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| <i>Revision number: 1.5</i> | <i>Last updated: July 2021</i> |
| <b>Catalog #</b>            | <b>AS-72113</b>                |
| <b>Kit Size</b>             | 1 Conjugation Reaction         |

- This kit is optimized for conjugating R-phycoerythrin (R-PE) to antibody.
- It provides ample materials to perform one conjugation reaction.
- One conjugation reaction can label up to 1 mg antibody.

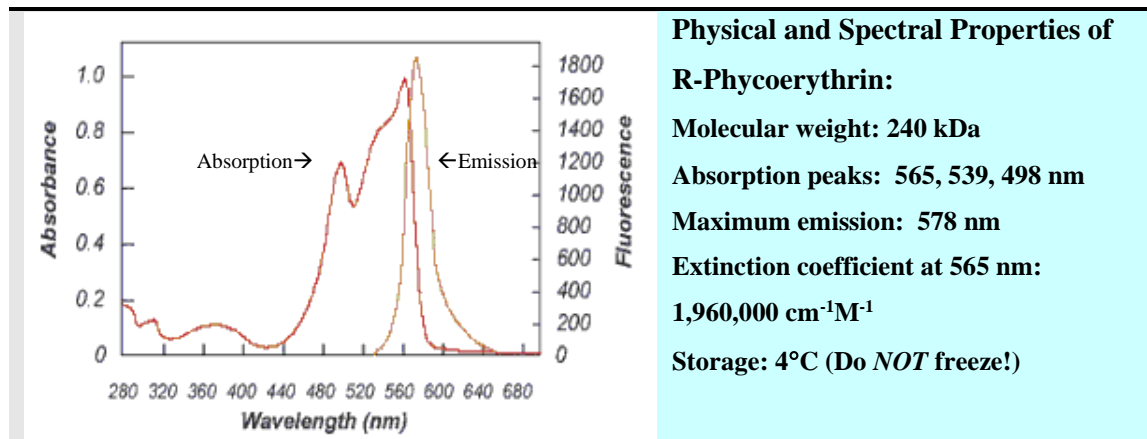
## Kit Components, Storage and Handling

| Component          | Function                                    | Quantity      |
|--------------------|---|---------------|
| A. Activated R-PE  | SMCC activated cross-linked R-phycoerythrin | 2 mg, 10mg/mL |
| B. DTT             | Reduces antibody                            | 0.1 mL        |
| C. Spin column     | Desalts protein by centrifugation           | 1 column      |
| D. Wash tube       | Collects buffer from the spin column        | 1 tube        |
| E. Collection tube | Collects sample from the spin column        | 1 tube        |
| F. Gravity column  | Desalts protein by gravity                  | 1 column      |
| G. DMSO            | Organic Solvent                             | 1 mL          |
| H. NEM             | Blocks free sulfhydryl groups               | 1 vial        |
| I. Reaction buffer | Buffer for conjugation of R-PE with protein | 100 mL        |

## Storage and Handling

- Store all kit components at 4°C.
- Components B and H may be frozen for best performance.
- Keep the Component A away from light, do not freeze.

## Introduction



The AnaTag™ R-PE Labeling Kit provides a convenient way to conjugate antibodies to R-phycoerythrin (R-PE). R-PE belongs to the phycobiliproteins family of highly soluble and fluorescent proteins derived from cyanobacteria and eukaryotic algae.<sup>1</sup> R-PE is made of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits and is present as  $(\alpha\beta)_6\gamma$ . The protein has broad absorption bands with peaks at 565 nm ( $\epsilon_M = 1.96 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$ ), 498 ( $\epsilon_M = 1.53 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$ ), and 539 nm ( $\epsilon_M = 1.62 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$ ); consequently, it can be excited with versatile excitation sources. The broad excitation spectrum provides the advantage for multi-color immunofluorescent staining or cell sorting. For example, a sample labeled with fluorescein and R-PE can be excited with a single light source at 488 nm but detected at 520 nm and 575 nm, respectively. R-PE and the closely related B-PE are significantly more sensitive and photostable than conventional organic fluorophores.<sup>2</sup> R-PE labeled primary and secondary antibody have been widely used in applications such as flow cytometry, live cell staining, and multi-color immunofluorescent staining.

The AnaTag™ R-PE Labeling Kit is a SH-reactive labeling kit. SMCC modified R-phycoerythrin has maleimide groups that easily react with SH groups of a target antibody without the need for an additional activation, thus simplifying the conjugation protocol. The maleimide group of R-PE and sulfhydryl group on the antibody form a covalent bond during conjugation.

The kit provides all the reagents, purification columns needed to label up to 1 mg of antibody, as well as a detailed step-by-step protocol.

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## Protocol

### 1. Preparing the antibody for conjugation

1.1 Adjust your antibody concentration to 2-10 mg/mL. A higher protein concentration is preferred.

Note 1: The protein can be dissolved in phosphate, carbonate, borate, Tris or MOPS buffer, pH 6.0-8.0, without oxidizing reagents, protein stabilizers (e.g. BSA) or other proteins.

Note 2: The conjugation efficiency is poor when the concentration of protein is less than 2 mg/mL. You may concentrate the protein solution using a speedvac or a centrifugal filter (Millipore, Cat# MRCPRT010).

1.2 Add 20 µL of DTT solution (Component B) per mL of IgG solution. Mix the reagents gently.

1.3 Incubate the reaction in a tightly capped tube at room temperature for 30 min without agitation.

1.4 If the reaction mixture is less than 120 µL, use a spin column (Component C) to desalt the reduced antibody (refer to Appendix I. Spin Column Procedures). If the reaction mixture is more than 120 µL, use a gravity column (Component F) to desalt the reduced antibody (refer to Appendix I. Gravity Column Procedures).

