AnaTag™ R-Phycoerythrin Protein Labeling Kit

Catalog #	71013
Unit Size	1 Kit
Kit Size	1 Conjugation Reaction

The AnaTag™ R-PE Protein Labeling Kit is optimized for labeling R-phycoerythrin to IgG. Instructions on labeling R-PE to proteins other than IgG are also included. This kit provides ample materials to label up to 1 mg of IgG.

- ***Convenient Format:*** Complete kit including all the labeling components.
- ***Optimized Performance:*** Optimal conditions for conjugation.
- ***Enhanced Value:*** Less expensive than the sum of individual components.
- ***High Speed:*** Minimal hands-on time.
- ***Assured Reliability:*** Detailed protocol and references are provided.

USA and Canada Ordering Information

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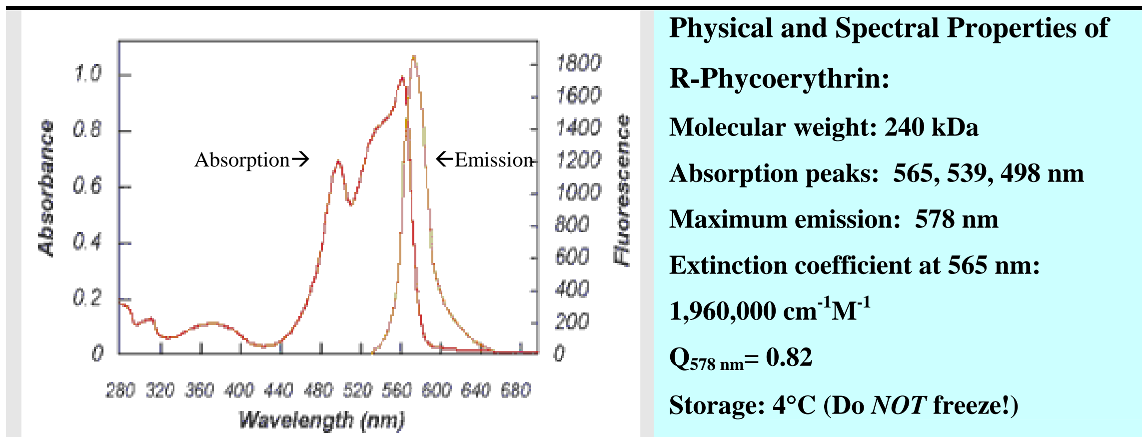
International Ordering Information

A list of international distributors is available at www.anaspec.com.

Introduction



Physical and Spectral Properties of R-Phycoerythrin:

Molecular weight: 240 kDa

Absorption peaks: 565, 539, 498 nm

Maximum emission: 578 nm

Extinction coefficient at 565 nm:

1,960,000 $cm^{-1}M^{-1}$

$Q_{578\ nm}$= 0.82

Storage: 4°C (Do *NOT* freeze!)

RPE (R-Phycoerythrin),[1] a fluorescent protein, belongs to the phycobiliproteins family, a family of highly soluble and fluorescent proteins derived from cyanobacteria and eukaryotic algae. RPE consists of α, β and γ subunits and is present as $(\alpha\beta)_6\gamma$. The polymer is very stable and does not dissociate even when diluted to 10^{-12} M. It has broad absorption bands with peaks at 565 nm (ε_M 1.96 X 10^6 $M^{-1}cm^{-1}$), 498 (ε_M 1.53 X 10^6 $M^{-1}cm^{-1}$), and 539 nm (ε_M 1.62 X 10^6 $M^{-1}cm^{-1}$), therefore can be excited with versatile excitation sources. The broad excitation spectrum also provides for multi-color immunofluorescent staining or cell sorting. For example, a sample labeled with fluorescein and RPE can be excited with a single light source at 488 nm and be detected at 520 nm and 575 nm. RPE and the closely related BPE are the most intensely fluorescent phycobiliproteins and 10-20 times brighter than conventional organic fluorophores.[2] Phycobiliproteins are also more photostable than their organic counterparts. RPE labeled biotin, avidin, primary and secondary antibody have been widely used in flow cytometry, live cell staining, and multi-color immunofluorescent staining.

