ATTO-TEC

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 and iodoacetamides. They react with thiol groups of proteins to form a stable thio-ether bond.

Preparation and Handling of Dye Stock-Solutions

For the preparation of dye stock-solutions a solvent recommendation for each dye is given in the Table 1 on page 5. To determine the concentration of a dye stock-solution we recommend to take an aliquot and dilute with acidified ethanol (0.1 vol.-% trifluoroacetic acid) to avoid dye aggregation and in some cases (ATTO 565 and ATTO 590) formation of a colorless spiro-lacton.
Depending on solvent quality such stock-solutions are not stable at room temperature and for storage purposes must be kept, protected from light, at -20 °C. Additionally, it may be difficult to avoid humidity entering a solution in continuous use. The reactive moiety may hydrolyze and become non-reactive. We advise to freshly prepare, whenever possible, the dye stock-solutions immediately before starting the labeling reaction. One should keep in mind that solvents like DMSO or DMF are never free of nucleophilic and/or basic impurities. Such compounds will react with the NHS-ester functionality and consequently reduce coupling efficiency. In some cases (ATTO 610, ATTO 647, ATTO 725, ATTO 740) they also undergo reactions with the dye chromophore resulting in dye-degradation.

Labeling Proteins with Amine-Reactive ATTO-Labels

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 unprotonated to a high degree and highly reactive towards the 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 acetonitrile (see Table 1). To determine the concentration of such a dye stock-solution we recommend to take an aliquot and dilute with acidified ethanol to avoid dye aggregation and in some cases (ATTO 565 and ATTO 590) formation of a colorless spiro-lacton. Depending on solvent quality such solutions are not stable at room temperature and for storage purposes must be kept, protected from light, at -20 °C. Additionally, 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. 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. For ATTO 565-NHS and ATTO 590-NHS we recommend an incubation time of 18 hours at ambient temperature for the reaction to be completed.

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 via gel filtration using Sephadex G-25 or equivalent. We recommend a column with 1 – 2 cm in diameter and 15 – 20 cm in length. For very hydrophilic dyes, e. g. ATTO 488, ATTO 532, ATTO 542, ATTO 594, the column has to be at least 30 cm in length to achieve a satisfactory result.
- 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.
- For re-use of the Sephadex column one can elute with either 0.01 % sodiumhydroxide solution and/or water/ethanol 80:20 to remove any residues of dye or dye-conjugate. The treatment is followed by exhaustive washing with water.

Labeling Proteins with Thiol-Reactive ATTO-Labels

ATTO maleimides (MAL) and iodoacetamides (IAA) readily react with thiol groups of proteins. The optimum acidity for thiol modification is pH 7.0 – 7.5 in the case of maleimides and pH 8.0 – 8.5 for the lesser reactive iodoacetamides. At these pH the thiol (sulfhydryl) group is deprotonated to a sufficient degree and readily reacts with the dye-maleimide or dye-iodoacetamide.

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 E**: Dissolve 1.0 mg of dye-maleimide in 50 – 200 µl of anhydrous, amine-free DMF or acetonitrile (see Table 1). Depending on solvent quality such solutions are not stable at room temperature and for storage purposes must be kept, protected from light, at -20 °C. Additionally, it may be difficult to avoid humidity entering a solution in continuous use. The maleimide moiety may hydrolyze and become non-reactive. We advise to freshly prepare, whenever possible, the dye-maleimide solution immediately before starting the labeling reaction.
- **Solution F**: Dissolve 1.0 mg of dye-iodoacetamide in 50 – 200 µl of anhydrous, amine-free DMF or acetonitrile (see Table 1). Depending on solvent quality such solutions are not stable at room temperature and for storage purposes must be kept, protected from light, at -20 °C. We strongly recommend to freshly prepare, whenever possible, the dye-iodoacetamide solution immediately before starting the labeling reaction.
- Gel filtration column filled with Sephadex G-25 or equivalent.

Conjugate Preparation

A. Maleimide Conjugation

- Dissolve 1 – 5 mg of protein in 1 ml of **Solution A** (PBS buffer, pH 7.4).
- The free thiol group 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.

