Introduction:

aladdin 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 $Na_2HPO_4 \cdot 2H_2O$, and 0.24 g KH_2PO_4, 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-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 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, dyeprotein 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

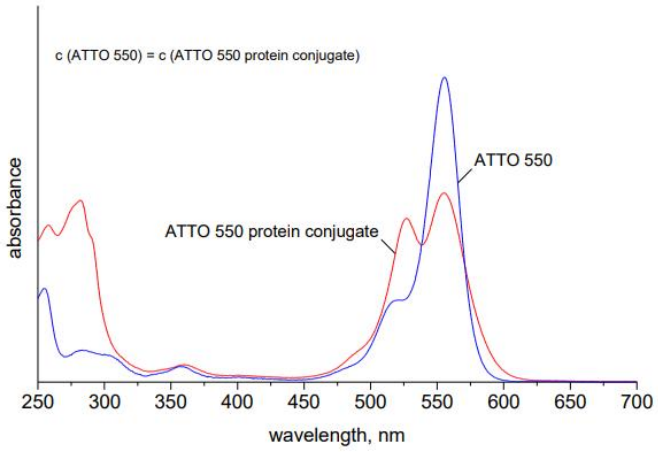
as much as possible.

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) × 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 (Amax) at the absorption maximum (abs) of the dye as well as the absorbance (A280) at 280 nm (absorption maximum of proteins). The concentration of bound dye is given by: c(dye) = Amax / 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 A280 must be corrected for the contribution of the dye. This is given by Amax × CF280. The values for the correction factor CF280 = ε 280 / ε max are listed in the table on p.4. It follows for the absorbance of the protein itself: Aprot = A280 − Amax × CF280. Then the concentration of protein is: c(protein) = Aprot / 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 with the above relations:

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

Note: The above equation is only valid if the extinction coefficient 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-d ye maleimides:

Dye	MW	M+	Δm	Δq	λ abs	λem	ε max	CF260	CF280	Solvent
ATTO 390	466	466	465.5	0	390	476	24000	0.46	0.09	DMF
ATTO 425	524	524	523.6	0	439	485	45000	0.19	0.17	DMF
ATTO 430LS	711	689	687.8	-1	436	545	32000	0.32	0.22	DMF
ATTO 465	518	418	418.5	1	453	506	75000	1.09	0.48	DMF
ATTO 488	1067	712	710.7	-1	500	520	90000	0.22	0.09	DMF
ATTO 495	574	474	474.6	1	498	526	80000	0.45	0.37	DMF
ATTO 490LS	818	796	795	-1	498	658	40000	0.39	0.21	DMF
ATTO Rho110	652	552	552.6	1	507	531	100000	0.21	0.14	DMF
ATTO 514	990	876	874.7	-1	511	532	115000	0.21	0.07	DMF
ATTO 520	589	489	489.6	1	517	538	110000	0.16	0.2	DMF
ATTO 532	1063	768	766.8	-1	532	552	115000	0.2	0.09	DMF
ATTO Rho6G	750	636	636.7	1	533	557	115000	0.19	0.16	DMF
ATTO 540Q	781	681	681.8	1	543		105000	0.27	0.26	DMF
ATTO 542	1150	1036	1033.1	-3	542	562	120000	0.18	0.08	DMF
ATTO 550	816	716	716.9	1	554	576	120000	0.23	0.1	DMF
ATTO 565	733	633	632.7	0	564	590	120000	0.27	0.12	DMF
ATTO Rho3B	764	664	664.8	1	566	589	120000	0.27	0.13	DMF
ATTO Rho11	788	688	688.8	1	572	595	120000	0.26	0.1	DMF
ATTO Rho12	872	772	773	1	577	600	120000	0.26	0.09	DMF
ATTO Thio12	724	624	624.8	1	582	607	110000	0.11	0.37	DMF
ATTO Rho101	812	712	712.9	1	587	609	120000	0.18	0.17	DMF
ATTO 575Q	833	733	733.8	1	582		120000	0.29	0.12	DMF
ATTO 580Q	917	817	818	1	587		110000	0.32	0.11	DMF
ATTO 590	813	713	712.8	0	593	622	120000	0.39	0.43	DMF
ATTO Rho13	868	768	769	1	603	626	120000	0.28	0.43	DMF
ATTO 594	1358	928	927.1	-1	603	626	120000	0.22	0.5	DMF
ATTO 612Q	913	813	814	1	615		115000	0.35	0.6	DMF
ATTO 620	734	634	634.8	1	620	642	120000	0.04	0.06	DMF
ATTO Rho14	1006	906	906.8	1	626	646	140000	0.26	0.47	DMF
ATTO 633	774	674	674.9	1	630	651	130000	0.04	0.05	DMF
ATTO 643	1072	958	957.2	-1	643	665	150000	0.05	0.04	DMF
ATTO 647	829	715	714.9	0	647	667	120000	0.08	0.04	ACN
ATTO 647N	868	768	769	1	646	664	150000	0.06	0.05	DMF
ATTO 655	812	650	649.8	0	663	680	125000	0.24	0.08	DMF
ATTO Oxa12	875	761	762	1	662	681	125000	0.32	0.12	DMF
ATTO 665	845	745	745.9	1	662	680	160000	0.07	0.06	DMF
ATTO 680	1024	648	647.8	0	681	698	125000	0.3	0.17	DMF
ATTO 700	971	688	687.8	0	700	716	120000	0.26	0.41	DMF
ATTO 725	638	538	538.7	1	728	751	120000	0.08	0.06	ACN
ATTO 740	690	590	590.8	1	743	763	120000	0.07	0.07	ACN
ATTO MB2	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; λabs: longest-wavelength absorption maximum in nm; λem: fluorescence maximum in nm; ε max: molar decadic extinction coefficient at the longest-wavelength absorption maximum in $M^{-1}cm^{-1}$; CF260 = ε260/ε max; CF280 = ε280/ε max