The AnaTag™ RPE Protein Labeling Kit is optimized for labeling antibodies with RPE. First, a cross-linker, SMCC, is used in modifying RPE. SMCC reacts with the primary amine on RPE through its N-hydroxysuccinimide (NHS) ester group and introduces maleimide groups to RPE. Second, another cross-linker, SATP, is used in modifying target proteins, e.g. IgG or streptavidin. SATP also reacts with the primary amine on the target proteins through NHS ester group and introduces a protected sulfhydryl group into the protein. Hydroxylamine.HCl is then used to deprotect (deacylate) the target protein and generate free sulfhydryl groups. The maleimide groups on the RPE and sulfhydryl groups on the target protein forms a stable thioether bond. All cross-linking reagents, reaction buffer, and purification columns are provided in this kit.

KIT COMPONENTS, STORAGE AND HANDLING

Note: Store kit components at 4℃.

Components	Function	Quantity
A. RPE	R-Phycoerythrin, fluorescence protein	10 mg/mL X 240 µL
B. EDTA	Chelate divalent metal cations.	50 µL
C. SMCC	Introduce maleimide to RPE	1 vial
D. Reaction buffer	Buffer for performing R-PE-protein conjugation reaction	100 mL
E. Hydroxylamine.HCl	Deacylation reagent	50 µL
F. SATP	Introduce protected sulfhydryl group to target protein	1 vial
G. Spin column	Desalt proteins by centrifugation.	3 columns
H. Wash tube	Collect elution from the spin column	3 tubes
I. Collection tube	Collect sample from the spin column	3 tubes
J. Gravity column	Desalting conjugates by gravity	1 column
K. NEM	Blocking free sulfhydryl group	1 vial
L. DMSO	Organic solvent	1 mL
M. Elution buffer	Solvent for eluting R-PE-protein conjugate	100 mL
O. Purification gel	Purify conjugates from free R-PE and proteins.	20 mL

OTHER MATERIALS REQUIRED (but not provided)

- The column for purification of RPE-protein conjugate: The column is not included in the kit and should be provided by the customer. The 10 mL disposable serological pipette or any commercial column of similar size may be used for purification of conjugates (Step 6).

PROTOCOL

1. Prepare the target protein solution (e.g. IgG)

- Adjust your target protein solution to a concentration of 2-10 mg/mL and pH 7.2-7.5. A higher protein concentration is preferred.
- Add EDTA (Component B) 1/50 (v/v) to the target protein solution.

Note 1: Protein can be dissolved in phosphate, carbonate, borate, triethanolamine or MOPS buffer, pH 7.2-7.5, without reducing reagents (e.g. DTT) or protein stabilizers (e.g. BSA). If the protein is dissolved in Tris or glycine buffer or any buffer containing ammonium salts (such as ammonium sulfate and ammonium acetate), it should be dialyzed against phosphate buffered saline (PBS), pH 7.2-7.5, or use gel filtration to get rid of free amines.

Note 2: Labeling efficiency is better at a higher protein concentration, but decreases when the concentration of protein is less than 2 mg/mL. Protein solution can be concentrated by using a speed vacuum or a centrifugal filter (Millipore, Cat# 42407).

2. Activate the target protein by thiolation

Note 1: Perform Steps 2 and 3 at the same time. Since sulfhydryl group and maleimide group are not stable, proteins modified with these groups should not exceed 24 hours.

Note 2: If the target protein contains native thiols (e.g. β-galactosidase), Step 2 can be omitted. Proceed to step 3 directly.

- Add 200 µL DMSO (Component L) into one vial of SATP (Component F). Dissolve SATP completely.

  Note: The concentration of SATP solution is 6.6 mM. Prepare SATP immediately before use. Discard unused portion.
- Add 10 µL of SATP solution per mg of IgG. Mix the reagents gently.

  Note: For target proteins other than IgGs, add SATP into the protein solution at a molar ratio of 5-10:1 (SATP:protein).
- Incubate the reaction at room temperature for 30 min with agitation.
- Add 1 µL hydroxylamine.HCl (Component E) per 100 µL protein solution and incubate at room temperature for 2 hrs.
- If the reaction mixture is less than 120 µL, use a spin column (component G) to desalt the thiolated proteins (refer to Appendix I. Spin Column Procedures). If the reaction mixture is more than 120 µL, please use a gravity column (component J) to desalt the thiolated protein (refer to Appendix I. Gravity Column Procedures).
- Calculate the concentration of the eluted protein based on the absorption at 280 nm. For protein solution with a small sample volume, you may estimate the protein concentration by the amount used at the beginning of the reaction and the volume of the eluent. If the concentration is less than 2 mg/mL, it is necessary to concentrate the protein solution to >2 mg/mL to get a better yield in the conjugation reaction step later.

