Recommended Procedures for Labeling

Introduction

ATTO-TEC offers a large variety of high-quality dyes for labeling amino and thiol groups. ATTO reactive dyes cover the spectral region from 350 nm in the UV to 750 nm in the NIR.
The most commonly used amine-reactive dye derivatives are N-hydroxysuccinimidyl(NHS)-esters. NHS-esters readily react with amine-modified oligonucleotides or amino groups of proteins, i.e. the ε-amino groups of lysines or the amine terminus, forming a chemically stable amide bond between the dye and the protein or oligo. However, the amino group ought to be unprotonated to be reactive. Therefore the pH of the solution must be adjusted sufficiently high to obtain a high concentration of unprotonated amino groups. On the other hand, the NHS-ester also reacts with the hydroxyl ions in the solution to yield free dye, which is no longer reactive. As the rate of this hydrolysis increases with the concentration of hydroxyl ions, the pH should be kept as low as possible. Buffering the solution at pH 8.3 has been found to be a good compromise between the contradicting requirements.
For labeling thiol groups the most popular and commonly used dye derivatives are maleimides. ATTO-maleimides react with thiol groups of proteins to form a stable thio-ether bond.

Labeling Proteins with Amine-Reactive ATTO-Labels (NHS-Esters)

ATTO NHS-esters readily react with amino groups of proteins. The optimum pH range for NHS-ester coupling is pH 8.0 – 9.0. At this pH amino groups of proteins, i.e. the ε-amino groups of lysines are to a high degree unprotonated and highly reactive towards dye-NHS-ester.

Required Materials

- **Solution A**: PBS buffer (Phosphate-Buffered Saline, pH 7.4): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2\ H_2O$, and 0.24 g KH_2PO_4, in 1 liter distilled water.
- **Solution B**: 0.2 M sodium bicarbonate solution, adjusted to pH 9.0 with 2 M sodium hydroxide.
- **Solution C**: To 20 parts of **Solution A** add 1 part of **Solution B** to obtain a labeling buffer of pH 8.3. Kept in an air-tight bottle, this solution will be stable for a long period of time.
- **Solution D**: Dissolve 1.0 mg of dye NHS-ester in 50 – 200 µl of anhydrous, amine-free DMSO or DMF. Due to the high quality of ATTO NHS-esters such solutions are stable for a long period of time (freeze and protect from light when not in use). However, it may be difficult to avoid humidity entering a solution in continuous use. In the presence of water NHS-esters readily hydrolyze and become non-reactive. Hence we advise to freshly prepare, whenever possible, the dye NHS-ester solution immediately before starting the labeling reaction.
- Gel filtration column filled with Sephadex G-25 or equivalent.

Conjugate Preparation

- Dissolve 1 – 5 mg of protein in 1 ml of **Solution C**. Protein solutions must be free of any amine-containing substances such as tris-(hydroxymethyl)-aminomethane (TRIS), free amino acids or ammonium ions. Antibodies that are dissolved in amine containing buffers should be dialyzed against **Solution A**, and the desired coupling pH of 8.3 will be obtained by the procedure given above for **Solution C**. The presence of sodium azide in low concentration (< 3 mM) will not interfere with the labeling reaction.
- To obtain a degree of labeling (DOL, dye-to-protein ratio) of 2 – 3 add, while gently shaking, a threefold molar excess of reactive dye(**Solution D**) to the protein solution. Variations due to different reactivities of both the protein and the labeling reagent may occur. This may necessitate optimization of the dye-to-protein ratio used in the reaction in order to obtain the desired DOL. To increase the degree of labeling a higher ratio of NHS-ester to protein has to be used and vice versa.
- Incubate the reaction mixture protected from light for up to 1 hour at room temperature. In most cases the labeling reaction will be complete within 5 – 10 minutes.

Conjugate Purification – Removal of Unbound Dye

- Due to an unavoidable side reaction part of the applied dye NHS-ester will hydrolyze during the labeling reaction and must be removed from the protein conjugate. We recommend using a Sephadex G-25 (or equivalent) gel filtration column (1 – 2 cm diameter and 10 – 20 cm length; for very hydrophilic dyes, e. g. ATTO 488, ATTO 532, ATTO 594, a 30 cm column is preferable) for separation of dye-protein conjugate from free dye.
- Preequilibrate the column with **Solution A**.
- Elute the dye-protein conjugate using **Solution A**.
- The first colored and fluorescent zone to elute will be the desired dye-protein conjugate. A second colored and fluorescent, but slower moving zone contains the unbound free dye (hydrolyzed NHS-ester).
- To prevent denaturation of the conjugate after elution, bovine serum albumin (BSA) or another stabilizer may be added.

