invitrogen™

MOLECULAR PROBES®

Alexa Fluor® 546 Protein Labeling Kit

Catalog no. A10237

Table 1. Contents and storage information.

<table>
<tr><th>Material</th><th>Amount</th><th>Storage*</th><th>Stability</th></tr>
<tr><td>Alexa Fluor® 546 reactive dye (Component A)</td><td>3 vials, each containing a magnetic stir bar</td><td>• ≤–20°C
• Dessicate
• Protect from light</td><td rowspan="9">When stored properly, the kit components are stable for at least 3 months.</td></tr>
<tr><td>Sodium bicarbonate (Component B, MW = 84)</td><td>84 mg</td><td>• 2–6°C
• Dessicate</td></tr>
<tr><td>Purification resin (Component C, Bio-Rad BioGel® P-30 fine size exclusion purification resin)</td><td>~25 mL in PBS, pH7.2, plus 2 mM sodium azide</td><td>• 2–6°C
• Do not freeze</td></tr>
<tr><td>Elution buffer (Component D)</td><td>~25 mL of 0.1 M potassium phosphate, 1.5 M NaCl, pH 7.2, plus 2 mM sodium azide (10X concentration)</td><td>• 2–6°C</td></tr>
<tr><td>Purification columns</td><td rowspan="4">3 each</td><td rowspan="5">• Room temperature</td></tr>
<tr><td>Column funnels</td></tr>
<tr><td>Foam column holders</td></tr>
<tr><td>Disposable pipets</td></tr>
<tr><td>Collection tubes</td><td>3 tubes, 4 mL each</td></tr>
<tr><td colspan="4">*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.</td></tr>
<tr><td colspan="4">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.</td></tr>
<tr><td colspan="4">Approximate fluorescence excitation/emission maxima: 554/570 nm for Alexa Fluor® 546 conjugate.</td></tr>
</table>

Introduction

Alexa Fluor® 546 Protein Labeling Kit provides a convenient means to label proteins with the Alexa Fluor® 546 dye. Alexa Fluor® 546 dye-labeled proteins have fluorescence excitation and emission maxima of approximately 554 nm and 570 nm, respectively, and are typically brighter than similar proteins labeled with the Cy®3 fluorophore.

The Alexa Fluor® 546 Protein Labeling Kit contains everything you need to perform three separate labeling reactions, and to purify the resulting conjugates. The Alexa Fluor® 546 reactive dye (Figure 1) has a succinimidyl ester moiety that reacts efficiently with primary amines of proteins to form stable dye–protein conjugates. Each of the three vials of reactive dye provided in the kit is sufficient for labeling ~1 mg of an IgG antibody, although you can also label other proteins.

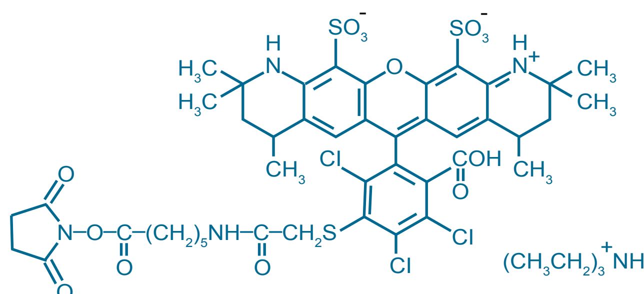

Figure 1. Alexa Fluor® 546 carboxylic acid, succinimidyl ester, triethylammonium salt (MW ~1,159.60).

Before You Begin

Preparing the Protein

Important

For optimal labeling efficiency, the purified protein should be in a buffer free of ammonium ions or primary amines. If the protein is in an unsuitable buffer (*e.g.*, Tris or glycine), replace the buffer with phosphate-buffered saline (PBS) by dialysis or another method. Impure proteins (*e.g.*, antibodies in crude serum or proteins 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.

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 *Tips for Using the Kit with Other Proteins and/or Concentrations* and *Troubleshooting*.

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 may store the bicarbonate solution, which will have a pH ~8.3, at 4°C for up to two weeks.

1.2 If the protein concentration is greater than 2 mg/mL, dilute the protein to 2 mg/mL in a suitable buffer (*e.g.*, PBS or 0.1 M sodium bicarbonate).

1.3 To 0.5 mL of the 2 mg/mL protein solution, add 50 µL of 1 M bicarbonate (prepared in step 1.1).

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.

1.4 Allow a vial of reactive dye to warm to room temperature. Transfer the protein solution from step 1.3 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.

Note: Because preparation of the purification column takes ~15 minutes, you may wish to begin pouring the column (see *Purifying the Labeled Protein*) during the labeling reaction.

