AnaTag™ AMCA-X
Microscale Protein Labeling Kit

Revision number: 1.3	*Last updated: May 2018*
Catalog #	**AS-72056**
Kit Size	3 Conjugation Reactions

- This kit is optimized to conjugate AMCA-X to proteins (e.g., IgG).
- It provides ample materials to perform three protein conjugations and purifications.
- One conjugation reaction can label up to 200μg protein.
- The entire process only takes about half an hour.

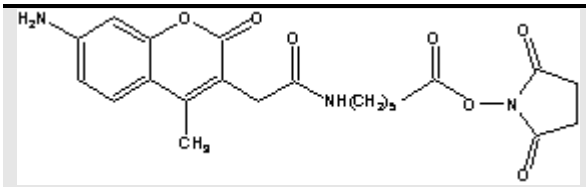
Kit Components, Storage and Handling

Component	Function	Quantity
A. AMCA-X SE	Amino-reactive dye	3 vials
B. Reaction buffer	For pH adjustment of the conjugation reaction	0.5 mL
C. Spin column	Purify dye-protein conjugate	3 pre-packed columns
D. DMSO	Solvent for preparing dye stock solution	150 μL
E. Elution buffer	Buffer for eluting dye-protein conjugate	20 mL
F. Wash tube	Holds buffer for Spin column	3 tubes
G.Collect tube	Collects dye-protein conjugate	3 tubes

Storage and Handling

- Store all kit components at 4°C.
- Keep Component A away from light and protect from moisture.
- Component A may be frozen.

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 ester (amine-reactive)**

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. 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. 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 (Figure 1). 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.

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.

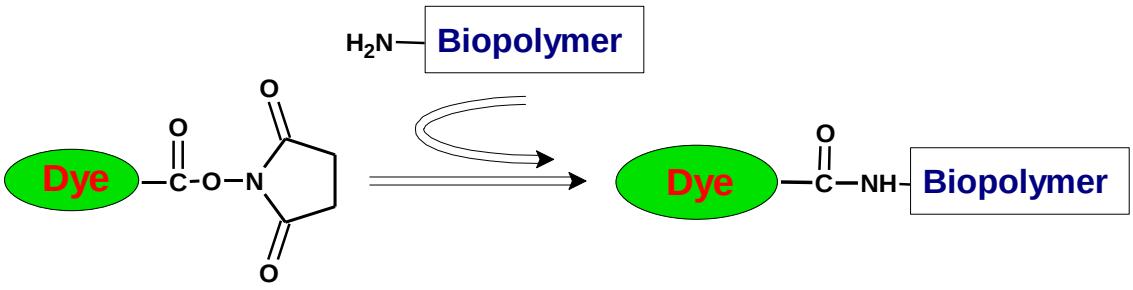


Figure 1. Labeling of an amino group (for instance, a lysine) on a biopolymer (i.e., a protein) with a succinimidyl ester of a dye.

Protocol

1. Preparing the protein solution

Add reaction buffer (component B) at 1/10 (v/v) ratio to your target protein (e.g. antibody) solution (3-10 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), protein stabilizers (e.g. BSA) or sodium azide. 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 3 mg/mL. Meanwhile, the purification column included in this kit can maximally purify 100 µl conjugate solution. You may concentrate the protein solution using a speed vacuum or a centrifugal filter (Millipore, Cat# MRCPRT010).

2. Preparing the dye solution

Add 10 µL of DMSO (component D) to one vial of AMCA-X SE (component A). This gives a 2 mM 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 must be kept from light.

3. Performing the conjugation reaction

Note: The procedure given here is optimized for IgG (MW ~ 150,000) labeling with AMCA-X SE. The dye: protein molar ratio is 10:1. For proteins other than IgG, the optimal dye/protein molar ratio may need to be determined. It will normally be between 2:1 and 20:1.

3.1 Add the dye solution to the solution of IgG or your protein at a dye to protein molar ratio of 10:1. For 200 µg IgG, add 6.6 µl of 2 mM dye solution.

Note: The molecular weight of IgG is 150 kDa.

3.2 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

4.1 Resuspend the gel in the spin column (component C) by inverting sharply several times. Avoid bubbles.

4.2 Remove the top cap of the column, and then cut its bottom tip. Place the column into a wash tube (component F) and centrifuge at 1,000 x g for 2 min. Discard the eluted buffer.

4.3 Exchange the gel-packing buffer by adding 500 µL of elution buffer (component E) to the spin column and centrifuge at 1,000 x g for 1 min. Discard the eluent. Repeat the above step three times.

4.4 Place the spin column into a clean collection tube (component G). Apply the reaction mixture from Step 3 to the center of gel bed surface. Centrifuge the column at 1,000 x g for 4 min.

4.5 The dye-protein conjugate is in the collection tube.

4.6 The degree of substitution (DOS) of the conjugate should be determined according to the Appendix.

Appendix. Characterizing The Dye-Protein Conjugate

The degree of substitution (DOS) is important for characterizing dye-labeled proteins. Proteins of lower DOS usually have weaker fluorescence intensity, but proteins of higher DOS (e.g. DOS>6) tend to have reduced fluorescence due to fluorescence quenching. The optimal DOS recommended for most antibodies is between 2 and 6. To determine the DOS of AMCA-X labeled proteins:

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

2. Calculating the DOS using the following equations for IgG labeling
Molar concentration of dye:

$[Dye] = (A_{347}/0.692 \times \text{dilution factor}) / \varepsilon_{AMCA\text{-}X}$ $\varepsilon_{AMCA\text{-}X} = 19{,}000\ cm^{-1}M^{-1}$

ε is the extinction coefficient.

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

Molar concentration of protein:

$[Protein] = ((A_{280} - 0.25 \times A_{347}) \times \text{dilution factor}) / \varepsilon_{protein}$ $\varepsilon_{IgG=}\ 203{,}000\ cm^{-1}M^{-1}$

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

DOS = [Dye]/[Protein]

Protein concentration in mg/mL for IgG:

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

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

Storage of Dye - 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). We recommend adding preservative (e.g. 0.01% sodium azide). The dye-labeled protein can be stored at 4°C for two months without significant changes if kept from light. For extended storage, it can be aliquoted or lyophilized and stored at -20°C in the dark.

References

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.
4. Banks PR, Paquette DM (1995). Comparison of three common amine reactive fluorescent probes used for conjugation to biomolecules by capillary zone electrophoresis. *Bioconjug Chem*. **6**, 447-58.