Labeling Proteins with Thiol-Reactive ATTO-Labels (Maleimides)

ATTO maleimides readily react with thiol groups of proteins. The optimum acidity for thiol modification with maleimides is pH 7.0 – 7.5. At this pH the thiol (sulfhydryl) group is deprotonated to a sufficient degree and readily reacts with the dye-maleimide.

Required Materials

- **Solution A**: PBS buffer (Phosphate-Buffered Saline, pH 7.4): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2\ H_2O$, and 0.24 g KH_2PO_4, in 1 liter distilled water.
  modification in an inert atmosphere to prevent oxidation of the thiols. It may also be advisable to deoxygenate all buffers and solvents used for the thiol conjugation.

- **Solution E**: Dissolve 1.0 mg of dye-maleimide in 50 – 200 µl of anhydrous, amine-free DMSO or DMF. Due to the high quality of ATTO maleimides such solutions are stable for a long period of time (freeze and protect from light when not in use). However, it may be difficult to avoid humidity entering a solution in continuous use. The maleimide moiety may hydrolyze and become non-reactive. Hence, we advise to freshly prepare, whenever possible, the dye-maleimide solution immediately before starting the labeling reaction.
- Gel filtration column filled with Sephadex G-25 or equivalent.

Conjugate Preparation

- Dissolve 1 – 5 mg of protein in 1 ml of Solution A (PBS buffer, pH 7.4).
- Free thiol groups will react with dye-maleimide by adding a 1.3 fold molar excess of reactive dye (**Solution E**) while gently shaking. Variations due to different reactivities of both the protein and the labeling reagent may occur.
- Incubate the reaction mixture protected from light for 2 hours at room temperature.

Note: If the protein contains disulfide bonds it may be desirable to reduce the disulfide before labeling. For reduction, reagents such as tris(2-carboxyethyl)phosphin (TCEP) or dithiothreitol (DTT) may be used. However, care has to be taken that any excess of these reducing agents has been removed (e.g. by dialysis) as they consume dye-maleimide themselves and in some cases even destroy the dye chromophore.

Conjugate Purification – Removal of Unbound Dye

- Part of the applied dye maleimide will hydrolyze during the labeling reaction. The unreacted maleimide and the hydrolyzed maleimide must be removed from the labeled protein. We recommend using a Sephadex G-25 (or equivalent) gel filtration column (1 – 2 cm diameter and 10 – 20 cm length; for very hydrophilic dyes, e. g. ATTO 488, ATTO 514, ATTO 532, ATTO 594, a 30 cm column is preferable) for separation of dye-protein conjugate from free dye.
- Preequilibrate the column with **Solution A**.
- Elute the dye-protein conjugate using **Solution A**.
- The first colored and fluorescent zone to elute will be the desired dye-protein conjugate. A second and maybe third colored and fluorescent, but slower moving zone contains the unreacted and/or hydrolyzed maleimide.
- To prevent denaturation of the conjugate after elution, bovine serum albumin (BSA) or another stabilizer may be added.

Storage of the Protein Conjugate

In general, conjugates should be stored under the same conditions used for the unlabeled protein. For storage in solution at 4 °C, sodium azide (2 mM final concentration) can be added as a preservative. Removal of preservatives prior to use may be necessary to avoid inhibitory effects in applications in which conjugates are added to live cell specimens. The conjugate should be stable at 4 °C for several months. For long-term storage, divide the solution into small aliquots and freeze at -20 °C. Avoid repeated freezing and thawing. Protect dye conjugates from light as much as possible.

Determining the Degree of Labeling (DOL)

