ATTO Antibody Labeling Kit

Protocol for labeling IgG antibodies with ATTO 488 NHS-ester

(Order #: ALK 488-3R)

DISCLAIMER: The following is an example protocol for labeling proteins, in particular IgG antibodies with ATTO 488 NHS-ester. It is intended as a guide and can be modified depending on your experimental requirements. Please read the entire protocol before starting.
In addition, this labeling protocol is not suitable for antibody- or protein-solutions containing bovine serum albumin (BSA), gelatin, glycerol, free amino acids, or ammonium salts.

Kit components:

Material	Order #	Quantity	Cap Color
Reactive dye	AD 488-3005	3 vials (60 nmol each)	transparent
DMSO, anhydrous	ALK DMSO-C01	1 mL	black
1X PBS, pH 7.4 containing 0.02% NaN_3	ALK PBS-C02	10.0 mL	colorless
Sodium bicarbonate salt	ALK BC-C03	3 vials	blue
Spin desalting columns*	ALK PSC-C04	6 columns	red
Collection tubes		12 tubes	colorless (to be removed)
Storage tubes (glass)		3 tubes	transparent with septum

*two for each reaction

Storage and Handling:

Stable for at least 6 months upon receipt, if the reactive dye is stored separately at -20°C. Store all other components at ambient temperature Do not freeze the spin desalting columns!

Number of Labeling Reactions:

Each of the three vials of reactive dye provided can be used for labeling ~100 µg of antibody. Alternatively, the procedure may be scaled up or down for other amounts of antibody (50-300 µg) if the ratios of the reagents are maintained. However, the labeling kit as provided is optimized for labeling of ~100 µg antibody per reaction.

Introduction:

The labeling kit contains everything you need to perform three separate labeling reactions and to purify the resulting conjugates. It provides a convenient way to label small amounts of antibodies (100 µg per reaction) with the superior dye ATTO 488. The reactive dye has a succinimidyl ester functionality, which readily reacts with amino groups of the protein, i.e., lysine residues, to form a stable amide linkage.

Note: The kit is not recommended for labeling of IgM antibodies. The kit can be used to label non-IgG proteins. The ratio of dye stock solution to protein amount may require optimization for different proteins. The spin desalting columns offer exceptional protein recovery characteristics. They are optimized for buffer exchanging proteins with a molecular weight > 40KDa and removing small molecules < 2000Da.

Degree of Labeling:

The labeling efficiency varies with antibody concentration. In general, the higher the antibody concentration, the higher the labeling efficiency of the reaction. The protocol is designed to achieve an optimal degree of labeling (DOL) of IgG antibodies with ATTO 488 of 4-5, i.e., 4-5 moles of dye per mole of antibody, when antibody concentrations of 1-2 mg/mL are used. For other proteins, the ratio of reactive dye to protein (D/P) may require optimization to achieve adequate DOLs.

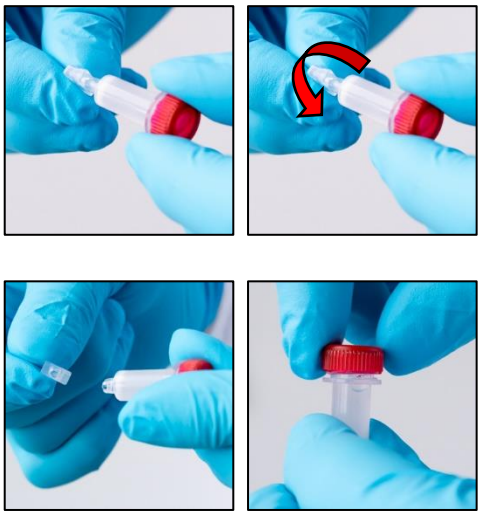
Experimental Protocol:

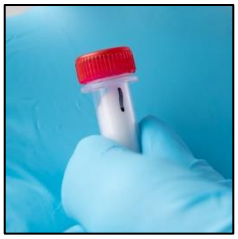
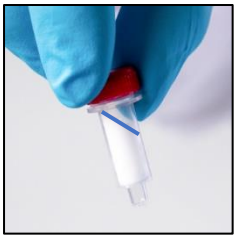
Allow all reagents to adjust to room temperature before use.

1. Preparing the antibody for labeling

If the antibody is already present in sodium bicarbonate solution at a concentration of ≥ 1 mg/mL and is free of any amino-containing chemicals or preservatives such as Tris, ammonium-salts, or free amino acids (e.g., glycine), proceed to step 2.
If the antibody is obtained in Tris or PBS buffer, it must be replaced by sodium bicarbonate buffer for optimal labeling.

Note: To guarantee a proper buffer exchange, the desalting resin must be equilibrated with sodium bicarbonate buffer before loading the antibody.

