Phycobiliproteins

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage*	Stability
allophycocyanin	0.5 mL	4 mg/mL solution in 60% saturated ammonium sulfate, 50 mM potassium phosphate, pH 7.0	• 2–6˚C • Protect from light • DO NOT FREEZE	When stored as directed the product is stable for at least 1 year.
B-phycoerythrin				
R-phycoerythrin				
allophycocyanin, crosslinked (APC-XL)	250 µL			
R-phycoerythrin, biotin-XX conjugate	0.5 mL	4 mg/mL solution in 0.1 M sodium phosphate, 0.1 M NaCl, 5 mM sodium azide, pH 7.5		When stored as directed the product is stable for at least 6 months.
R-phycoerythrin, pyridyldisulfide derivative	1 mL	2 mg/mL solution in 0.1 M sodium phosphate, 0.1 M NaCl, 5 mM sodium azide, pH 7.5		
Approximate fluorescence excitation/emission maxima: See Table 2.				

Introduction

Phycobiliproteins are stable and highly soluble proteins derived from cyanobacteria and eukaryotic algae that possess a mono-disperse population of prosthetic fluorophores. Their biological role as light collectors requires maximal absorbance and fluorescence without susceptibility to internal or external fluorescence quenching. Consequently, their absorption and fluorescence characteristics are exceptional—quantum yields up to 0.98 and molar extinction coefficients of up to 2.4×10^6 have been reported. Phycobiliproteins have been covalently conjugated to proteins such as antibodies and other molecules to make probes with greatly enhanced detectability.[1] Molecular Probes offers three highly purified phycobiliproteins—B-phycoerythrin (B-PE, Cat. no. P800), R-phycoerythrin (R-PE, Cat. no. P801), and allophycocyanin (APC, Cat. no. A803)—for these applications, in addition to phycobiliprotein-labeled secondary antibodies and avidin/biotin probes. All allophycocyanin conjugates are made using allophycocyanin stabilized by chemical crosslinking (APC-XL, Cat. no. A819) to avoid dissociation of the molecule in dilute solutions. Phycobiliproteins and derivatives are available from Molecular Probes in bulk at a considerable savings.

On a molar basis, phycobiliproteins have a fluorescence yield that is equivalent to at least 30 fluorescein or 100 rhodamine molecules at comparable wavelengths. The broad excitation spectra, particularly of the R-phycoerythrin conjugates, permits simultaneous detection of two cell structures or subpopulations by microscopy[2] and analysis and sorting by flow cytometry of fluorescein- and phycoerythrin-labeled probes using a single excitation source. Fluorescein and phycoerythrin conjugates are both excited at 488 nm (a spectral line of the

argon-ion laser), and fluorescence measurements are made at approximately 520 nm for fluorescein and >575 nm for B- or R-phycoerythrin.

By using a second excitation wavelength near 590 nm, one can excite both allophycocyanin and the Texas Red® or Alexa Fluor® 594 fluorophores for four-color applications. Allophycocyanin conjugates are ideal for use with helium–neon (He–Ne) laser excitation (633 nm).

Properties

The principal absorption and fluorescence characteristics of phycobiliproteins are summarized in Table 2 and illustrated in Figures 1 and 2. The relative (percentage) absorbances are compared in Table 3 for the primary wavelengths of the argon-ion (488 nm), krypton-ion (568 nm), and He–Ne (633 nm) lasers. From this table, one can predict that, at equal degrees of substitution, the greatest sensitivity will be achieved with R-PE conjugates for 488 nm excitation, with B-PE for 545 or 568 nm excitation and with APC at 633 nm excitation.

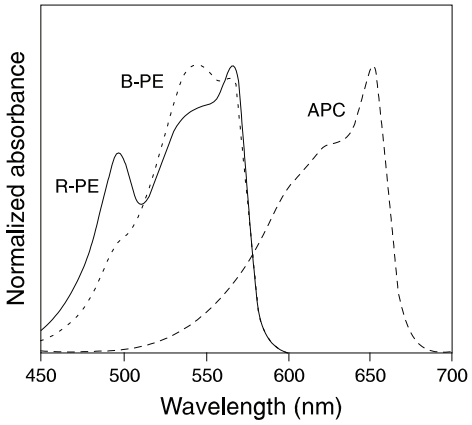

Figure 1. Normalized absorbance spectra of B-PE, R-PE, and APC.

