Introduction:

aladdin offers a large variety of high-quality amine-reactive dyes for labeling proteins and other aminecontaining substrates. The dyes cover the spectral region from 350 nm in the UV to 750 nm in the NIR. The most commonly used amine-reactive reagents are N-hydroxysuccinimidyl(NHS)-esters. NHS-esters readily react with compounds containing amino groups, forming a chemically stable amide bond between the dye and e.g. a protein. However, the amino group ought to be unprotonated to be reactive. Hence 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 unavoidable 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.

Labeling Proteins with ATTO 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 unprotonated to a degree sufficiently high for fast coupling.

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 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 an-hydrous, amine-free DMSO or acetonitrile. For detailed information on preparation and handling of dye stock-solutions see page 2.
- 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 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 2 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 dyedegradation.

Conjugate Preparation

• Dissolve 1 - 5 mg of protein in 1 ml of Solution C. Protein solutions must be free of any aminecontaining 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 an average 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 from the protein conjugate. We recommend using a Sephadex G-25 (or equivalent) gel filtration column of 1 - 2 cm diameter and 10 - 20 cm length. For very hydrophilic dyes, e. g. ATTO 488, ATTO 532, ATTO 542, ATTO 594, ATTO 643 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 NHSester).
• 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

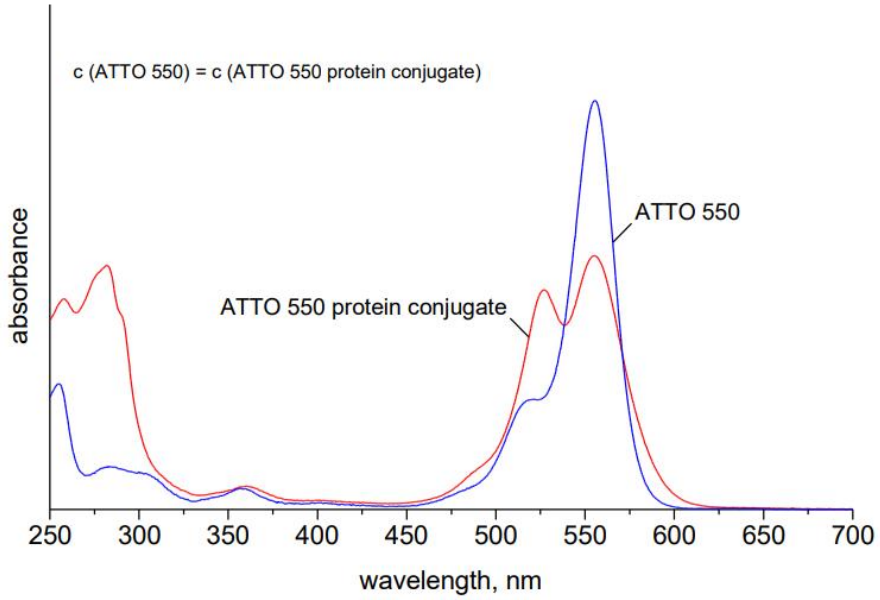
In general, conjugates should be stored under the same conditions used for the unlabeled protein. For storage in solution at 4 ° C, sodium azide (2 mM final concentration) can be added as a preservative. Removal of preservatives prior to use may be necessary to avoid inhibitory effects in applications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

The average degree of labeling (DOL, dye-to-protein ratio) 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 (UVtransparent) 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 (λabs) 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-dye NHS-esters