The degree of labeling (DOL, dye-to-protein ratio) obtained by the above procedures can be determined by absorption spectroscopy making use of the Lambert-Beer law: Absorbance (A) = extinction coefficient (ε) × molar concentration × path length (d). Simply measure the UV-VIS spectrum of the conjugate solution as obtained after gel filtration in a quartz (UV-transparent) cell. You may need to dilute the solution, if it turns out to be too concentrated for a correct absorbance measurement. Determine the absorbance (A_{max}) at the absorption maximum (λ_{abs}) of the dye and the absorbance (A_{280}) at 280 nm (absorption maximum of proteins). The concentration of bound dye is given by: c(dye) = A_{max} / ε_{max} × d, where ε_{max} is the extinction coefficient of the dye at the absorption maximum. The protein concentration is obtained in the same way from its absorbance at 280 nm. As all dyes show some absorption at 280 nm, the measured absorbance A_{280} must be corrected for the contribution of the dye. This is given by A_{max} × CF_{280}. The values for the correction factor CF_{280} = ε_{280} / ε_{max} are listed in the table on p.76. It follows for the absorbance of the protein itself:
A_{prot} = A_{280} – A_{max}× CF_{280}. Then the concentration of protein is:
c(protein) = A_{prot} / ε_{prot}× d, where ε_{prot} is the extinction coefficient of the protein at 280 nm.
It follows for the degree of labeling, i.e. the average number of dye molecules coupled to a protein molecule: DOL = c(dye) / c(protein) and with the above relations:

$$DOL = \frac{A_{max} / \varepsilon_{max}}{A_{prot} / \varepsilon_{prot}} = \frac{A_{max} \cdot \varepsilon_{prot}}{(A_{280} - A_{max} \cdot CF_{280}) \cdot \varepsilon_{max}}$$

Note: The above equation is only valid if the extinction coefficient of the free dye λ_{max} at the absorption maximum is the same as the extinction coefficient of the conjugated dye at this wavelength. Due to dye aggregation effects this is frequently not the case. Hence the value calculated for DOL may be too low by 20 % or more. This is illustrated by direct comparison of the absorption spectra of ATTO 550 as free, i.e. unbound, dye (blue curve) and the same amount of dye, conjugated to a protein (red curve).

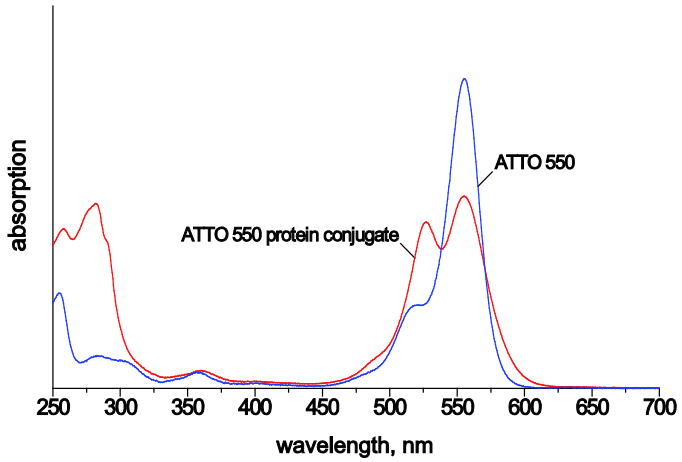

Table: Optical properties of ATTO-Labels

Dye	MW, g/mol		λ_{abs}, nm	ε_{max}, M^{-1} cm^{-1}	CF_{260}	CF_{280}
	NHS	Mal.				
ATTO 390	440	465	390	2.4×10^4	0.52	0.08
ATTO 425	498	523	436	4.5×10^4	0.27	0.23
ATTO 465	493	518	453	7.5×10^4	1.12	0.54
ATTO 488	981	1067	501	9.0×10^4	0.25	0.10
ATTO 495	549	574	495	8.0×10^4	0.57	0.39
ATTO 5114	1111	989	511	1.15×10^5	0.21	0.08
ATTO 520	564	589	516	1.1×10^5	0.40	0.40
ATTO 532	1081	1063	532	1.15×10^5	0.22	0.11
ATTO Rho6G	711	849	535	1.15×10^5	0.22	0.19
ATTO 540Q	756	781	542	1.05×10^5	0.22	0.24
ATTO 550	791	816	554	1.2×10^5	0.24	0.12
ATTO 565	708	733	563	1.2×10^5	0.34	0.16
ATTO Rho3B	642	764	565	1.2×10^5	0.28	0.14
ATTO Rho11	763	788	571	1.2×10^5	0.25	0.09
ATTO Rho12	847	872	576	1.2×10^5	0.27	0.09
ATTO Thio12	699	824	579	1.1×10^5	0.10	0.37
ATTO Rho101	787	812	586	1.2×10^5	0.24	0.19
ATTO 580Q	892	917	586	1.1×10^5	0.36	0.13
ATTO 590	788	813	594	1.2×10^5	0.42	0.44
ATTO 594	1389	1358	601	1.2×10^5	0.26	0.51
ATTO Rho13	843	867	600	1.25×10^5	0.38	0.44
ATTO 610	588	613	615	1.5×10^5	0.02	0.05
ATTO 611X	592	617	611	1.0×10^5	0.05	0.07
ATTO 612Q	888	913	615	1.15×10^5	0.35	0.57
ATTO 620	709	734	619	1.2×10^5	0.05	0.07
ATTO Rho14	981	1019	625	1.4×10^5	0.29	0.46
ATTO 633	749	774	629	1.3×10^5	0.05	0.06
ATTO 647	811	832	645	1.2×10^5	0.08	0.04
ATTO 647**N**	843	868	644	1.5×10^5	0.06	0.05
ATTO 655	887	812	663	1.25×10^5	0.24	0.08
ATTO Oxa12	835	874	663	1.25×10^5	0.24	0.08
ATTO 680	828	1024	680	1.25×10^5	0.30	0.17
ATTO 700	837	971	700	1.2×10^5	0.26	0.41