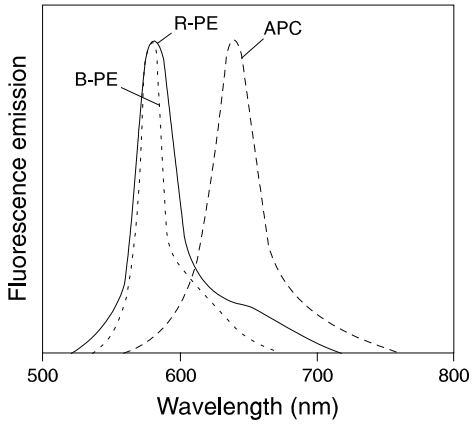

Figure 2. Normalized fluorescence emission spectra of B-PE, R-PE, and APC.

Table 2. Properties of phycobiliproteins.

Cat. no.	Protein	Mol. weight	Ex (nm)*	Em (nm)*	ε_{max} (cm^{-1} M^{-1})	QY
P800	B-phycoerythrin	240,000	546, 565	575	2,410,000	0.98
P801	R-phycoerythrin	240,000	480, 546, 565	578	1,960,000	0.82
A803, A819	allophycocyanin	104,000	650	660	700,000	0.68

Ex/Em = fluorescence excitation and emission maximum, ε_{max} = Maximum molar extinction coefficient, QY = Fluorescence quantum yield.

Table 3. Relative (percentage) absorbance of phycobiliproteins at commonly used excitation wavelengths.*

Protein	488 nm	568 nm	633 nm
B-phycoerythrin	33	97	0
R-phycoerythrin	63	82	0
allophycocyanin	0.5	20	56

*Percentages shown are relative to the peak absorption for each phycobiliprotein.

Before Starting

Before use, centrifuge pure phycobiliproteins at 5,000 rpm for 10 minutes. Dissolve the pellet in 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5, and then dialyze against several changes of the same buffer, avoiding dilutions.

The phycobiliprotein conjugates and modified phycobiliproteins may be used as received.

Conjugation of Phycobiliproteins to Other Proteins

Use of the exceptional fluorescence of phycobiliproteins usually requires chemical coupling of the phycobiliproteins to other proteins. Molecular Probes offers a custom service for conjugation of phycobiliproteins to antibodies supplied by customers. Alternatively, Molecular Probes supplies all reagents necessary for you to prepare their own phycobiliprotein conjugates. These reagents are supplied individually or as part of our convenient Protein–Protein Crosslinking Kit (Cat. no. P6305), which contains a detailed protocol in addition to the necessary reagents. A general protocol for preparing phycobiliprotein-labeled probes is given below.

Overview

Some published methods for preparing phycobiliprotein conjugates with antibodies and other proteins yield a reductively unstable disulfide linkage.[1,3,4] We recommend an alternative and easier procedure that gives a more stable thioether protein–protein linkage. In this procedure (see Figure 3), the phycobiliprotein is converted to a pyridyldisulfide derivative with SPDP (Cat. no. S1531),[5] then reduced to a thiol with dithiothreitol (DTT, Cat. no. D1532). For your convenience, the pyridyldisulfide conjugate of R-phycoerythrin (Cat. no. P806) is also available from Molecular Probes. Lysines of the second protein are converted to thiolreactive maleimides with a heterobifunctional crosslinking reagent. SMCC (Cat. no. S1534) is most commonly used for this purpose.[6]

Procedure for Coupling Phycobiliproteins to Proteins without Free Thiols

Use this procedure to couple phycobiliproteins to proteins without free thiols (e.g., most IgG antibodies). If the protein you wish to label has free thiols (e.g., ß-galactosidase), see **Procedure for Coupling Phycobiliproteins to Proteins with Free Thiols**. To determine whether a protein has free thiols, Molecular Probes offers a Thiol and Sulfide Quantitation

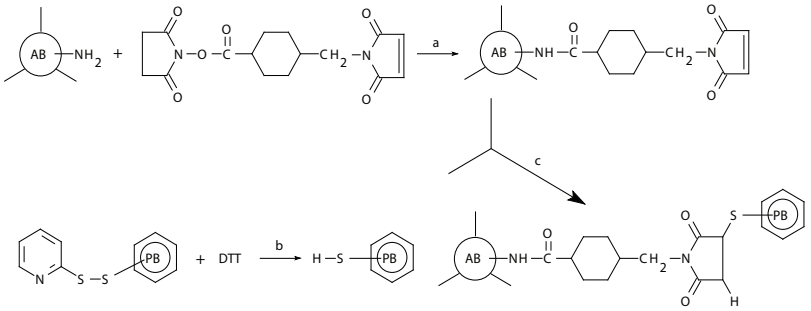

Figure 3. Schematic outlining the coupling chemistry used to crosslink a phycobiliprotein to another protein. Step a. The antibody (AB) or other protein is treated at pH 7.5 with a succinimidyl ester maleimide derivative such as SMCC (shown in schematic). This converts some lysine residues of the antibody to thiol-reactive maleimides. Step b. Thiolated phycobiliprotein (HS-PB) is prepared by reduction of the appropriate SPDP-modified phycobiliprotein with dithiothreitol (DTT) or with tris-(2-carboxyethyl)phosphine (TCEP). Step c. After dialysis, the above two protein conjugates are mixed to yield a stable thioether crosslink. The phycobiliprotein conjugate is then chromatographically separated from unreacted proteins.

