

Atto 532 Protein Labeling Kit

Fluorescent labeling of thiol groups

Cat.-No.	Amount
FP-202-532	3 reactions

For *in vitro* use only!
Protect from light – work under low light conditions!

Kit Contents

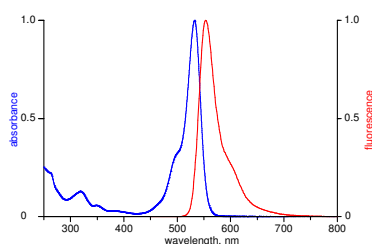
- **Atto 532 Maleimide**
1 vial containing 1 mg
- **Dimethylformamide (DMF)**
100 μ l
- **Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP)**
3 vials containing 1 mg each
- **Glutathione (GSH)**
3 vials containing 1 mg each
- **ultra-pure water**
1 ml

Storage and Stability

Upon receipt store the dye at -20°C . The other components may be stored at 4°C .
If stored as recommended, Jena Bioscience guarantees optimal performance of this kit for 12 months.

Spectroscopic data of Atto 532

Excitation maximum: $\lambda_{\text{Ex}} = 532 \text{ nm}$
Emission maximum: $\lambda_{\text{Em}} = 553 \text{ nm}$
Extinction coefficient: $\epsilon_{\text{max}} = 115,000 \text{ cm}^{-1} \text{ M}^{-1}$
Correction factor: $\text{CF}_{280 \text{ nm}} = 0.11$



Atto 532 excitation and emission spectra

Introduction

Fluorescence techniques have become a major tool in biological sciences. Fluorescent proteins such as GFP and DsRed fused to the protein of interest (POI) allow expression analysis and *in vivo* protein localization. Their application however, is limited due to the large molecular weight of those fusion proteins. Furthermore, the associated molecular biology is tedious and time-consuming.

Small fluorophores, covalently attached to the POI, may help to overcome this problem. The most common labeling method for proteins is amine modification. Amine-reactive fluorophores such as NHS-esters readily react with lysines, present at the surface of the POI, resulting in a stable covalent bond. (Please note: Jena Bioscience offers a ready-to-use kit for labeling of lysine residues with high-quality small fluorophores (Cat.# FP-201) for application in immunochemistry and other assays that require extraordinary stability of the conjugate).

Although fluorescent labeling of lysine residues is a reliable method, the alternative labeling of the thiol group of cysteines is often preferred for investigating structure, function and interactions of the POI. Since many enzymes contain a cysteine in their catalytic domain, protein activity and function may be conveniently investigated by targeting cysteines involved in the catalytic process or binding site.^{1,2}

In general, thiol labeling is more specific than amine labeling due to the relatively low abundance of cysteines compared to lysines. Since however, appearance of cysteines and lysines varies among different proteins, the amino acid sequence and tertiary structure of the POI must be analyzed prior to deciding on the labeling strategy.

This Jena Bioscience Protein Labeling Kit is designed for labeling the cysteines of a POI with a small fluorophore resulting in a fluorescent protein-fluorophore conjugate. It contains all reagents required for performing 3 separate labeling reactions of 1 mg of POI.

¹ Skegro et al. (2007) N-terminal and C-terminal Domains of Arrestin Both Contribute in Binding to Rhodopsin. *Photochem. Photobiol.* **83**: 385-392

² Kamata et al. (2005) Reactive Oxygen Species Promote TNF α -Induced Death and Sustained JNK Activation by Inhibiting MAP Kinase Phosphatases. *Cell.* **120**: 649-661

Protocol

General notes

The protein concentration should be at least 2 mg/ml, since labeling efficiency suffers from lower concentrations. We recommend using about 1 mg protein per labeling reaction.