  Note: Protein solution can be concentrated by using a speed vacuum or a centrifugal filter (Millipore, Cat# 42407).

3. Prepare maleimidylated RPE

- Add 100 µL DMSO (Component L) into one vial of SMCC (Component C). Dissolve SMCC completely.

  Note: Prepare SMCC immediately before use. Discard unused portion.
- Add 17 µL of SMCC solution into the vial containing 240 µL RPE (Component A). Mix the reagents gently.

- Cover the vial with aluminum foil. Incubate the reaction at room temperature for 60 min under agitation.
- Desalt the maleimidylated RPE using two spin column (component G) (refer to Appendix I. Spin Column Procedures). Divide the maleimidylated RPE sample and apply ~120 µL to each spin column.

4. **Conjugate the thiolated target protein with maleimidylated RPE**

   Note: The purification column for Step 6 may be set up at this time since the purification gel needs to completely settle down overnight.

   - Add 240 µL of maleimidylated RPE (10 mg/mL) per mg of thiolated IgG. Incubate overnight at 4°C with agitation and protect from light.

     Note 1: The conjugation reaction can also be completed by incubating at room temperature for 5-6 hr.

     Note 2: For target proteins other than IgGs, calculate the amount of maleimidylated RPE needed based on the molar ratio of 1-1.5:1(RPE: target protein).

5. **Block excess free thiols**
   - Add 10 µL of DMSO (Component L) into one vial of NEM (Component K).
   - Add 1 µL of NEM solution per 100 µL of conjugation mixture from Step 4 and mix completely. Incubate at room temperature for 30 min and keep away from light.

   Note: If protein contains native free thiols (e.g. β-Galactosidase), NEM should not be used and Step 5 should be omitted.

6. **Purify RPE-protein conjugate from unconjugated proteins and RPE.**
   - Briefly centrifuge the reaction mixture for 30 seconds to pellet insoluble conjugate aggregates. Collect the supernatant, which contains the soluble RPE-protein conjugate.
   - Set up the purification column. Shake the purification gel (Component O) well and pour it into the column, avoiding bubbles. Close the outlet at the bottom of column and let the gel settle down in the column. It takes several hours to overnight for the gel to completely settle down. Open the outlet at the bottom of the column. Add a total of 30-40 mL of elution buffer (Component M) into the column to wash the purification gel. Keep the fluid running through the column and do not let the gel dry. Discard all the eluent.
   - When the elution buffer is just running below the gel surface, add the conjugate solution into the surface of gel bed.
   - When the conjugate solution is just running below the gel surface, add 10-20 mL of elution buffer (Component M) gently on the top of the gel bed.
   - Collect the red-colored eluent every 0.5-1 mL per fraction. Measure A_{280nm} and A_{565nm} of all fractions. Refer to Appendix II for characterizing the conjugate.

     Note: *The RPE-Ig G conjugate (390 kDa) has the highest molecular weight and will come out in the earlier fractions. Free RPE (240 kDa) will come out in the middle fractions. The free IgG (150 kDa) will come out in the later fractions. The fractionation range/exclusion limit of the purification gel is between10,000-1,500,000.*

7. **Storage**
   - If the concentration of the RPE-conjugate is less than 1 mg/mL, you can either concentrate the conjugate or add bovine serum albumin (BSA) to a final concentration of 1 mg/mL as a stabilizer.
   - The elution buffer (Component M) is compatible with the buffer system used in most cellular biological assays (e.g. flow cytometry, immunofluorescent staining). For long-term storage, the RPE-protein conjugate solution can be sterilized by filtering through a 0.22 µM filter or by adding preservatives (e.g. 2 mM sodium azide). These conjugates can then be stored at 4°C for up to six months. Keep conjugate away from light and avoid freezing.
   - If there is aggregation in the conjugate solution, centrifuge it briefly for 30 sec and use the supernatant only.

Appendix I.

Spin Column Procedures

Note: The spin column can desalt a sample with a volume of 20-120 μL. The MW exclusion size is 6,000.