1.5 Calculate the concentration of the eluted protein based on absorption at 280 nm. For antibody solution with a small 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 antibody solution to >2 mg/mL for better yield in the conjugation reaction.

Note 1: To confirm the fractions containing the antibody, use Dye Reagent Concentrate (Bio-Rad Protein Assay, Cat#500-0006)

Note 2: Antibody solution can be concentrated by using a speedvac or a centrifugal filter (Millipore, Cat# MRCPRT010).

### 2. Performing the conjugation reaction

2.1 Add 1.6 mg of SMCC activated R-PE (Component A) per mg of reduced IgG. Incubate 1 hour at room temperature with agitation and protect from light.

2.2 Block excess free thiols

- Add 10 µL of DMSO (Component G) into one vial of NEM (Component H).
- Add 1 µL of NEM solution per 100 µL of conjugation mixture from Step 2.1 and mix completely. Incubate at room temperature for 30 min and keep away from light.

2.3 Conjugation is complete. The reaction mixture should contain mostly the R-PE-antibody conjugate, with small amounts of free R-PE and antibody. Unconjugated R-PE should not interfere with the assay. If purification is necessary, use a Bio-gel A 1.5 m (Bio-rad Cat# 151-0450) or an affinity column for IgG.

2.4 For protein concentration calculation, please refer to Appendix II. Characterization of R-PE-Antibody Conjugate.

### 3. Storage of conjugates

- 3.1 Store the R-PE-labeled antibody at concentration > 0.5 mg/mL or add 1-10 mg/mL of BSA as a stabilizer.
- 3.2 Add preservative (e.g. 0.01% sodium azide).
- 3.3 Store the conjugate solution at 4°C in the dark for two months. DO NOT FREEZE.
- 3.4 If there is aggregation in the conjugate solution, centrifuge briefly for 30 sec and use the supernatant only.

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## Appendix I. Desalting of the reduced antibody

### 1. 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 C) by inverting sharply 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 D) and centrifuge at 1,000x g for 2 min. Discard the eluted buffer.
- Exchange the gel-packing buffer by adding 500 µL of reaction buffer (Component I) to the spin column and centrifuge at 1,000x g for 1 min. Discard the eluent. Repeat the above step three times.
- Place the spin column into a clean collection tube (Component E). Apply the reduced antibody solution from Step 1.3 to the center of gel bed surface. Centrifuge the column using at 1,000x g for 4 min.
- The antibody is in the collection tube.

### 2. Gravity Column Procedures

Note: The gravity column can desalt a sample with a volume up to 3 mL. The MW exclusion size is 6,000.

- Hold the desalting column (Component F) upright. Remove the top cap of the column, and then cut its bottom tip. Pour off the excess buffer above the top frit.
- Add 25 mL of reaction buffer (Component I) to pre-equilibrate the column.
- Allow the buffer to drain to the top frit. The column will not run dry. Flow will stop when the buffer level reaches the top frit. Load the column with the reduced antibody solution from Step 1.3.
- Allow entire sample to enter the column, add 10 mL reaction buffer (Component I) into the column.
- Using clean tubes, immediately start collecting the eluted fractions (500 µL per fraction). To confirm the fractions containing the thiolated protein use Dye Reagent Concentrate (Bio-Rad Protein Assay, Cat#500-0006) or measure the absorbance. Combine fractions, which contain the reduced antibody.

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## Appendix II. Characterizing the R-PE-Antibody Conjugate

The degree of constitution (DOS) of the conjugates represents the amount of R-PE molecules conjugated to one target antibody molecule. The degree of substitution (DOS) is important for characterizing R-PE-protein conjugates. To determine the DOS of R-PE labeled antibody:

### 1. Read absorbance at 280 nm ( $A_{280}$ ) and 565 nm ( $A_{565}$ )

For most spectrophotometers, dilute a small portion of conjugate solution in PBS so that the absorbance readings are in the 0.1 to 0.9 ranges. The maximal absorption of antibody is at 280 nm ( $A_{280}$ ). The maximal absorption of R-PE is approximately at 565 nm ( $A_{565}$ ).

### 2. Calculating the DOS using the following equations for R-PE labeled IgG

Molar concentration of R-PE:

$$[\text{R-PE}] = (A_{565} \times \text{dilution factor}) / \epsilon_{\text{R-PE}}$$

$$\epsilon_{\text{R-PE}} = 1,960,000 \text{ cm}^{-1}\text{M}^{-1}$$

$\epsilon$  is the extinction coefficient

Molar concentration of antibody:

$$[\text{IgG}] = ((A_{280} - 0.18 \times A_{565}) \times \text{dilution factor}) / \epsilon_{\text{IgG}}$$

\* 0.18 is correction factor for the fluorophore's contribution to  $A_{280}$

$$\epsilon_{\text{IgG}} = 203,000 \text{ cm}^{-1}\text{M}^{-1}$$

$$\text{DOS} = [\text{R-PE}] / [\text{IgG}]$$

The total concentration of conjugate (mg/mL):

$$\text{Conjugate (mg/mL)} = [\text{IgG}] \times 150,000 + [\text{R-PE}] \times 240,000$$

$$\text{MW}_{\text{IgG}} = 150,000$$

$$\text{MW}_{\text{R-PE}} = 240,000$$

For effective labeling, the degree of substitution should fall within 1-2 moles of R-PE per one mole of antibody.

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## References

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