

## 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<sub>2</sub>HPO<sub>4</sub> • 2 H<sub>2</sub>O, and 0.24 g KH<sub>2</sub>PO<sub>4</sub>, 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)phosphine (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.

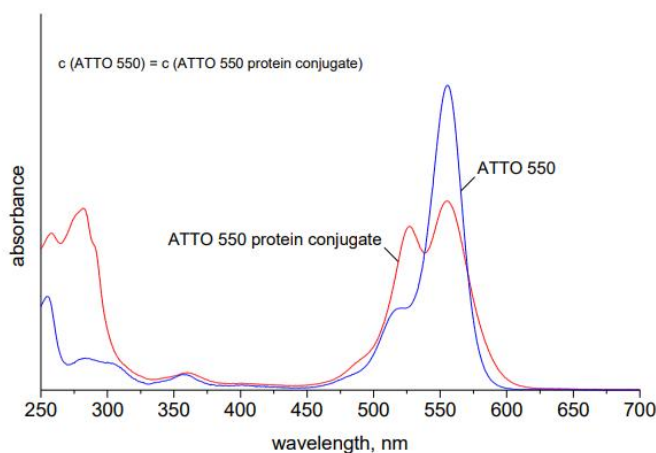
Avoid repeated freezing and thawing. Protect dye conjugates from light as much as possible.

### 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 ( $\epsilon$ )  $\times$  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 (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  $\epsilon_{max}$  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  $\epsilon_{prot}$  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  $\epsilon_{max}$  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*   | $\Delta m$ | $\Delta q$ | $\lambda$<br>abs | $\lambda$ em | $\epsilon$ 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);  $\Delta m$ : increase of molecular mass on conjugation with ATTO-dye maleimides;  $\Delta q$ : increase of electrical charge on conjugation with ATTO-dye maleimides;  $\lambda_{abs}$ : longest-wavelength absorption maximum in nm;  $\lambda_{em}$ : fluorescence maximum in nm;  $\epsilon_{max}$ : molar decadic extinction coefficient at the longest-wavelength absorption maximum in  $M^{-1}cm^{-1}$ ;  $CF_{260} = \epsilon_{260} / \epsilon_{max}$ ;  $CF_{280} = \epsilon_{280} / \epsilon_{max}$