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Product Information: Thiol-Reactive ATTO-Label (Maleimides)

Compound	Storage information	Shelf Life
Thiol-reactive label Lyophilized or crystalline solid	Freeze upon receipt < -20 °C Protect from light and moisture	When stored as indicated, ATTO maleimides are stable for at least 3 years.

For optical properties see table on page 4.

Introduction:

ATTO-TEC offers a large variety of high-quality thiol-reactive dyes for labeling proteins, affibodies or other sulfhydryl (thiol)-containing compounds. The dyes cover the spectral region from 350 nm in the UV to 750 nm in the NIR.

The most commonly used thiol-reactive reagents are maleimides which readily react with compounds containing sulfhydryl groups, forming a chemically stable thio-ether bond between the dye and e.g. a protein. The optimum pH for the modification of thiols with maleimides is pH 7.0 - 7.5. We recommend the reaction to be carried out in phosphate buffered saline (PBS) pH 7.4. At this pH the thiol group is deprotonated to a sufficient degree to react with the dye-maleimide, whereas the amino groups of the protein show only little reactivity. Hence it is possible to selectively couple thiol groups in the presence of amino groups.

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

Required Materials

- **Solution A:** PBS buffer (Phosphate-Buffered Saline, pH 7.4): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ · 2 H₂O, and 0.24 g KH₂PO₄, in 1-liter distilled water.
- **Solution E:** Dissolve 1.0 mg of dye-maleimide in 50 – 200 µl of anhydrous, amine-free DMF. For the preparation and handling of dye stock-solutions see the section below.
- Gel filtration column filled with Sephadex G-25 or equivalent.

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 on page 4. To determine the concentration of a dye stock-solution we recommend taking 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-lactone.

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 DMF are never free of nucleophilic and/or basic impurities. Such compounds will react with the maleimide 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.

Conjugate Preparation

- Dissolve 1 – 5 mg of protein in 1 ml of **Solution A** (PBS buffer, pH 7.4).
- Free thiol will react with dye-maleimide by adding a 1.3-fold molar excess of reactive dye (**Solution E**) per sulfhydryl group 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 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-maleimide themselves and in some cases (ATTO 725, ATTO 740, ATTO 610, ATTO 647) 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 with 1 – 2 cm in diameter and 10 – 20 cm length for the separation of dye-protein conjugate from free dye. For very hydrophilic dyes, e. g. ATTO 488, ATTO 514, ATTO 532, ATTO 594 the column should be 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 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.
- 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 unlabeled protein, dye-protein conjugate, and unreacted dye maleimide. The treatment is followed by exhaustive washing with water.

Storage of the Protein Conjugate

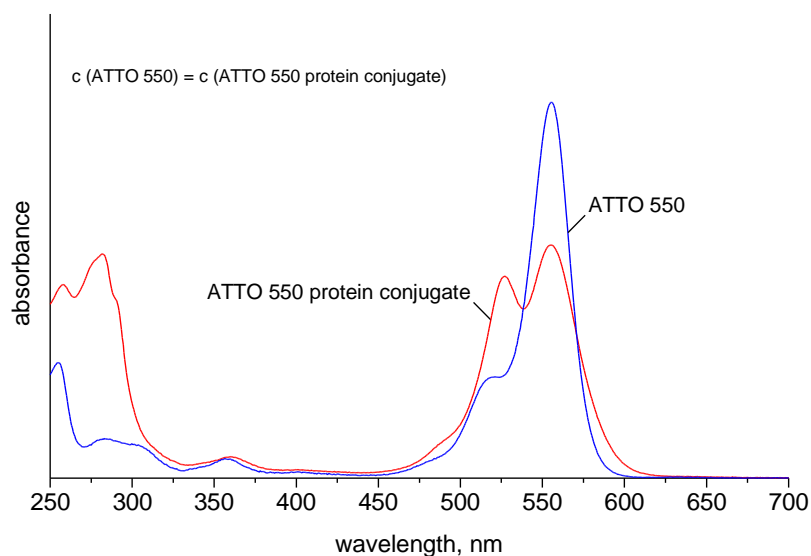
In general, conjugates should be stored under the same conditions used for the unlabeled protein. For storage in solution at 4 °C, sodium azide (2 mM final concentration) can be added as a preservative. Removal of preservatives prior to use may be necessary to avoid inhibitory effects in applications 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 Average Degree of Labeling (DOL)

