AnaTag™ AMCA-X Protein Labeling Kit

Catalog #	71000
Unit Size	1 Kit
Kit Size	5 Conjugation Reactions

This kit is optimized to conjugate AMCA-X to proteins (e.g., IgG). It provides ample materials to perform five protein conjugations and purifications. One conjugation reaction can label up to 5 mg protein. The entire process only takes 30 minutes.

- ***Convenient Format:*** Complete kit including all the assay components.
- ***Optimized Performance:*** Optimal conditions for conjugation and purification.
- ***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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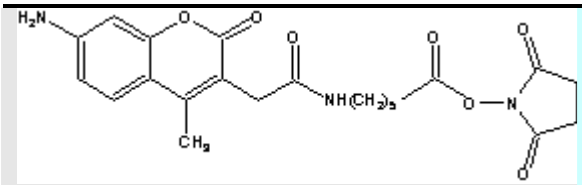
International Ordering Information

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

Introduction



Physical and Spectral Properties of AMCA-X, SE:

Fluorescent color: Blue

Molecular weight: 443.46

Maximum absorption: 347 nm

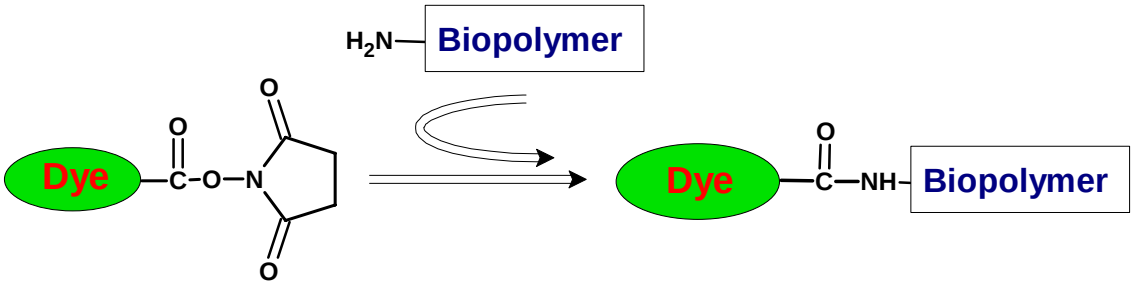
Maximum emission: 447 nm

Reactive form: Succinimidyl esters (amino-reactive)

AMCA-X, SE (6-((7-Amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid, succinimidyl ester) is one of the most popular blue fluorophores used to label proteins. AMCA-X succinimidyl ester contains a seven-atom aminohexanoyl spacer, the so-called 'X' spacer, between the fluorophore and the reactive group. It is well known that the spacer between the fluorophore and the reactive group, which separates the fluorophore from the biomolecule to which it is conjugated, potentially reduces the quenching that typically occurs upon conjugation. In some cases, the 'X' spacer allows the dye to be more available for recognition by secondary detection reagents.

The AnaTag™ AMCA-X Protein Labeling Kit provides a convenient way to label proteins by using the succinimidyl ester (SE) reactive form of AMCA-X. Succinimidyl ester shows good reactivity and selectivity with aliphatic amines of the protein and forms a carboxamide bond, which is identical to, and is as stable as the natural peptide bond (Scheme 1). AMCA-X-protein conjugates are very stable and are suitable for immunofluorescent staining, fluorescence *in situ* hybridization, flow cytometry and other biological applications.

The kit has all the essential components for performing the conjugation reaction and for purifying the AMCA-X--protein conjugates.



Scheme 1: The succinimidyl ester group of fluorophore reacts with amine groups on the protein to form a stable carboxamide bond.

KIT COMPONENTS, STORAGE AND HANDLING

Note: Store component A at -20℃ with desiccant. Store all the rest kit components at room temperature.

Component	Function	Quantity
A. AMCA-X, SE	Amino-reactive dye	5 vials
B. Reaction buffer	For pH adjustment of conjugation reaction	1X0.5 mL
C. Desalting column	Purify dye-protein conjugate	5 pre-packed columns
D. DMSO	Solvent for preparing dye stock solution	2 X 1mL
E. 10X Elution buffer	Solvent for eluting protein	30 mL
F. Buffer reservoir	Hold buffer for desalting column	1

Standard Operating Protocol (SOP)

1. Preparing the protein solution

Add reaction buffer (component B) at 1/10 (v/v) ratio to your target protein (e.g. antibody) solution (2-10 mg/mL is the recommended concentration range).

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, it should be dialyzed against 0.01 M phosphate buffer saline, pH 7.2-7.4 to get rid of free amines. Ammonium salts (such as ammonium sulfate and ammonium acetate) that are widely used for protein precipitation must also be removed before performing the dye conjugations.
Note 2: The conjugation efficiency is poor when the concentration of protein is less than 2 mg/mL.

2. Preparing the dye solution

Add 20 µL of DMSO (component D) to one vial of AMCA-X, SE (component A) to get 10 mg/mL dye solution. Completely dissolve all the dye contents by vortexing.

Note: Dye solution must be prepared fresh for each conjugation reaction. Extended storage of the dye solution may reduce dye activity. Any solutions containing the dye should be kept away from light.