B. Iodoacetamide Conjugation

- Dissolve 1 – 5 mg of protein in 1 ml of **Solution C** (PBS buffer, pH 8.3).
- The free thiol group will react with 1.3 fold molar excess of reactive dye (**Solution F**) while gently shaking. Incubate the reaction mixture, protected from light for 2 hours at **37 °C**. The slight rise in temperature speeds up the conjugation reaction drastically. At room temperature it may take more than 10 hours to complete conjugation.

Note: While dye-maleimides and iodoacetamides react readily with thiol (mercapto or sulfhydryl) groups, there is absolutely no reaction with disulfides. If the protein contains disulfide bonds and labeling at their position is desired, it is necessary to reduce the disulfide to thiol groups before labeling. For reduction, reagents such as tris(2-carboxyethyl)phosphin (TCEP) or dithiothreitol (DTT)

may be used. However, great care has to be taken that any excess of these reducing agents has been removed (e.g. by dialysis) as they consume dye-maleimidethemselves and in some cases (ATTO 725, ATTO 740, ATTO 610, ATTO 647) even destroy the dye chromophore.

Conjugate Purification – Removal of Unbound Dye

- Excess and hydrolyzed dye must be removed from the protein conjugate via gel filtration using Sephadex G-25 or equivalent. We recommend a column with 1 – 2 cm in diameter and 15 – 20 cm in length. For very hydrophilic dyes, e. g. ATTO 488, ATTO 532, ATTO 542, ATTO 594, the column has to be at least 30 cm in length to achieve a satisfactory result.
- 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 or hydrolyzed dye
- To prevent denaturation of the conjugate after elution, bovine serum albumin (BSA) or another stabilizer may be added.
- For re-use of the Sephadex column one can elute with either 0.01 % sodiumhydroxide solution and/or water/ethanol 80:20 to remove any residues of dye or dye-conjugate. The treatment is followed by exhaustive washing with water.

Storage of the Protein Conjugate

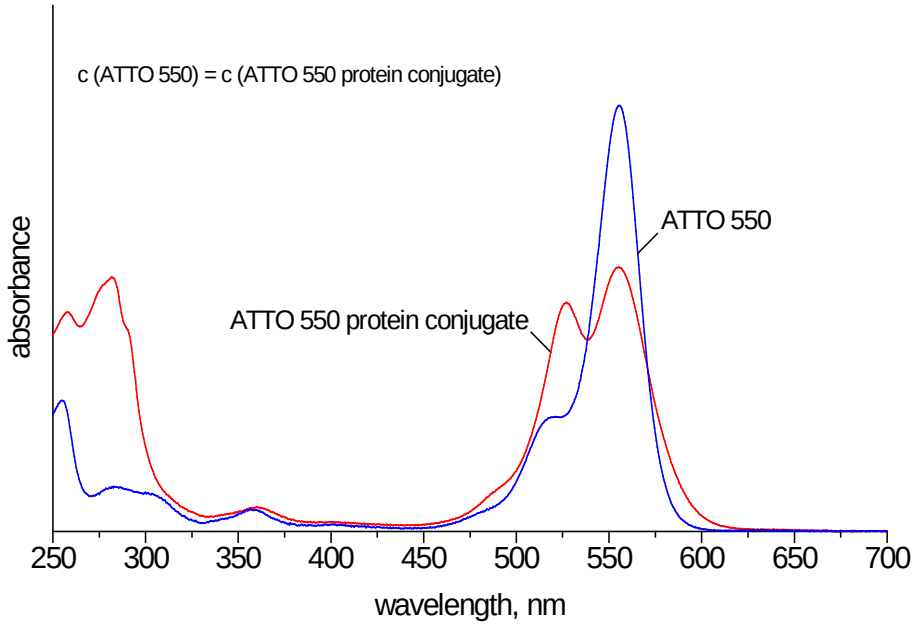
In general, conjugates should be stored under the same conditions used for the unlabeled protein. For storage in solution at 4 °C, sodium azide (2 mM final concentration) can be added as a preservative. Removal of preservatives prior to use may be necessary to avoid inhibitory effects in applications 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 (c) × 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: $c(dye) = A_{max} / \varepsilon_{max} \times d$, 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 $A_{max} \times CF_{280}$. The values for the correction factor $CF_{280} = \varepsilon_{280} / \varepsilon_{max}$ are listed in the Table 1 on page 6. It follows for the absorbance of the protein itself:
$A_{prot} = A_{280} - A_{max} \times CF_{280}$. Then the concentration of protein is:
$c(protein) = A_{prot} / \varepsilon_{prot} \times d$, 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 ε_{max} of the free dye 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).