Purifying the Labeled Protein

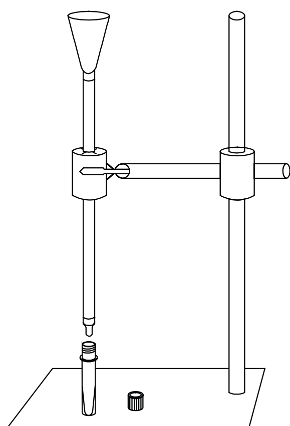

Figure 2. Column assembly.

2.1 Assemble the column and position it upright (see Figure 2): Attach a funnel to the top of a column. Gently insert the column through the X-cut in one of the provided foam holders to avoid damaging the column. Using the foam holder, secure the column with a clamp to a ringstand. Carefully remove the cap from the bottom of the column.

2.2 Prepare elution buffer by diluting the room temperature 10X stock (Component D) 10-fold in deionized water. Typically, less than 10 mL is required for each purification. Set 1X elution buffer aside until step 2.5.

Note: The 10X elution buffer (10X PBS) contains 0.1 M potassium phosphate, 1.5 M NaCl, pH 7.2, with 2 mM sodium azide. Warm the 10X stock to room temperature prior to use to ensure that the buffer is fully dissolved. Sufficient elution buffer is included to allow washing of the columns for reuse, if desired.

For maintaining optimal stability of your labeled protein, you may substitue other elution buffers for 1X PBS.

2.3 Using one of the provided pipets, stir the purification resin (Component C) thoroughly to ensure a homogeneous suspension. Pipet the resin into the column, allowing excess buffer to drain away into a small beaker or other container. Pack the resin into the column until the resin is ~3 cm from the top of the column.

Note: Component C, Bio-Rad BioGel® P-30 Fine size exclusion purification resin, is designed to separate free dye from proteins with MW > 40,000. This is packaged in PBS containing 2 mM sodium azide. For smaller proteins, select gel filtration media of a suitable molecular weight cutoff. You may separate labeled peptides from free dye by TLC or HPLC.

2.4 Allow the excess buffer to drain into the column bed. Do not worry about the column drying out, since the matrix will remain hydrated. Make certain the buffer elutes through the column with a consistently even flow prior to adding the reaction mixture. If the flow of buffer is slow or stalled, repack the column. Carefully load the reaction mixture from step 1.4 onto the column. You may wish to remove the column funnel to load the sample. Allow the mixture to enter the column resin. Rinse the reaction vial with ~100 µL of elution buffer and apply to the column. Allow this solution to enter the column.

2.5 Replace the funnel if it was removed for sample loading. Slowly add elution buffer (prepared in step 2.2), taking care not to disturb the column bed. Continue adding elution buffer until the labeled protein has been eluted (typically about 30 minutes).

Important: Collect, and retain as fractions, all of the eluted buffer.

2.6 As the column runs, periodically illuminate the column with a hand-held UV lamp. The two colored bands observed represent the separation of labeled protein from unincorporated dye.

Collect the first colored band, which contains the labeled protein, into one of the provided collection tubes. If desired, you can use a foam holder to support the collection tube. Add elution buffer to the column as necessary. Do not collect the slower moving band, which consists of unincorporated dye.

Once you have successfully collected the fraction containing the labeled protein, you may discard all other fractions of eluted buffer. In rare instances where there is no discernable band corresponding to labeled protein, you can use the retained fractions to recover any unlabeled protein.

Determining the Degree of Labeling

3.1 Measure the absorbance of the conjugate solution at 280 nm and 554 nm (A_{280} and A_{554}) in a cuvette with a 1 cm pathlength. It may be necessary to dilute the sample.

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, in cm^{-1} M^{-1}, of a typical IgG, 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 cm^{-1} 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 protein—which will be in PBS, pH 7.2, containing ~2 mM sodium azide—at 2–6°C, **protected from light**. If the final concentration of purified protein 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.**

It is a good practice to centrifuge solutions of conjugates in a microcentrifuge before use; use only the supernatant in the experiment. This step removes any aggregates that may have formed during storage.

Tips for Using the Kit 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 you cannot concentrate the protein to ~2 mg/mL, use less than 1 mg protein per reaction to increase the molar ratio of dye to protein.

Using a dilute protein solution, especially at <1 mg/mL, makes 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 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. Based on the initial results, you can optimize the labeling conditions, if desired.

Troubleshooting

Under-Labeling

If calculations indicate that the protein is labeled with significantly less than two moles of fluorophore per mole of 145,000 dalton protein, 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. See *Proteins at Less Than 2 mg/mL*.

- The addition of sodium bicarbonate (step 1.3) 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 145,000 dalton 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 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.

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

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

For country-specific contact information, visit www.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 Service 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 No. 223: Labeling and Detection Technology
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) 335-0354.

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 2009, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.