The average 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) \times molar concentration \times 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 as well as the absorbance (A_{280}) at 280 nm (absorption maximum of proteins). The concentration of bound dye is given by: $c(\text{dye}) = A_{max} / \epsilon_{max} \times d$, 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 $CF_{280} = \epsilon_{280} / \epsilon_{max}$ are listed in the table on p.4. It follows for the absorbance of the protein itself: $A_{prot} = A_{280} - A_{max} \times CF_{280}$. Then the concentration of protein is: $c(\text{protein}) = A_{prot} / \epsilon_{prot} \times d$, 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 with the above relations:

$$DOL = \frac{c(\text{dye})}{c(\text{protein})} = \frac{A_{max}/\epsilon_{max}}{A_{prot}/\epsilon_{prot}} = \frac{A_{max} \cdot \epsilon_{prot}}{(A_{280} - A_{max} \cdot CF_{280}) \cdot \epsilon_{max}}$$

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: Properties of available ATTO-dye maleimides:

Dye	Order #		MW	M ⁺	Δm	Δq	λ_{abs}	λ_{em}	ϵ_{max}	CF ₂₆₀	CF ₂₈₀	Solvent
	1 mg	5 mg										
ATTO 390	AD 390-41	AD 390-45	466	466	465.5	0	390	476	24000	0.46	0.09	DMF
ATTO 425	AD 425-41	AD 425-45	524	524	523.6	0	439	485	45000	0.19	0.17	DMF
ATTO 430LS	AD 430LS-31	AD 430LS-35	711	689	687.8	- 1	436	545	32000	0.32	0.22	DMF
ATTO 465	AD 465-41	AD 465-45	518	418	418.5	+ 1	453	506	75000	1.09	0.48	DMF
ATTO 488	AD 488-41	AD 488-45	1067	712	710.7	- 1	500	520	90000	0.22	0.09	DMF
ATTO 495	AD 495-41	AD 495-45	574	474	474.6	+ 1	498	526	80000	0.45	0.37	DMF
ATTO 490LS	AD 490LS-31	AD 490LS-35	818	796	795.0	- 1	498	658	40000	0.39	0.21	DMF
ATTO Rho110	AD Rho110-41	AD Rho110-45	652	552	552.6	+ 1	507	531	100000	0.21	0.14	DMF
ATTO 514	AD 514-41	AD 514-45	990	876	874.7	- 1	511	532	115000	0.21	0.07	DMF
ATTO 520	AD 520-41	AD 520-45	589	489	489.6	+ 1	517	538	110000	0.16	0.20	DMF
ATTO 532	AD 532-41	AD 532-45	1063	768	766.8	- 1	532	552	115000	0.20	0.09	DMF
ATTO Rho6G	AD Rho6G-41	AD Rho6G-45	750	636	636.7	+ 1	533	557	115000	0.19	0.16	DMF
ATTO 540Q	AD 540Q-41	AD 540Q-45	781	681	681.8	+ 1	543		105000	0.27	0.26	DMF
ATTO 542	AD 542-41	AD 542-45	1150	1036	1033.1	- 3	542	562	120000	0.18	0.08	DMF
ATTO 550	AD 550-41	AD 550-45	816	716	716.9	+ 1	554	576	120000	0.23	0.10	DMF
ATTO 565	AD 565-41	AD 565-45	733	633	632.7	0	564	590	120000	0.27	0.12	DMF
ATTO Rho3B	AD Rho3B-41	AD Rho3B-45	764	664	664.8	+ 1	566	589	120000	0.27	0.13	DMF
ATTO Rho11	AD Rho11-41	AD Rho11-45	788	688	688.8	+ 1	572	595	120000	0.26	0.10	DMF
ATTO Rho12	AD Rho12-41	AD Rho12-45	872	772	773.0	+ 1	577	600	120000	0.26	0.09	DMF
ATTO Thio12	AD Thio12-41	AD Thio12-45	724	624	624.8	+ 1	582	607	110000	0.11	0.37	DMF
ATTO Rho101	AD Rho101-41	AD Rho101-45	812	712	712.9	+ 1	587	609	120000	0.18	0.17	DMF
