



## AnaTag™ Alkaline Phosphatase Protein Labeling Kit

<b>Catalog #</b>	<b>71009</b>
<b>Unit Size</b>	1 Kit
<b>Kit Size</b>	1 Conjugation Reaction

The AnaTag™ Alkaline Phosphatase Protein Labeling Kit is optimized for labeling proteins with alkaline phosphatase. This kit provides ample materials to label and desalt 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

#### AnaSpec Corporate Headquarter

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### International Ordering Information

A list of international distributors is available at [www.anaspec.com](http://www.anaspec.com).

## Introduction

Alkaline phosphatase is a popular label enzyme for protein detection. It has fast turnover rates over a variety of chromogenic, fluorogenic and chemiluminescent synthetic substrates. Alkaline phosphatase-conjugated secondary antibody and streptavidin have been widely applied to ELISA and immunohistochemistry.

The AnaTag™ Alkaline Phosphatase Protein Labeling Kit is optimized for labeling proteins with alkaline phosphatase. The alkaline phosphatase in this kit is a 140 kDa protein isolated from calf intestine with the activity >2200 U/mg. It has been pre-activated by SMCC in order to make the conjugation reaction timesaving and convenient. A cross-linking reagent, SATP, is used to add a protected sulfhydryl group on the target proteins. After de-protected by hydroxylamine, the free sulfhydryl group on the target protein can then react with the maleimide group on pre-activated alkaline phosphatase to form a stable thioether bond.

## **KIT COMPONENTS, STORAGE AND HANDLING**

*Note: Store components C, F and K at -20 °C. Store the rest of the kit components at 4 °C, and keep component A away from light.*

Components	Function	Quantity
A. Maleimidyl-AP	Pre-activated alkaline phosphatase.	3 mg
B. EDTA	Chelate divalent metal cations.	50 µL
C. 2-MEA	Mild disulfide reducing agent	1 vial
D. Reaction buffer	Buffer for performing AP-protein conjugation reaction	100 mL
E. Hydroxylamine.HCl	Deacylation reagent	50 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	2 columns
K. NEM	Block free sulfhydryl group	1 vial
L. DMSO	Organic solvent	1 mL
M. Elution buffer	Solvent for eluting AP-protein conjugate	100 mL
N. Buffer Reservoir	Reserve buffer for gravity column	1

## **Standard Operating Protocol**

### **1. Prepare the target protein solution (e.g. IgG)**

- Adjust your target protein solution to a concentration of 1-10 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 1 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: There are two protocols to prepare thiolated proteins. Protocol A is for labeling proteins without native thiol groups and protocol B is designed for labeling protein with native thiol groups.*

**Protocol A:** For Ig Gs and target proteins without native thiol groups.

- Add 200  $\mu$ L 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  $\mu$ L of SATP solution per mg of IgG. The molar ratio of SATP: Ig G is 10:1. Mix the reagents gently.

*Note 1: For streptavidin, a recommended molar ratio of SATP: streptavidin is 5:1. For other target proteins, the molar ratio of SATP: protein should be determined experimentally.*

*Note 2: The MW of Ig G is 150 kDa and streptavidin is 55 kDa.*

- Incubate the reaction at room temperature for 30 min with agitation.
- Add 1  $\mu$ L hydroxylamine.HCl (component E) per 100  $\mu$ L protein solution and incubate at room temperature for 2 hrs.
- If the reaction mixture is less than 120  $\mu$ L, use a spin column (component G) to purify the thiolated proteins (refer to [Appendix I. Spin Column Procedures](#)). If the reaction mixture is more than 120  $\mu$ L, please use a gravity column (component J) to purify the thiolated protein (refer to [Appendix I. Gravity Column Procedures](#)).
- Calculate the concentration of the eluted protein based on the absorption at 280 nm. If the concentration is less than 1 mg/mL, it is necessary to concentrate the protein solution to 1-10 mg/mL for better yield in the conjugation reaction step later.

