AnaTag™ APC Protein Labeling Kit

Catalog #	71011
Unit Size	1 Kit
Kit Size	1 Conjugation Reaction

The AnaTag™ APC Protein Labeling Kit is optimized for labeling antibodies with cross-linked Allophycocyanin. Instructions on labeling other proteins are also included. This kit provides ample materials to label up to 1 mg of Ig G.

- ***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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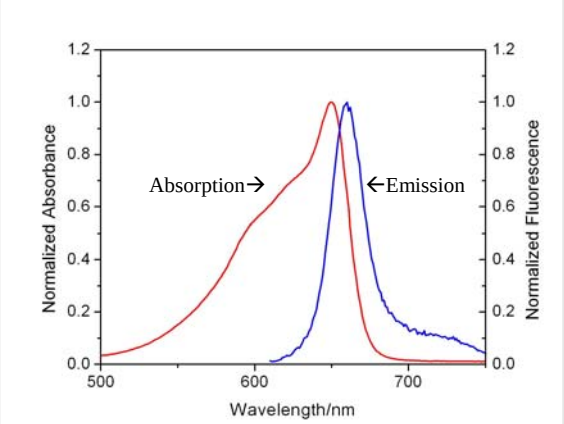
International Ordering Information

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

Introduction



Physical and Spectral Properties of Allophycocyanin:

Molecular weight: 104 kDa

Maximum absorption: 650±5 nm

Maximum emission: 660±5 nm

Fluorescence quantum yield at 660±5 nm: 0.68

Storage: 4°C (Do *NOT* freeze!)

APC (Allophycocyanin)[1-3], a near-infrared fluorescent protein, belongs to the phycobiliproteins family of highly soluble and fluorescent proteins derived from cyanobacteria and eukaryotic algae. APC is made up of alpha and beta subunits and is present as a trimer $(\alpha\beta_3)^4$. The trimer is unstable and susceptible to dissociation at low concentrations including concentrations at which some assays are performed. The monomer, αβ, has a lower fluorescence quantum yield compared to the trimer and the maximal absorption is also shifted to 620 nm[5]. In this kit, we have provided a chemically cross-linked APC trimer (APC-XL) that is much more stable than the native APC trimer, but still retains comparable spectroscopic properties as the native APC trimer.

APC is an ultra-sensitive fluorescent tracer because of its high emission quantum yields. Its near-infrared fluorescence is relatively free of interference from the autofluorescence of cellular components and other biological materials. It is greater than 100 times more sensitive than conventional organic fluorophores and has been used in applications such as flow cytometry, homogeneous FRET assay and other immunoassays[6;7].

The AnaTag™ APC Protein Labeling Kit is optimized for labeling antibodies with APC-XL. First, a cross-linker, SMCC, is chosen to modify APC. SMCC reacts with the primary amine on APC through its N-hydroxysuccinimide (NHS) ester group and introduces maleimide groups into APC. Second, another cross-linker, SATP, is chosen to modify target proteins, e.g. IgG or streptavidin. SATP reacts with the primary amine on the target proteins also through NHS ester group and introduces a protected sulfhydryl group into the protein. Then hydroxylamine.HCl is used to deprotect (deacylate) the target protein and generate free sulfhydryl groups. Then, the maleimide groups on the APC and sulfhydryl groups on the target protein will form 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. APC-XL	Cross-linked allophycocyanin	10 mg/mL X 100 µL
B. EDTA	Chelate divalent metal cations.	50 µL
C. SMCC	Cross-linker	1 vial
D. Reaction buffer	Buffer for performing APC-protein conjugation reaction	100 mL
E. Hydroxylamine.HCl	Deacylation reagent	40 µL
F. SATP	Introduce protected sulfhydryl group to target protein.	1 vial
G. Spin column	Desalt proteins by centrifugation.	2 columns
H. Wash tube	Collect eluent from the spin column	2 tubes
I. Collection tube	Collect sample from the spin column	2 tubes
J. Gravity column	Desalt conjugates by gravity	1 column
K. NEM	Block free sulfhydryl group	1 vial
L. DMSO	Organic solvent	1 mL
M. Elution buffer	Solvent for eluting APC-XL-protein conjugate	100 mL
O. Purification gel	Purify conjugates from free APC-XL 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) into your target protein solution.

Note 1: The 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: The labeling efficiency is better at a higher protein concentration, but poor 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 step 2 and 3 at the same time. Since sulfhydryl group and maleimide group are not stable, proteins modified with these groups should go on conjugation reaction within 24 hours.