3. Performing the conjugation reaction

3.1 Calculate the needed volume of AMCA-X solution for conjugation reaction. For labeling IgG, we usually use a molar ratio of 10:1 (AMCA-X: IgG). Table 1 gives a quick reference for labeling IgG. Add the needed amount of dye solution to a clean tube.

Table 1. The volume of dye solution needed for different amount of IgG.

Ig G	Dye solution	Ig G	Dye solution
0.5 mg	1.5 µL	3 mg	8.8 µL
1 mg	3.0 µL	3.5 mg	10.4 µL
1.5 mg	4.4 µL	4 mg	11.8 µL
2 mg	5.9 µL	4.5 mg	13.3 µL
2.5 mg	7.4 µL	5 mg	14.8 µL

Note 1: The molecular weight of IgG is 150 kDa.

Note 2: The dye: protein molar ratio 10:1 is optimized for labeling IgG with AMCA-X, SE. For proteins other than IgG, the optimal dye/protein molar ratio needs to be experimentally determined. The desired dye/protein molar ratio usually should be between 2:1 and 20:1.

3.2 According to the volume of your protein solution, add DMSO (component D) at 10% of your protein solution to the above tube containing dye solution.

3.3 Then add this diluted dye solution into your protein solution.

Note: Since AMCA-X has poor water solubility, 10% DMSO is necessary to present in the conjugation reaction mixture.

3.4 Keep the reaction mixture away from light and shake for 15 min at room temperature on a rotator or a shaker.

4. Purify dye-protein conjugates

Note: Desalting column (component C) is best suited for purifying proteins of MW>5,000. For smaller proteins, we recommend using Sephadex LH-20 or dialysis. HPLC may also be used to purify the smaller protein conjugates.

4.1 Dilute 10X elution buffer (component E) to 1X in deionized water.

4.2 Hold the desalting column (component C) upright. Remove the top cap of the column, and then cut its bottom tip. Place the buffer reservoir (component F) on the top of the column.

4.3 Add 25 mL 1X elution buffer into the buffer reservoir to pre-equilibrate the column.

4.4 As soon as the liquid runs just below the top solid surface, load the column with the reaction mixture (directly from step 3.4.).

4.5 As soon as the reaction mixture runs just below the top gel surface, add 10 mL 1X elution buffer into the column.

4.6 When the reaction mixture runs down the column, collect all fractions every 0.5-1 mL. Use bio-rad protein assay reagent or the reading at $UV_{280\ nm}$ to determine the fractions containing protein. If you use $UV_{280\ nm}$, please note the first peak is protein conjugate; the second peak is free AMCA. Do not collect the second peak.

4.7 Combine the fractions containing protein.

4.8 The degree of substitution (DOS) of the conjugate can be determined according to Appendix I.

Appendix I. Characterizing the Dye-Protein Conjugate

The Degree of substitution (DOS) is the most important factor for characterizing dye-labeled protein. Proteins of lower DOS usually have weaker fluorescence intensity, but proteins of higher DOS (e.g. DOS>6) tend to have reduced fluorescence as well. The optimal DOS recommended for most antibodies is between 2 and 6. We used the following method to determine the DOS of AMCA-X labeled proteins.

1. Read absorbance at 280 nm (A_{280}) and A_{max} of AMCA-X

For most spectrophotometers, dilute a small portion of conjugate elution in phosphate buffered saline so that the absorbance readings are in the 0.1 to 0.9 range. The absorbance at 280 nm (A_{280}) is the maximal absorption of protein. The maximal absorption of AMCA-X amide (A_{max}) is approximately at 347 nm.

2. Calculating DOS using the following equations for IgG labeling

The molar concentration of dye:

$[dye] = A_{max} / 0.692 / \varepsilon_{AMCA\text{-}X}$ X dilution factor $\qquad \varepsilon_{AMCA\text{-}X} = 19{,}000\ cm^{-1}M^{-1}$

Note: The absorption of AMCA is environmental sensitive. The constant, 0.692, is used to convert the absorption of AMCA in phosphate buffered saline to methanol. ε is the extinction coefficient.

The molar concentration of protein:

$[protein] = (A_{280} - 0.368 \times A_{max}) / \varepsilon_{protein}$ X dilution factor $\qquad \varepsilon_{IgG=} 203{,}000\ cm^{-1}M^{-1}$

DOS = [dye]/[protein]

Protein concentration (mg/mL);

Ig G (mg/mL)=[Ig G] X 150,000 $\qquad MW_{Ig\ G}=150{,}000$

For effective labeling, the degree of substitution should fall within 2-6 moles of AMCA-X to one mole of protein.

Storage of protein conjugates

The dye-labeled protein should be stored at > 0.5 mg/mL or in the presence of a carrier protein (e.g., 0.1% bovine serum albumin), and the addition of preservatives (e.g. 0.1% sodium azide) is highly recommended. The labeled protein can be stored at 4°C for two months without significant change if kept from light. For extended storage, it should be divided into aliquots or lyophilized and stored at -20°C in the dark.

Reference

1. Hermanson GT (1996). *Bioconjugate Techniques*, Academic Press, New York.
2. Haugland RP (1995). Coupling of monoclonal antibodies with fluorophores. *Methods Mol Biol* **45,** 205-21.
3. Brinkley M (1992). A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjug Chem* **3,** 2-13.