*Note 1: Protein solution can be concentrated by using a speed vacuum or a centrifugal filter (Millipore, Cat# 42407).*

*Note 2: The concentration of Ig G (mg/mL) =  $A_{280nm} / 1.35 \times$  dilution fold*

*The concentration of Streptavidin (mg/mL) =  $A_{280nm} \times 0.313 \times$  dilution fold.*

**Protocol B:** For proteins containing native thiol groups (e.g.  $\beta$ -Galactosidase)

- Prepare 2-MEA solution by adding 50  $\mu$ L reaction buffer (component D) into one vial of 2-MEA (component C).
- Add 2-MEA solution at 1/10 (v/v) into protein solution prepared in Step 1 and incubate the reaction at 37°C for 90 minutes. Mix the reaction mixture once in a while during the incubation.

*Note: Prepare fresh 2-MEA solution for the experiment. Discard any unused portion.*

- Equilibrate the reaction mixture to room temperature. If the reaction mixture is less than 120  $\mu$ L, purify the reduced protein by using a spin column (component G, refer to [Appendix I. Spin](#)

Column Procedures for details). If the reaction mixture is more than 120  $\mu\text{L}$ , please use a gravity column (component J) to purify the protein (refer to Appendix I. Gravity Column Procedures).

- Calculate the concentration of eluted protein based on the absorption at 280 nm. If the concentration is less than 1 mg/mL, it is necessary to concentrate the protein solution to 1-10 mg/mL to obtain a better yield in the conjugation reaction later.

*Note: Protein solution can be concentrated by using a speed vacuum or a centrifugal filter (Millipore, Cat# 42407).*

### 3. Conjugate the thiolated target protein with maleimide-alkaline phosphatase.

- Add the thiolated protein solution (e.g. 1 mg IgG) to the vial containing maleimidyl-AP (3 mg lyophilized powder, component A). Incubate the reaction mixture for 1-2 hrs at room temperature with agitation.

*Note: For proteins other than IgG, mix the protein with maleimidyl-AP at 1:3 molar ratio. You may dissolve the maleimidyl-AP (3 mg lyophilized powder, component A) into 300  $\mu\text{L}$  reaction buffer (component D) first. Then add appropriate amount of this 10 mg/mL maleimidyl-AP solution into your protein solution.*

### 4. Block excess free thiols

- Add 10  $\mu\text{L}$  of DMSO (component L) into one vial of NEM (component K).
- Add 1  $\mu\text{L}$  of NEM solution per 100  $\mu\text{L}$  of conjugation mixture from Step 3 and mix completely. Incubate at room temperature for 30 min and keep away from light.

*Note: If protein contains native free thiols (e.g.  $\beta$ -Galactosidase), NEM should not be used and Step 4 should be omitted.*

### 5. Desalt alkaline phosphatase-protein conjugate.

- Briefly centrifuge the reaction mixture for 30 seconds to pellet insoluble conjugate aggregates. Collect the supernatant, which contains the soluble alkaline phosphatase-protein conjugate.
- If the reaction mixture is less than 120  $\mu\text{L}$ , use a spin column (component G) to purify the thiolated proteins (refer to Appendix I. Spin Column Procedures). If the reaction mixture is more than 120  $\mu\text{L}$ , please use a gravity column (component J) to purify the thiolated protein (refer to Appendix I. Gravity Column Procedures).

### 6. Storage

- For long-term storage, the alkaline phosphatase-protein conjugate solution should be sterilized by filtrating through 0.22  $\mu\text{M}$  filter. These conjugates can then be stored at 4°C for up to six months.

*Note: Alkaline phosphate is sensitive to thaw-freeze cycle. Do not freeze the alkaline phosphatase-protein conjugates.*

## **Appendix I.**

### **Spin Column Procedures**

*Note: The spin column can desalt a sample with a volume of 20-120  $\mu\text{L}$ . 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, add 500  $\mu\text{L}$  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.  
When purifying the protein conjugate in Step 5, add 500  $\mu\text{L}$  of elution buffer (component M) 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 Step 5 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  $\mu\text{L}$ . 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.
- Put the buffer reservoir (component N) on the top of the column. 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.  
When purifying the protein conjugate in Step 5, wash the column with 25 mL of elution buffer (component M) and discard the eluent.
- Load the reaction mixture from Step 2 or Step 5 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.  
When purifying the protein conjugate in Step 5, add 6 mL of elution buffer (component M) to elute the protein.
- Immediately start collecting the eluted fractions (500  $\mu\text{L}$  per fraction) using clean tubes. Measure the absorbance of each fraction at 280 nm to decide which fraction contains the proteins. Or use Bio-rad protein assay kit to decide which fraction contains the proteins. Combine the protein-containing fractions.