ATTO 575Q	AD 575Q-41	AD 575Q-45	833	733	733.8	+ 1	582		120000	0.29	0.12	DMF
ATTO 580Q	AD 580Q-41	AD 580Q-45	917	817	818.0	+ 1	587		110000	0.32	0.11	DMF
ATTO 590	AD 590-41	AD 590-45	813	713	712.8	0	593	622	120000	0.39	0.43	DMF
ATTO Rho13	AD Rho13-41	AD Rho13-45	868	768	769.0	+ 1	603	626	120000	0.28	0.43	DMF
ATTO 594	AD 594-41	AD 594-45	1358	928	927.1	- 1	603	626	120000	0.22	0.50	DMF
ATTO 610	AD 610-41	AD 610-45	613	513	513.7	+ 1	616	633	150000	0.03	0.06	ACN
ATTO 612Q	AD 612Q-41	AD 612Q-45	913	813	814.0	+ 1	615		115000	0.35	0.60	DMF
ATTO 620	AD 620-41	AD 620-45	734	634	634.8	+ 1	620	642	120000	0.04	0.06	DMF
ATTO Rho14	AD Rho14-41	AD Rho14-45	1006	906	906.8	+ 1	626	646	140000	0.26	0.47	DMF
ATTO 633	AD 633-41	AD 633-45	774	674	674.9	+ 1	630	651	130000	0.04	0.05	DMF
ATTO 643	AD 643-41	AD 643-45	1072	958	957.2	- 1	643	665	150000	0.05	0.04	DMF
ATTO 647	AD 647-41	AD 647-45	829	715	714.9	0	647	667	120000	0.08	0.04	ACN
ATTO 647N	AD 647N-41	AD 647N-45	868	768	769.0	+ 1	646	664	150000	0.06	0.05	DMF
ATTO 655	AD 655-41	AD 655-45	812	650	649.8	0	663	680	125000	0.24	0.08	DMF
ATTO Oxa12	AD Oxa12-41	AD Oxa12-45	875	761	762.0	+ 1	662	681	125000	0.32	0.12	DMF
ATTO 665	AD 665-41	AD 665-45	845	745	745.9	+ 1	662	680	160000	0.07	0.06	DMF
ATTO 680	AD 680-41	AD 680-45	1024	648	647.8	0	681	698	125000	0.30	0.17	DMF
ATTO 700	AD 700-41	AD 700-45	971	688	687.8	0	700	716	120000	0.26	0.41	DMF
ATTO 725	AD 725-41	AD 725-45	638	538	538.7	+ 1	728	751	120000	0.08	0.06	ACN
ATTO 740	AD 740-41	AD 740-45	690	590	590.8	+ 1	743	763	120000	0.07	0.07	ACN
ATTO MB2	AD MB2-41	AD MB2-45	591	478	478.5	+ 1	668		110000	0.08	0.24	ACN

MW: molecular weight of the dye including counterions in g/mol; M⁺: molecular weight of dye cation (HPLC_MS acetonitrile/water 0.1 vol-% trifluoroacetic acid); Δm : increase of molecular mass on conjugation with ATTO-dye maleimides; Δq : increase of electrical charge on conjugation with ATTO-dye maleimides; λ_{abs} : longest-wavelength absorption maximum in nm; λ_{em} : fluorescence maximum in nm; ϵ_{max} : molar decadic extinction coefficient at the longest-wavelength absorption maximum in M⁻¹ cm⁻¹; CF₂₆₀ = $\epsilon_{260}/\epsilon_{\text{max}}$; CF₂₈₀ = $\epsilon_{280}/\epsilon_{\text{max}}$

Note: Mass and, frequently, electrical charge of a biomolecule will be different after conjugation with a dye. The table shows the mass (Δm) and charge (Δq) increase that occur on coupling with an ATTO-dye maleimide. Because biomolecules as well as ATTO-dyes may carry basic (-NH₂) and acidic (-COOH, -SO₃H) 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 (-NH₂), and deprotonated acid groups (-COO⁻, -SO₃⁻). 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 Δm and Δq will be higher by one, compared to the values given in the table.

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