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| <i>Revision number: 1.5</i> | <i>Last updated: July 2021</i> |
| Catalog # | AS-72111 |
| Kit Size | 1 Conjugation Reaction |

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

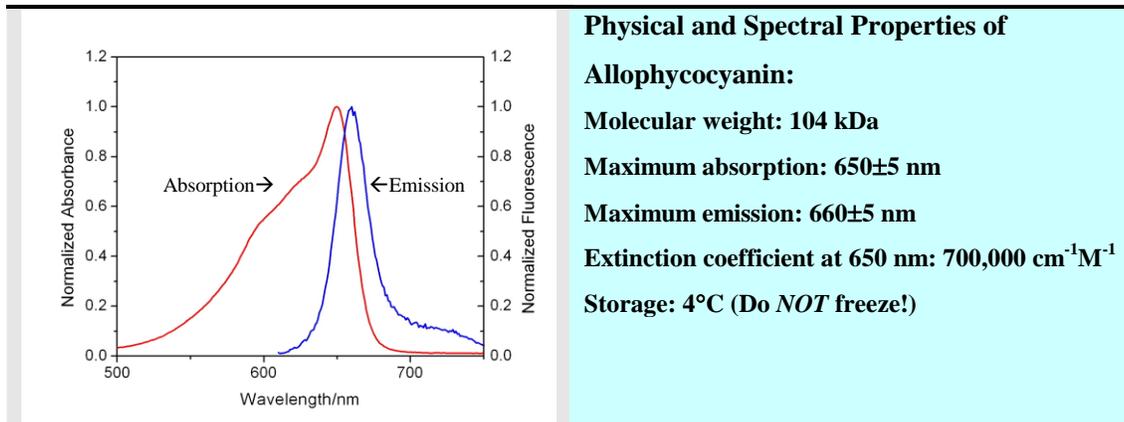
Kit Components, Storage and Handling

| Component | Function | Quantity |
|---------------------|---|---------------|
| A. Activated CL-APC | SMCC activated cross-linked allophycocyanin | 1 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 APC 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



Physical and Spectral Properties of

Allophycocyanin:

Molecular weight: 104 kDa

Maximum absorption: 650±5 nm

Maximum emission: 660±5 nm

Extinction coefficient at 650 nm: 700,000 cm⁻¹M⁻¹

Storage: 4°C (Do NOT freeze!)

The AnaTag™ APC Labeling Kit provides a convenient way to conjugate antibodies to allophycocyanin (APC). APC belongs to the phycobiliproteins family of highly soluble and fluorescent proteins derived from cyanobacteria and eukaryotic algae.¹⁻³ APC, an ultra-sensitive fluorescent tracer with high emission quantum yields, emits in the near-infrared fluorescence range. It therefore avoids interference from autofluorescence of cellular components and other biological materials. APC is significantly more sensitive than conventional organic fluorophores and has been used in applications such as flow cytometry, homogeneous FRET assay and immunoassays.^{4,5}

The AnaTag™ APC Labeling Kit is a SH-reactive labeling kit. SMCC modified allophycocyanin 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 APC and sulfhydryl group on the antibody form a covalent bond during conjugation.

The AnaTag™ APC Labeling Kit contains a chemically cross-linked APC (CL-APC) that is much more stable than the native APC, yet still retaining the original spectroscopic properties. 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# MRCPR010).

1.2. Add 20 μ L 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 μ L, 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 μ L, 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# MRCPR010).

2. Performing the conjugation reaction

2.1. Add 0.693 mg of SMCC activated APC (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 μ L of DMSO (Component G) into one vial of NEM (Component H).
- Add 1 μ L of NEM solution per 100 μ L 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 APC-antibody conjugate, with small amounts of free APC and antibody. Unconjugated APC 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 APC-Antibody Conjugate](#).

3. Storage of conjugates

- 3.1. Store the APC-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.

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 μ L. 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 μ l 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 μ L per fraction). To confirm the fractions containing the protein use Dye Reagent Concentrate (Bio-Rad Protein Assay, Cat#500-0006) or measure the absorbance. Combine fractions, which contain the reduced antibody.

Appendix II. Characterizing the APC-Antibody Conjugate

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

1. Read absorbance at 280 nm (A_{280}) and 650 nm (A_{650})

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 APC is approximately at 650 nm (A_{650}).

2. Calculating the DOS using the following equations for APC labeled IgG

Molar concentration of APC:

$$[\text{APC}] = (A_{650} \times \text{dilution factor}) / \epsilon_{\text{APC}}$$

$$\epsilon_{\text{APC}} = 700,000 \text{ cm}^{-1}\text{M}^{-1}$$

ϵ is the extinction coefficient.

Molar concentration of antibody:

$$[\text{IgG}] = ((A_{280} - 0.22 \times A_{650}) \times \text{dilution factor}) / \epsilon_{\text{IgG}}$$

* 0.22 is correction factor for the fluorophore's contribution to A_{280}

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

$$\text{DOS} = [\text{APC}] / [\text{IgG}]$$

The total concentration of conjugate (mg/mL):

$$\text{Conjugate (mg/mL)} = [\text{IgG}] \times 150,000 + [\text{APC}] \times 104,000$$

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

$$\text{MW}_{\text{APC}} = 104,000$$

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

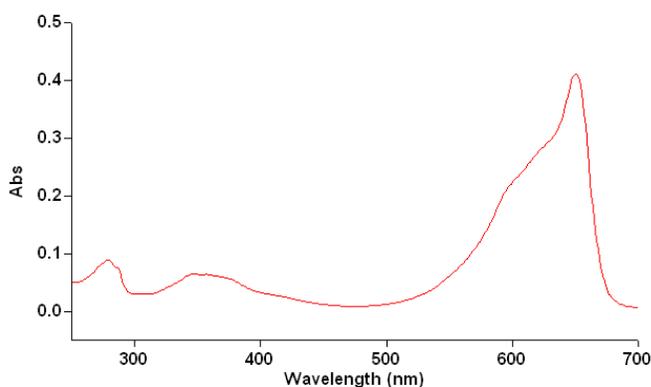


Figure 1. The absorption spectrum properties of APC-IgG conjugate.

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