In such cases it is recommended to determine the DOL by measuring the amount of uncoupled dye. Therefore it is necessary to collect the second colored zone during gel filtration containing the unbound dye. The molar amount of dye can be calculated by measuring the absorbance of this solution and applying the Lambert-Beer law. Due to the tendency of hydrophobic dyes to form aggregates it needs to be assured that the absorbance of the dye solution does not exceed A = 0.04 (pathlength: 1 cm). Otherwise it is mandatory to dilute the solution accordingly. The difference in the initial molar amount of dye and the molar amount of unbound dye represents the molar amount of bound dye. The ratio of bound dye and the amount of deployed protein yields the DOL by eliminating the absorbance of the dye coupled to the biomolecule.

Table 1: Optical properties of ATTO-Labels

Dye	MW, g/mol			λ_{abs}, nm	ε_{max}, M^{-1} cm^{-1}	CF_{260}	CF_{280}	Solvent recommendation	
	NHS	MAL	IAA					NHS	MAL/IAA
ATTO 390	440	465	553	390	2.4×10^4	0.46	0.09	DMSO	DMF
ATTO 425	498	523		439	4.5×10^4	0.19	0.17	DMSO	DMF
ATTO 430LS	686	711		436	3.2×10^4	0.32	0.22	DMSO	DMF
ATTO 465	493	518		453	7.5×10^4	1.09	0.48	DMSO	DMF
ATTO 488	981	1067	914	500	9.0×10^4	0.22	0.09	DMSO	DMF
ATTO 490LS	793	818		495	4.0×10^4	0.39	0.21	DMSO	DMF