Kit (Cat. no. T6060). A detailed protocol for protein coupling is available with our Protein–Protein Crosslinking Kit (Cat. no. P6305). Also, see the reference by Rosaria P. Haugland.[7]

Steps 1.1 and 1.2 should be undertaken separately but simultaneously to avoid loss of the maleimide reactivity in step 1.1 and to minimize air oxidation of the thiolated phycobiliprotein in step 1.2. Depending on the availability and expense of the materials, usually one to two moles of the antibody or other protein per mole of phycobiliprotein are used in the procedure below. If possible, use at least 4 mg of phycobiliprotein and 2.5 mg of antibody to obtain a reasonable yield of conjugate.

For most applications, microgram quantities or less of conjugate are used so that once the conjugate is formed it should be sufficient for a large number of tests.

1.1 The antibody or other protein is dissolved at about 5 mg/mL in phosphate buffer, pH 7.5. SMCC [6] is dissolved at about 5 mM in dimethylformamide or dimethylsulfoxide. An amount of SMCC or SMB stock solution to give a 10- to 20-fold molar excess is added to the protein solution. This mixture is incubated for approximately two hours at room temperature. The product is separated from excess reagents by exhaustive dialysis, with care being taken to avoid excessive dilution.

1.2 Phycobiliprotein containing an average of about 2 moles of pyridyldisulfide per mole of phycobiliprotein (prepared from SPDP and the phycobiliprotein [1,3,5] or available directly from Molecular Probes) is treated with 50 mM (approximately 8 mg/mL) final concentration of DTT by adding the compound as a powder while stirring and then incubating for 15 minutes at room temperature. The excess DTT is then eliminated by dialysis at 4°C with five changes of a large volume of buffer at pH 7.5 within 24 hours.

Alternatively, tris-(2-carboxyethyl)phosphine (TCEP, Cat. no. T2556) can be used to reduce the pyridyldisulfide-modified phycobiliprotein. Note that because excess TCEP does not need to be removed by dialysis, perform reduction with TCEP just prior to beginning step 1.3. First, a 1 mg/mL stock solution of TCEP is prepared in phosphate buffer, pH 7.5. An amount of this TCEP solution to give a five-fold molar excess is then added to the modified phycobiliprotein solution. After mixing, the solution is incubated at room temperature for 15 minutes.

1.3 The two protein solutions prepared in steps 1.1 and 1.2 are mixed and incubated at 4°C for 16 to 20 hours to form the coupled protein conjugate. The reaction is terminated by addition of a 20-fold molar excess of N-ethylmaleimide to "cap" remaining thiols. This step avoids any possibility of antibody disulfide bond reduction or formation of phycobiliprotein dimers via air oxidation. The conjugate is separated from nonconjugated proteins by either of the chromatographic procedures described below.

Procedure for Coupling Phycobiliproteins to Proteins with Free Thiols

Materials that already contain intrinsic thiol residues, including partially reduced antibodies, can be conjugated to phycobiliproteins by reacting the unmodified form of phycobiliprotein with the succinimidyl ester maleimide SMCC. This reaction is performed by following the procedure outlined in step 1.1 above, substituting the phycobiliprotein in place of the antibody or other protein described in the protocol. The modified phycobiliprotein is then reacted with the unmodified, free thiol-containing protein as described in step 1.3. Dilution must be avoided because the cross-conjugation follows bimolecular kinetics—where dilutions of both components to half causes a fourfold decrease in the rate of crosslinking—while the reactions that destroy the maleimide or thiolated intermediate are likely pseudo first order.

Destruction of the maleimide occurs most rapidly above pH 8.0. To concentrate phycobiliprotein solutions, we have used CX-30 immersible filters from Millipore or adsorption on hydroxyapatite from dilute phosphate followed by elution with higher molarity phosphate.