Experimental protocol

1. Dissolve your protein in a suitable buffer at pH 7.2 such as PBS, Tris, or HEPES.
2. Dissolve 1 vial TCEP by adding 100 μ l ultra-pure water. TCEP solution should be prepared freshly prior to use.
3. Add 500 μ l protein solution (2 mg/ml) and 30 μ l TCEP to an appropriate vial. Vortex carefully!
4. Prepare the dye by adding 100 μ l DMF resulting in a dye concentration of 10 mg/ml. Vortex until the dye is completely dissolved! We recommend preparing the solution shortly prior to use however, it is stable for one week at -80°C . Whenever handling the fluorophore or conjugate, work under low light conditions!
5. Add 33 μ l dye (10 mg/ml) to your protein solution. Vortex carefully and centrifuge briefly to collect the reaction mixture at the bottom of the tube.
6. Incubate for two hours in a shaker at 20°C . Protect from light!
7. Dissolve 1 vial GSH by adding 100 μ l ultra-pure water. GSH solution must be prepared freshly prior to use.
8. Add 30 μ l GSH to the reaction mixture and incubate for 15 minutes in a shaker at 20°C . Protect from light!
9. Purify the conjugate using standard gel filtration columns such as Sephadex G-25 or similar. Alternatively, the free dye may be separated from the conjugate by dialysis or appropriate spin concentrators.

Please note that protein purification materials are not provided with the kit!

Concentration of the conjugate

Since there may occur a certain loss of protein during labeling and particularly during purification, measuring the concentration of the conjugate is important for further application.

The concentration of a protein is commonly determined by measuring its absorbance at 280 nm. As shown in the excitation spectrum of Atto 532 however, the fluorescent dye absorbs at 280 nm as well, thereby increasing the A_{280} for the conjugate. Therefore, a correction factor (CF) is required to eliminate the contribution of the dye at 280 nm.

The CF of each dye is given in the *Spectroscopic Data* section, and hence the concentration of the conjugate is calculated according to:

$$c \left(\frac{\text{mg}}{\text{ml}} \right) = \frac{A_{280} - A_{\text{max}} \times CF}{\epsilon_{280}} \times MW(\text{protein})$$

A_{280} absorbance of the conjugate solution measured at 280 nm

A_{max} absorbance of the conjugate solution measured at λ_{Ex}

λ_{Ex} , ϵ_{max} , CF intrinsic properties of the Atto dye, please refer to *Spectroscopic Data*

ϵ_{280} , MW intrinsic properties of your protein, if not known they can be obtained from web sources such as ExPASy Proteomics Server³

Degree of labeling (DOL)

The DOL specifies the average number of fluorophore molecules per molecule of conjugate. It is an important parameter of the conjugate, significantly affecting further applications.

The DOL is calculated according to:

$$DOL = \frac{A_{\text{max}} \times \epsilon_{280}(\text{protein})}{(A_{280} - A_{\text{max}} \times CF) \times \epsilon_{\text{max}}}$$

A_{280} absorbance of the conjugate solution measured at 280 nm

A_{max} absorbance of the conjugate solution measured at λ_{Ex}

λ_{Ex} , ϵ_{max} , CF intrinsic properties of the Atto dye, please refer to *Spectroscopic Data*

ϵ_{280} , MW intrinsic properties of your protein, if not known they can be obtained from web sources such as ExPASy Proteomics Server³

³ www.expasy.ch/tools/protparam.html

Data Sheet

Example for determining the conjugate concentration and the DOL of BSA (Bovine serum albumin), labeled with Atto 488 maleimide

<u>BSA</u>	<u>Atto 488</u>
$\epsilon_{280} = 42,925 \text{ cm}^{-1} \text{ M}^{-1}$	$\lambda_{\text{Ex}} = 501 \text{ nm}$
MW = 66,433 Da	$\epsilon_{\text{max}} = 90,000 \text{ cm}^{-1} \text{ M}^{-1}$
	CF = 0.1

After labeling and purification, the absorption of the conjugate solution is measured at 280 and 501 nm, respectively.

	absorption
A_{280}	0.29
A_{501}	0.5

The conjugate concentration and the DOL are calculated:

$$c \left(\frac{\text{mg}}{\text{ml}} \right) = \frac{A_{280} - A_{\text{max}} \times CF}{\epsilon_{280}(\text{protein})} \times MW(\text{protein})$$

$$c \left(\frac{\text{mg}}{\text{ml}} \right) = \frac{0.29 - 0.5 \times 0.1}{42,925} \times 66,433$$

$$c \left(\frac{\text{mg}}{\text{ml}} \right) = 0.37$$

$$DOL = \frac{A_{\text{max}} \times \epsilon_{280}(\text{protein})}{(A_{280} - A_{\text{max}} \times CF) \times \epsilon_{\text{max}}}$$

$$DOL = \frac{0.5 \times 42,925}{(0.29 - 0.5 \times 0.1) \times 90,000}$$

$$DOL = 0.99$$

In this experiment the concentration of the conjugate is 0.37 mg/ml and each protein molecule is labeled with approximately one Atto 488 molecule.

