive dye (Figure 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.

Before You Begin

Protein Preparation

Each reaction is optimized to label 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.

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

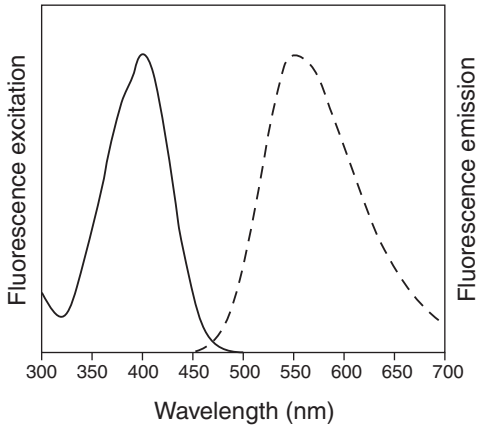

Figure 1. Normalized excitation and emission spectra of Pacific Orange™ dye conjugated to goat anti-mouse IgG in pH 7.2 buffer.

Experimental Protocol

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 2–6°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*), 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.

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.

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.

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

Purification of the Labeled Protein

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

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

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.

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) 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.

Determination of 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 both 280 nm (A_{280}) and 397 nm (A_{397}).

3.2 Calculate the concentration of protein in the sample:

$$\text{Protein concentration (M)} = \frac{[A_{280} - A_{397} \times 0.6] \times \text{dilution factor}}{203{,}000}$$

where 203,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient (ϵ) of a typical IgG at 280 nm and 0.6 is a correction factor for the fluorophore's contribution to the absorbance at 280 nm.

3.3 Calculate the degree of labeling:

$$\text{Moles dye per mole protein} = \frac{A_{397} \times \text{dilution factor}}{24{,}500 \times \text{protein concentration (M)}}$$

where 24,500 $cm^{-1}M^{-1}$ is the approximate molar extinction coefficient of the Pacific Orange™ dye at 397 nm. For IgGs, we find that labeling with 12–20 moles of Pacific Orange™ dye per mole of antibody is optimal.

Storage of Conjugates

Store the labeled protein at 2–6°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 2–6°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 ten 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 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) 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. 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 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 twenty 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 next time, either 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 *Determination of Degree of Labeling*). 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 #	Product Name	Unit Size.
P30014	Pacific Orange™ Monoclonal Antibody Labeling Kit *5 labelings*	1 kit

Contact Information

Molecular Probes, Inc.
29851 Willow Creek Road,
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:
6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
probesorder@invitrogen.com

Toll-Free Ordering for USA:
Order Phone: (800) 438-2209
Order Fax: (800) 438-0228

Technical Service:
8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probestech@invitrogen.com

Invitrogen European Headquarters
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Assistance Department in Eugene, Oregon.

Molecular Probes products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Limited Use Label License
For research use only. Not intended for any animal or human therapeutic or diagnostic use. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402. Tel: (541) 465-8300. Fax: (541) 335-0504.

Several Molecular Probes products and product applications are covered by U.S. and foreign patents and patents pending. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Copyright 2005, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.