Table 4. Pure phycobiliprotein absorbance (A) ratios.*

Protein	Absorbance ratio
B-phycoerythrin**	$A_{565\ nm}/A_{280}$ nm greater than 5
R-phycoerythrin	$A_{545\ nm}/A_{280\ nm}$ greater than 5
allophycocyanin	$A_{650\ nm}/A_{280\ nm}$ greater than 4

*These values should be checked prior to conjugation on your individual lot of phycobiliprotein because they will vary slightly with each batch and also with light scattering and instrumental factors.
**This value does not apply to the pyridyldisulfide derivative.

Purifying Phycobiliprotein Conjugates

Chromatography on Sephacryl® S-300 or Agarose 1.5 m.

An antibody–phycobiliprotein conjugate will have a molecular weight considerably higher than either of its components. Thus, gel filtration using a material, such as Sephacryl S-300 or Agarose 1.5 m, with the ability to separate high molecular weight materials will elute the conjugate first, followed by the unconjugated phycobiliprotein, followed in most cases by the antibody.[3] Visualization and quantitation is considerably facilitated by the visible color of the conjugates. Collect small fractions and analyze using the absorption ratios for the pure phycobiliproteins listed in Table 4 to determine the approximate antibody-to-phycobiliprotein ratio. Since the antibody in the conjugate contributes to the absorption at 280 nm but not to the absorption at the long wavelength, the fractions with the highest ratio of antibody to phycobiliprotein, which will be eluted first from the column, will have the lowest ratio of long-wavelength absorption to 280 nm absorption. If separation between the unconjugated phycobiliprotein and unconjugated antibody is incomplete, then later fractions may also have absorption ratios less than those reported in Table 4. To determine the utility of the resultant phycobiliprotein-labeled antibody, evaluate the column fractions separately in the desired biological application to determine the effect of the antibody:phycobiliprotein ratio of the conjugate. Modifications can then be made in the synthetic method (e.g., changing ratios of protein to phycobiliprotein added to the reaction) to optimize the degree of conjugation.

Chromatography on hydroxyapatite

This method was suggested by Drs. Randy Hardy and David Parks of Stanford University and is a good method to purify and concentrate phycobiliprotein conjugates. First the crude conjugate from step 1.3 is dialyzed against 1 mM sodium phosphate in 0.1 M NaCl, pH 7.0, then adsorbed onto a small hydroxyapatite column in the same buffer. For both B- and R-phycoerythrin, a gradient from 1 mM to 50 mM phosphate in 0.1 M NaCl elutes first the unconjugated phycobiliprotein (and presumably unconjugated antibody) followed by the conjugates in order of increasing molecular weight. The highest molecular weight conjugates may require ≥50 mM phosphate to elute. The fractions are analyzed using the absorption ratio table as described above in step 1.1. Allophycocyanin conjugates require higher phosphate concentrations for elution from hydroxyapatite.

Storing Phycobiliprotein Conjugates

Store phycobiliprotein conjugates at 4°C. **Do not freeze.** The addition of sodium azide to a final concentration of 5 mM is recommended to avoid bacterial contamination. Removal of the azide may be necessary before use of conjugates with live cells.

Custom Phycobiliprotein Conjugation

Molecular Probes performs conjugation of phycobiliproteins to antibodies and other proteins supplied by customers. Contact our Custom and Bulk Sales Department for further information.

References

1. J Cell Biol 93, 981 (1982); **2.** Science 239, 771 (1988); **3.** Clin Chem 29, 1582 (1983); **4.** Biophys J 43, 383 (1983); **5.** Biochem J 173, 723 (1978); **6.** Eur J Biochem 101, 395 (1979); **7.** Methods in Monoclonal Biology, Vol. 45: Monoclonal Antibody Protocols, W.C. Davis, Ed., Humana Press (1995) pp. 205–221.

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

Cat. no.	Product Name	Unit Size
A803	allophycocyanin *4 mg/mL*	0.5 mL
A819	allophycocyanin, crosslinked (APC-XL) *4 mg/mL*	250 µL
P800	B-phycoerythrin *4 mg/mL*	0.5 mL
P801	R-phycoerythrin *4 mg/mL*	0.5 mL
P811	R-phycoerythrin, biotin-XX conjugate *4 mg/mL*	0.5 mL
P806	R-phycoerythrin, pyridyldisulfide derivative *2 mg/mL*	1 mL
Related Products		
D1532	dithiothreitol (DTT)	1 g
P6305	Protein-Protein Crosslinking Kit *3 conjugations*	1 kit
S1531	succinimidyl 3-(2-pyridyldithio)propionate (SPDP)	100 mg
S1534	succinimidyl *trans*-4-(maleimidylmethyl) cyclohexane-1-carboxylate (SMCC)	100 mg
T2556	tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP)	1 g
T6060	Thiol and Sulfide Quantitation Kit *50–250 assays*	1 kit

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