Please note that the spectroscopic determination of the concentration and the DOL of the conjugate is not absolutely correct. The spectroscopic characteristics of the free dye are not exactly the same as of the dye bound to the POI. Also, the spectroscopic characteristics of the native protein at 280 nm differ from those of the conjugate.

In general, these changes are negligibly small and therefore, spectroscopic determination of the concentration and the DOL of the conjugate is the most frequently used method.⁴

In addition to the spectroscopic measurements, one may analyze the conjugate by SDS-PAGE and subsequent fluorescence scanning of the gel. Only one band (consisting of the fluorescently labeled protein) should be visible in the fluorescent scan. If there is an additional band at very low molecular weight, the conjugate solution still contains free dye and must be purified again.

Storage of the Conjugate

Protect from light! Store the conjugate just like the unlabeled protein. We recommend dividing the solution into small aliquots and freeze at -20°C or -80°C . Avoid repeated freezing and thawing!

Troubleshooting

Inefficient Labeling

- Concentration of protein solution
The assay is optimized for labeling of 1 mg protein at a concentration of 2 mg/ml. Increase the amount of dye proportionally for protein concentrations above 2 mg/ml. If your protein concentration is very low ($< 1 \text{ mg/ml}$), apply spin concentrators to achieve a final concentration of 2 mg/ml. The efficiency of labeling is strongly concentration dependent and varies among different proteins. Thus, in every single case optimization might be necessary to obtain the desired results.
- Impact of the pH
Check the pH of your protein solution! The reference range is 7.0 – 7.5. At this pH range the thiol groups are sufficiently nucleophilic, thus react well with the maleimide resulting in a stable covalent bond.

⁴ Brinkley (1992) A Brief Survey of Methods for Preparing Protein Conjugates with Dyes, Haptens, and Cross-Linking Reagents. *Bioconjugate Chem.* **3**: 2-13

The primary amino groups of the protein are protonated at this pH range and therefore, do not react with the maleimide.

Higher pH values – particularly above 8.0 – negatively affect the labeling reaction, since the hydrolysis rate of maleimides increases with the pH of the solution, resulting in non-reactive dye.

- Reduction of disulfide bonds
Increase the amount of TCEP (reducing agent) in case you use more than 1 mg of protein per labeling reaction. Make sure that at least a 10-fold molar excess of TCEP is added to your protein solution prior to the labeling reaction.

Note that labeling efficiency does not only depend on the surrounding conditions, but also on the protein characteristics such as tertiary structure and the number of cysteines.

Overlabeling

Reasons for overlabeling can be a high number of cysteines on the surface of your protein.

To prevent overlabeling, decrease the amount of dye or increase the protein concentration. You may also reduce the reaction time.

Purification of the conjugate

The maleimide is unstable in aqueous solutions. Thus, a certain amount of free dye is produced during each labeling reaction and needs to be separated from the conjugate.

If your purified conjugate still contains traces of free dye, apply it to a second purification step. Check the purity of your conjugate by SDS-PAGE or thin layer chromatography.

Increase the amount of GSH proportionally in case you increase the amount of maleimide per labeling reaction. GSH is essential for consuming excess thiol-reactive dye after the labeling reaction is completed. Reactive dye molecules might interfere with the purification of the conjugate and thus, an excess of GSH must be added subsequent to the labeling reaction.

Separation of free dye from the conjugate becomes more difficult at higher concentrations of free dye. The column or membrane might even be blocked by the dye at very high concentrations, so try to optimize the labeling reaction in order to decrease the concentration of free dye. Keep in mind that overall yield suffers from additional purification steps.

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