Dye	MW, g/mol			λ_{abs}, nm	ε_{max}, M^{-1} cm^{-1}	CF_{260}	CF_{280}	Solvent recommondation	
	NHS	MAL	IAA					NHS	MAL/IAA
ATTO 495	549	574		498	8.0×10^4	0.45	0.37	DMSO	DMF
ATTO 514	1111	989	1078	511	1.15×10^5	0.21	0.07	DMSO	DMF
ATTO 520	564	589		517	1.1×10^5	0.16	0.20	DMSO	DMF
ATTO 532	1081	1063	970	532	1.15×10^5	0.20	0.09	DMSO	DMF
ATTO Rho6G	711	849		533	1.15×10^5	0.19	0.16	DMSO	DMF
ATTO 540Q	756	781		543	1.05×10^5	0.27	0.26	DMSO	DMF
ATTO 542	1125	1150		542	1.2×10^5	0.18	0.08	DMSO	DMF
ATTO 550	791	816	980	554	1.2×10^5	0.23	0.10	DMSO	DMF
ATTO 565	708	733	835	564	1.2×10^5	0.27	0.12	DMSO	DMF
ATTO Rho3B	642	764		566	1.2×10^5	0.28	0.14	DMSO	DMF
ATTO Rho11	763	788		572	1.2×10^5	0.27	0.13	DMSO	DMF
ATTO Rho12	847	872		577	1.2×10^5	0.26	0.09	DMSO	DMF
ATTO Thio12	699	824		582	1.1×10^5	0.11	0.37	DMSO	DMF
ATTO 575Q	808	833		582	1.2×10^5	0.29	0.12	DMSO	DMF
ATTO Rho101	787	812		587	1.2×10^5	0.18	0.17	DMSO	DMF
ATTO 580Q	892	917		587	1.1×10^5	0.32	0.11	DMSO	DMF
ATTO 590	788	813	931	593	1.2×10^5	0.39	0.43	DMSO	DMF
ATTO 594	1389	1358	1129	603	1.2×10^5	0.22	0.50	DMSO	DMF
ATTO Rho13	843	867		603	1.25×10^5	0.28	0.43	DMSO	DMF
ATTO 610	588	613		616	1.5×10^5	0.03	0.06	**ACN**	**ACN**
ATTO 612Q	888	913		615	1.15×10^5	0.35	0.60	DMSO	DMF
ATTO 620	709	734		620	1.2×10^5	0.04	0.06	DMSO	DMF
ATTO Rho14	981	1019		626	1.4×10^5	0.26	0.47	DMSO	DMF
ATTO 633	749	774	876	630	1.3×10^5	0.04	0.05	DMSO	DMF
ATTO 643	954	1071		643	1.5×10^5	0.05	0.04	DMSO	DMF
ATTO 647	811	832		647	1.2×10^5	0.08	0.04	**ACN**	**ACN**
ATTO 647**N**	843	868	970	647	1.5×10^5	0.04	0.03	DMSO	DMF
ATTO 655	887	812	852	663	1.25×10^5	0.24	0.08	DMSO	DMF
ATTO Oxa12	835	874		662	1.25×10^5	0.32	0.12	DMSO	DMF
ATTO 665	820	845		662	1.6×10^5	0.07	0.06	DMSO	DMF
ATTO 680	828	1024	850	681	1.25×10^5	0.30	0.17	DMSO	DMF
ATTO 700	837	971		700	1.2×10^5	0.26	0.41	DMSO	DMF
ATTO 725	613	638		728	1.2×10^5	0.08	0.06	**ACN**	**ACN**
ATTO 740	665	690		743	1.2×10^5	0.07	0.07	**ACN**	**ACN**
ATTO MB2	553	591		668	1.0×10^5	0.08	0.24	DMSO	DMF

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**, Table 2 shows the increase in mass (Δm) and charge (Δq) 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. This reflects the situation given in a close-to-neutral environment (pH 6 – 8). It is worth mentioning that under more 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 valid for pH 6 – 8.

Table 2: Increase of Molecular Mass (Δm) and Charge (Δq) on Conjugation with ATTO-Labels

ATTO-Label	Δm (NHS : amine)	Δm (Mal : thiol)	Δm (IAA : thiol)	Δq
ATTO 390	325.4	465.5	425.5	0
ATTO 425	383.4	523.6		0
ATTO 430LS	547.7	687.8		-1
ATTO 465	278.4	418.5		+1
ATTO 488	570.6	710.7	670.7	-1
ATTO 490LS	654.8	687.8		-1
ATTO 495	334.4	474.6		+1
ATTO 514	734.6	874.7	834.8	-1
ATTO 520	349.5	489.6		+1
ATTO 532	626.7	766.8	726.8	-1
ATTO Rho6G	496.6	636.7		+1
ATTO 542	893.0	1033.1		-3
ATTO 550	576.8	716.9	678.9	+1
ATTO 565	492.6	632.7	593.7	+1
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 590	572.7	712.8	673.8	+1
ATTO Rho13	628.8	769.0		+1
ATTO 594	786.9	927.1	831.9	-1
ATTO 610	373.5	513.7		+1
ATTO 620	494.7	634.8		+1

ATTO-Label	Δm (NHS : amine)	Δm (MAL : thiol)	Δm (IAA : thiol)	Δq
ATTO Rho14	766.6	906.8		+1
ATTO 633	534.7	674.9	634.8	+1
ATTO 643	814.0	953.2		-1
ATTO 647	574.8	714.9		0
ATTO 647**N**	628.9	769.0	729.0	+1
ATTO 655	509.6	649.8	610.8	0
ATTO Oxa12	621.9	762.0		0
ATTO 665	605.7	745.9		+1
ATTO 680	507.6	647.8	608.7	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 575Q	594.7	733.8		+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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