Note 2: If the target protein contains native thiols (e.g. β-galactosidase), step 2 can be omitted. Continue 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 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 protein solution to >2 mg/mL for 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 APC-XL.

- 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 12 µL of SMCC solution into the vial containing APC-XL (component A). Mix the reagents gently.

- Cover the vial with alumina foil. Incubate the reaction at room temperature for 60 min under agitation.
- Desalt the maleimidylated APC-XL by a spin column (component G) (refer to Appendix I. Spin Column Procedures).

4. Conjugate the thiolated target protein with maleimidylated APC-XL

Note: You may set up the purification column now (step 6), since the purification gel needs overnight to completely settle down.

- Add 100 μL of maleimidylated APC-XL (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 APC-XL needed based on the molar ratio of 1-1.5:1(APC-XL: 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 it 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 APC-XL-protein conjugate from unconjugated proteins and APC-XL.

- Briefly centrifuge the reaction mixture for 30 seconds to pellet insoluble conjugate aggregates. Collect the supernatant, which contains the soluble APC-XL-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 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 blue-colored eluent every 0.5-1 mL per fraction. Measure A_{280nm} and A_{650nm} of all fractions. Refer to Appendix II for characterizing the conjugate.

  Note: *The APC-XL-Ig G conjugate (over 254 kDa) has the highest molecular weight and will come out in the earlier fractions. The free Ig G (150 kDa) will come out in the middle fractions. The free APC-XL (104 kDa) will come out in the later fractions. For the proteins other than Ig G, if their molecular weight is smaller than APC-XL, they will come out later than APC-XL. The fractionation range/exclusion limit of the purification gel is <10,000-1,500,000.*

7. Storage

- If the concentration of the APC-XL-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 APC-XL-protein conjugate solution can be sterilized by filtrating through 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 the 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 by 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 the fractions, which contains the thiolated protein.
- For protein concentration calculation, please refer to **Appendix II Characterization of APC-XL-Protein Conjugate.**

Appendix II.

Characterization of APC-XL--Protein Conjugate

- The proteins (APC-XL-Ig G 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 Ig G,

  $Protein\ (mg/mL)=A_{280\ nm}/\varepsilon_{protein\ at\ 280nm}$ X $MW_{protein}$ X dilution factor
  (MW: molecular weight)
- The concentration of APC-XL in the elution from Step 6, can be calculated with the following formula:

  $[APC\text{-}XL]\ (M)= (A_{max}\ /700{,}000)$ 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 650 ±5 nm.
- The concentration of IgG in the eluent from Step 6 can be calculated with the following formula:

  $[IgG]\ (M)=[(A_{280\ nm} - 0.22\ X\ A_{650\ nm}) / 203{,}000]$ X dilution factor
- The degree of constitution (DOS) of the conjugates represents the amount of APC-XL molecules conjugated to one Ig G molecule.

  DOS = [APC-XL]/ [Ig G]

  Note: The optimal DOS of APC-XL-IgG conjugates should be within 0.7-1.5. Collect all the fractions that have the optimal DOS for further immunofluorescence application. Discard the fractions with DOS too high or too low.
- The total concentration of APC-XL-IgG conjugate in the eluent from Step 6 can be calculated with the following formula:

  Total protein concentration (mg/mL)=[IgG] X 150,000 + [APC-XL] X 104,000

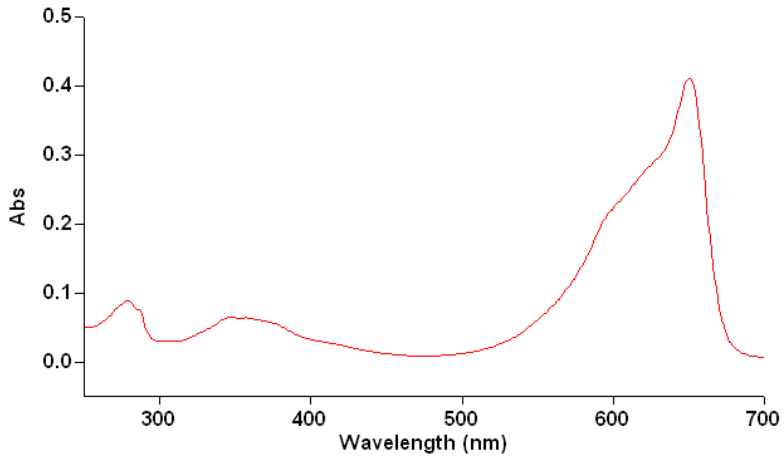


Figure 1. The absorption spectrum properties of APC-XL-Ig G conjugate.

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