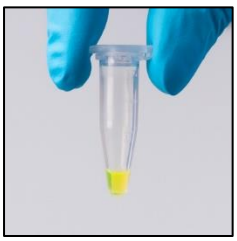
1.1	Dissolve sodium bicarbonate salt (blue cap) in 1.5 mL ultra-pure water.	
1.2	Remove the bottom closure of the spin column and loosen the red cap. Do not remove the cap.	

1.3	Take a collection tube and cut off the cap. Place the column into the collection tube, and centrifuge at 1500 × *g* in a bench-top microcentrifuge for 1 min to remove the storage buffer.	   
1.4	Discard the flow-through and centrifuge again for 1 minute to ensure that the storage buffer is completely removed.	
1.5	Mark the mating direction of the spin column to ensure that the inclined resin of the column is always placed into the centrifuge in the same direction!	 
1.6	Add 300 µL sodium bicarbonate buffer slowly on top of the inclined resin bed. Replace the red cap - do not fully tighten. Put the column back into the collection tube, centrifuge again at 1500 × *g* for 1 min, and discard the flow-through. Repeat this step two additional times, with the time set to **2 min** in the **last** centrifugation step.	 
Note: After each spin, the resin should appear white and free of liquid. If liquid is present, make sure you are using the correct centrifugation speed and time. Incomplete centrifugation may result in poor sample recovery or sample dilution.		
1.7	Blot the bottom of the column to remove excess liquid. Transfer the column to a **new** collection tube (cut of the cap and **keep the cap**).	

1.8	Add 50-200 µL antibody solution (~100 µg) slowly on the **top end** of the inclined resin bed. In case of a **sample volume < 70 µL** add the antibody solution as described above and let the solution sink into the resin. Add an additional amount of 15 µL sodium bicarbonate buffer in the same way. Screw the red cap back onto the vial but do not fully tighten. Centrifuge at 1500 × *g* for **2 min**. Pay attention that the column is placed in the same orientation. **Retain** the flow-through containing the antibody in sodium bicarbonate buffer. **Discard the used column**.	  

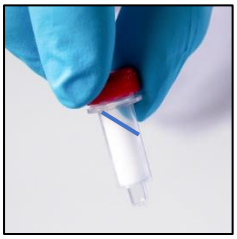
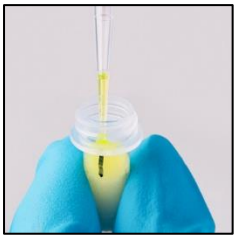
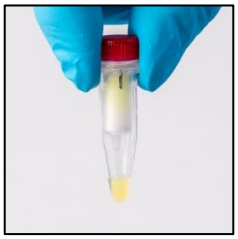
2. Labeling the protein

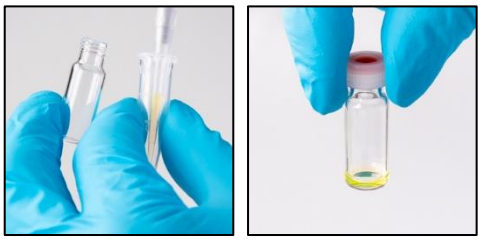
2.1	Allow the sachet containing the vial of reactive dye to warm to room temperature before opening. Open the sachet, take out the vial (transparent cap) with reactive dye and briefly tap the bottom of the vial on the bench to ensure that all dye (solid) is collected at the bottom of the vial.	 
2.2	Add 30 µL anhydrous DMSO (black cap) to the vial and vortex to dissolve the dye. Make sure that all the dye has dissolved. Centrifuge briefly to collect the dye solution at the bottom of the vial. The dye stock solution has a molar concentration of 2 mM.	   

2.3	Add **2 µL** of the dye stock solution from Step 2.2 to the antibody solution prepared in Step 1.8 (D/P 6:1), close the tube with the cap and mix well. Protect the reaction mixture from light and incubate for 1 hour at ambient temperature.	 

3. Purifying the labeled antibody

3.1	Remove the bottom closure of the spin column and loosen the red cap. Do not remove the cap.	   
3.2	Take a new collection tube and cut off the cap. Place the column into the collection tube, and centrifuge at 1500 × *g* in a bench-top microcentrifuge for 1 min to remove the storage buffer.	   
3.3	Discard the flow-through and centrifuge again for 1 minute to ensure that the storage buffer is completely removed.	