- Resuspend the gel in the spin column (Component G) by inverting vigorously several times. Avoid bubbles.
- Remove the top cap of the column, and then cut its bottom tip. Place the column into a wash tube (Component H) and centrifuge using a swinging bucket centrifuge at 1,000 x g for 2 min. Discard the eluted buffer.
- Exchange the gel-packing buffer with an appropriate buffer:

  When purifying the thiolated protein in Step 2 and maleimided protein in Step 3, add 500 μL of reaction buffer (Component D) to the spin column and centrifuge using a swinging bucket at 1,000 x g for 1 min. Discard the eluent. Repeat this step three times.
- Place the spin column into a clean collection tube (Component I). Apply the reaction mixture from Step 2 or 3 to the center of gel bed surface. Centrifuge the column using a swinging bucket at 1,000 x g for 4 min.
- The protein is in the eluted buffer.

Gravity Column Procedures

Note: The gravity column can desalt a sample with a volume of 130-2500 μL. The MW exclusion size is 5,000.

- Hold the column upright. Remove the top cap of the column, and then cut its bottom tip. Discard the eluted liquid.
- Exchange the gel-packing buffer with an appropriate buffer as follows:

  When purifying the thiolated protein in Step 2, wash the column with 25 mL of reaction buffer (Component D) and discard the eluent.
- Load the reaction mixture from Step 2 to the center of gel bed in the column.

  When purifying the thiolated protein in Step 2, add 6 mL of reaction buffer (Component D) to elute the protein. Using clean tubes, immediately start collecting the eluted fractions (500 μL per fraction). Measure the absorbance of each fraction to decide which fractions contain the thiolated protein. Combine fractions, containing the thiolated protein.
- For protein concentration calculation, please refer to **Appendix II: Characterization of RPE-Protein Conjugate.**

Appendix II: Characterization of R-PE-Protein Conjugate

- The proteins (R-PE-IgG conjugates or other protein conjugates) can be diluted in phosphate-buffered saline or equivalents, pH 7.2-7.4 before measuring absorption.
- The concentration of IgG in the elution from Step 2 can be calculated with the following formula:

  $$[IgG]\ (mg/mL)=(A_{280\ nm}\ /1.35)\ X\ dilution\ factor$$

  For proteins other than IgG,

  $$Protein\ (mg/mL)=A_{280\ nm}/\varepsilon_{protein\ at\ 280nm}\ X\ MW_{protein}\ X\ dilution\ factor$$

  (MW: molecular weight)
- The concentration of R-PE in the elution from Step 6, can be calculated with the following formula:

  $$[R\text{-}PE]\ (M)=(A_{max}\ /1{,}960{,}000)\ X\ dilution\ factor$$

  Note: Adjust the sample concentration, A_{max} should be between 0.3-0.8. The maximal absorption (A_{max}) is at the wavelength of 565 ±5 nm.
- The concentration of IgG in the elution from Step 5 can be calculated with the following formula:

  $$[IgG]\ (M)=[(A_{280\ nm}-0.18\ x\ A_{565\ nm})\ /\ 203{,}000]\ X\ dilution\ factor$$

  For proteins other than Ig G,

  $$[Protein]\ (M)=[(A_{280\ nm}-0.18\ x\ A_{565\ nm})/\varepsilon_{protein\ at\ 280nm}]\ X\ dilution\ factor$$

- The degree of constitution (DOS) of the conjugates represents the amount of R-PE molecules conjugated to one Ig G molecule.

  DOS = [R-PE]/ [Ig G]

  For proteins other than Ig G,

  DOS = [R-PE]/ [Protein]

  Note: The optimal DOS of R-PE-IgG conjugates should be within 0.7-2. Collect all the fractions that have the optimal DOS. Discard the fractions with DOS too high or too low.
- The total concentration of R-PE-IgG conjugate in the elution from Step 5 can be calculated with the following formula:

  Total protein concentration (mg/mL)=[IgG] X 150,000 + [R-PE] X 240,000

  For proteins other than Ig G,

  Total protein concentration (mg/mL)=[Protein] X $MW_{protein}$ + [R-PE] X 240,000

References

1. Glazer, AN. and L. Stryer, *Methods Enzymol.* **184**, 188 (1990).
2. Oi, VT. et al. *J. Cell Biol.* **93**, 981 (1982).