Dye-NHS	Solvent	MW	M^+	Δm	Δq	λabs	λem	εmax	CF_{260}	CF_{280}
ATTO 390	DMSO	440	441	325.4	0	390	476	24000	0.46	0.09
ATTO 425	DMSO	498	499	383.4	0	439	485	45000	0.19	0.17
ATTO 430LS	DMSO	686	664	547.7	-1	436	545	32000	0.32	0.22
ATTO 465	DMSO	493	393	278.4	1	453	506	75000	1.09	0.48
ATTO 488	DMSO	981	687	570.6	-1	500	520	90000	0.22	0.09
ATTO 495	DMSO	549	449	334.4	1	498	526	80000	0.45	0.37
ATTO 490LS	DMSO	793	771	654.8	-1	498	658	40000	0.39	0.21
ATTO Rho110	DMSO	627	527	412.5	1	507	531	100000	0.21	0.14
ATTO 514	DMSO	1111	851	734.6	-1	511	532	115000	0.21	0.07
ATTO 520	DMSO	564	464	349.5	1	517	538	110000	0.16	0.2
ATTO 532	DMSO	1081	743	626.7	-1	532	552	115000	0.2	0.09
ATTO Rho6G	DMSO	711	611	496.6	1	533	557	115000	0.19	0.16
ATTO 540Q	DMSO	756	656	541.6	1	543		105000	0.27	0.26
ATTO 542	DMSO	1125	1011	893	-3	542	562	120000	0.18	0.08
ATTO 550	DMSO	791	691	576.8	1	554	576	120000	0.23	0.1
ATTO 565	DMSO	708	608	492.6	0	564	590	120000	0.27	0.12
ATTO Rho3B	DMSO	642	639	524.7	1	566	589	120000	0.27	0.13
ATTO Rho11	DMSO	763	664	548.7	1	572	595	120000	0.26	0.1
ATTO Rho12	DMSO	847	747	632.9	1	577	600	120000	0.26	0.09
ATTO Thio12	DMSO	699	600	484.6	1	582	607	110000	0.11	0.37
ATTO Rho101	DMSO	787	687	572.7	1	587	609	120000	0.18	0.17
ATTO 575Q	DMSO	808	708	591.7	1	582		120000	0.29	0.12
ATTO 580Q	DMSO	892	792	677.9	1	587		110000	0.32	0.11
ATTO 590	DMSO	788	688	572.7	0	593	622	120000	0.39	0.43
ATTO Rho13	DMSO	843	743	628.8	1	603	626	120000	0.28	0.43
ATTO 594	DMSO	1389	903	786.9	-1	603	626	120000	0.22	0.5
ATTO 610	ACN	588	488	373.5	1	616	633	150000	0.03	0.06
ATTO 612Q	DMSO	888	788	673.8	1	615		115000	0.35	0.6
ATTO 620	DMSO	709	609	494.7	1	620	642	120000	0.04	0.06
ATTO Rho14	DMSO	981	881	766.6	1	626	646	140000	0.26	0.47
ATTO 633	DMSO	749	649	534.7	1	630	651	130000	0.04	0.05
ATTO 643	DMSO	955	933	817.1	-1	643	665	150000	0.05	0.04
ATTO 647	ACN	811	690	574.8	0	647	667	120000	0.08	0.04
ATTO 647N	DMSO	843	743	628.9	1	646	664	150000	0.06	0.05
ATTO 655	DMSO	887	625	509.6	0	663	680	125000	0.24	0.08
ATTO Oxa12	DMSO	835	736	621.9	1	662	681	125000	0.32	0.12
ATTO 665	DMSO	820	720	605.7	1	662	680	160000	0.07	0.06
ATTO 680	DMSO	828	623	507.6	0	681	698	125000	0.3	0.17
ATTO 700	DMSO	837	663	547.7	0	700	716	120000	0.26	0.41
ATTO 725	ACN	613	513	398.5	1	728	751	120000	0.08	0.06
ATTO 740	ACN	665	565	450.6	1	743	763	120000	0.07	0.07
ATTO MB2	ACN	553	453	338.4	1	668		110000	0.08	0.24

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 NHS-ester; Δq: increase of electrical charge on conjugation with ATTO-dye NHS-ester; 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

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 NHS-ester. 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 ($-NH_2$), and deprotonated acid groups ($-COO^-$, $-SO_3^-$). 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.