3.4	Mark the mating direction of the column to ensure that the inclined resin of the column is always placed into the centrifuge in the same direction!	 
3.5	Add 300 µL PBS, pH 7.4 containing 0.02% NaN_3 slowly on the top end of the inclined resin bed. Replace the red cap - do not fully tighten. Put the column back into the collection tube, centrifuge at 1500 × *g* for 1 min and discard flow-through. Repeat this step two additional times, with the time set to **2 min** in the **last** centrifugation step.	 
Note: After each spin, the resin should appear white and free of liquid. If liquid is present, make sure you are using the correct centrifugation speed and time according to the manual of the device. Incomplete centrifugation may result in poor sample recovery or sample dilution.		
3.6	Blot the bottom of the column to remove excess liquid. Take the last unused collection tube and cut off the cap and transfer the column to the new collection tube.	
3.7	Add the protein labeling solution from Step 2.3 slowly on the **top end** of the resin and ensure that the resin is still inclined. In case of a sample volume **< 70 µL** add the antibody solution in the way described above and let the solution sink into the resin. Add an **additional amount of 15 µL** PBS buffer in the same way. Screw the red cap back onto the vial but do not fully tighten. Centrifuge at 1500 × *g* for **2 min**. Pay attention that the column is placed in the same orientation. **Retain** the flow-through. **The collected liquid contains the labeled antibody dissolved in PBS.** The yellow upper zone of the column contains the free, unconjugated, hydrolyzed NHS-ester of ATTO 488.	   

We recommend transferring the labeled antibody solution into the provided glass vial (transparent cap with septum). For storage recommendations see Step 5. **Discard the spin column.**	
Note: Free dye should not flow through the column and should remain in the upper part of the column. The separation of the free dye is most effective if the reaction volume is between 50-100 µL.	

4. Determining the Degree of Labeling (DOL)

4.1 Take a small amount of the purified labeled antibody conjugate from Step 3.7 in PBS and measure the absorbance of the solution at 280 nm (A_{280}) and at 503 nm (A_{503}), the absorption maximum of the ATTO 488-IgG conjugate (λ_{max}, *503 nm*). Please make sure that the value of A_{503} is below 1.5 in a 1 mm equivalent pathlength. Otherwise dilute the sample.

4.2 Calculate the DOL, i.e., the average number of dye molecules coupled to the antibody, using the equation below. As all dyes show some absorption at 280 nm, the measured absorbance at 280 nm (A_{280}) must be corrected for the contribution of the dye by $A_{503} \times CF_{280}$, where $CF_{280} = \varepsilon_{280} / \varepsilon_{max}$. For ATTO 488 use the following values:

λ_{max} = 503 nm
ε_{max} = 9.0 × 10^4 M^{-1} cm^{-1}
CF_{280} = 0.09

$$DOL = \frac{A_{503}/\varepsilon_{max}}{A_{protein}/\epsilon_{protein}} = \frac{A_{503} \times \varepsilon_{protein}}{(A_{280} - A_{503} \times CF_{280}) \times \epsilon_{max}}$$

where $\varepsilon_{protein}$ of a typical IgG antibody at 280 nm is 203,000 M^{-1} cm^{-1}. The same extinction coefficient is suitable for IgA, IgD, and IgE.

Note: If the sample has been diluted, the dilution factor must be accounted for in the calculation!

5. Storing the labeled protein conjugate

Store the labeled protein at 2-8°C, protected from light, for up to two weeks. For long term storage, divide the conjugate into suitable aliquots and freeze at -20°C. Avoid repeated freezing and thawing. Certain proteins might require specific conditions, such as cryoprotectants. Please refer to the antibody manufacturer guidelines.

6. Troubleshooting

6.1 Under-labeling, i.e., conjugate exhibits a DOL significantly lower than expected.

Under-labeling can be caused by:

- trace amounts of compounds containing primary amino-groups (e.g., Tris or glycine) reacting with the dye and decrease the labeling efficiency.

- low protein concentrations (≤ 1 mg/mL).

- pH values lower than 8.0, since succinimidyl esters react most efficiently with primary amines at slightly alkaline pH.

To increase labeling efficiency, use higher protein concentrations and sodium bicarbonate buffer at **pH 8,3-9.0**.

Because different proteins react with succinimidyl esters at different rates and retain biological activity at different DOL, the standard protocol may not always result in optimal labeling. To increase the DOL, you can label a new protein sample using a higher dye-to-protein ratio (D/P). The reactive dye provided per vial is sufficient to label additional protein samples.

6.2 Over-labeling, i.e., conjugate exhibits a DOL significantly higher than expected.

Over-labeling can cause aggregation of the protein conjugate and reduce the binding specificity of the antibody for its antigen resulting in a higher background signal. It can also result in fluorescence quenching of the conjugate. To reduce the DOL, decrease the dye-to-protein ratio.

6.3 Inefficient purification of labeled antibody

Although very unlikely, it is possible that trace amounts of free dye flow through the column and remain in the conjugate solution after purification. In the presence of free dye, the calculation of the DOL erroneously results in higher values. Remaining traces of free dye can be removed by applying the conjugate to another column or by external dialysis.

6.4 Inefficient elution of protein from spin column

If the protein did not fully elute during centrifugation, do not add additional buffer to the column. Instead, re-centrifuge one or more times at the recommended centrifugation speed of 1500 x g to elute the protein.