Dye	MW, g/mol		λ_{abs}, nm	ε_{max}, M^{-1} cm^{-1}	CF_{260}	CF_{280}
	NHS	Mal.				
ATTO 725	613	638	729	1.2×10^5	0.10	0.08
ATTO 740	665	690	740	1.2×10^5	0.11	0.10
ATTO MB2	553	591	658	1.0×10^5	0.11	0.28

Increase of Molecular Mass and Charge on Conjugation with ATTO-Labels

Although ATTO-dye molecules are small compared to biomolecules like proteins, DNA etc., they will affect their properties to a certain degree. Notably mass and, frequently, electrical charge of the biomolecule will be different after conjugation with a dye. To aid in the analysis of biomolecule-dye conjugates, the table below shows the mass and charge increase that occur on coupling with an ATTO-dye. Because biomolecules as well as ATTO-dyes may carry basic ($-NH_2$) and acidic (-COOH, $-SO_3H$) substituents, both mass and electrical charge depend on pH. The data given in the table are based on the assumption of non-protonated amino groups ($-NH_2$), deprotonated acid groups ($-COO^-$, $-SO_3^-$) and neutral thiol groups. It is worth mentioning that under acidic conditions (pH < 4) the additional, non-reactive, carboxylic acid group of dyes like ATTO 565 and ATTO 590 will be protonated. As a consequence both mass and charge will be higher by one unit than the values given in the table, which are based on the assumption of deprotonated carboxylic acid groups.

Increase of Molecular Mass (Δm) and Charge (Δq) on Conjugation with ATTO-Labels

ATTO-Label	Δm (NHS-ester : amine)	Δm (maleimide : thiol)	Δq
ATTO 390	325.4	465.5	0
ATTO 425	383.4	523.6	0
ATTO 465	278.4	418.5	+ 1
ATTO 488	570.6	710.7	- 1
ATTO 495	334.4	474.6	+ 1
ATTO 514	734.6	874.7	- 1
ATTO 520	349.5	489.6	+ 1
ATTO 532	626.7	766.8	- 1
ATTO Rho6G	496.6	636,7	+ 1
ATTO 550	576.8	716.9	+ 1
ATTO 565	492.6	632.7	0
ATTO Rho3B	524.7	664.8	+ 1
ATTO Rho11	548.7	688.8	+ 1
ATTO Rho12	632.9	773.0	+ 1
ATTO Thio12	484.6	624.8	+ 1
ATTO Rho101	572.7	712.9	+ 1

ATTO-Label	Δm (NHS-ester : amine)	Δm (maleimide : thiol)	Δq
ATTO 590	572.7	712.8	0
ATTO Rho13	628.8	769.0	+ 1
ATTO 594	786.9	927.1	- 1
ATTO 610	373.5	513.7	+ 1
ATTO 620	494.7	634.8	+ 1
ATTO Rho14	766.6	906.8	+ 1
ATTO 633	534.7	674.9	+ 1
ATTO 647	574.8	714.9	0
ATTO 647**N**	628.9	769.0	+ 1
ATTO 655	509.6	649.8	0
ATTO Oxa12	621.9	762.0	+ 1
ATTO 665	605.7	745.9	+ 1
ATTO 680	507.6	647.8	0
ATTO 700	547.7	687.8	0
ATTO 725	398.5	538.7	+ 1
ATTO 740	450.6	590.8	+ 1
ATTO 540Q	541.6	681.8	+ 1
ATTO 580Q	677.9	818.0	+ 1
ATTO 612Q	673.8	814.0	+ 1
ATTO MB2	338.4	478.5	+ 1

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