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.

Among the most frequently used thiol-reactive reagents are haloalkyl derivatives such as iodoacetamides 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 iodoacetamide is pH 8 - 8.5. At this pH the thiol group is deprotonated to a sufficient degree to react with the dye-iodoacetamide. Note that iodoacetamides are not as thiol-selective as maleimides as they might react with histidine or methionine.

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

Required Materials

• **Solution A**: PBS buffer (phosphate-buffered saline, pH 7.4): Dissolve 8 g/l NaCl (137 mM), 0.2 g/l KCl (2.7 mM), 1.44 g/l Na2HPO4 • 2 H2O (8 mM), and 0.24 g/l KH2PO4 (1.8 mM), in 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-iodoacetamide in 50 - 200 µl of anhydrous, amine-free DMSO or DMF. Please note that iodoacetamide solutions are not stable for a long period of time. Only prepare as much dye solution as is needed immediately prior to use. In addition, iodoacetamide are extremely light-sensitive and solutions must be protected from irradiation as much as possible. More information on the preparation and handling of dye stock-solution can be found on page 2.

• **Solution F:** Dissolve 1.0 mg of dye-iodoacetamide in 50 - 200 µl of anhydrous, amine-free DMF. For the preparation and handling of stock-solutions see page 2. 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.

• Stabilizer to prevent denaturation after elution: bovine serum albumin (BSA).

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 iodoacetamide functionality and consequently reduce coupling efficiency.

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-iodoacetamide 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. This may necessitate optimization of the dye-to-protein ratio used in the reaction to obtain the desired DOL.

• 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 for the conjugation to complete.

Note: If the protein contains disulfide bonds it may be disireable to reduce the disulfide before labeling. For reduction, reagents such as tris(2-carboxyethyl)phosphin (TCEP) or dithiothreitol (DTT) may be used. However, one has to take into account that an excess of these reducing agents has to be removed (e.g. with dialysis) prior to conjugation.

Conjugate Purification – Removal of Unbound Dye

• The unreacted iodoacetamide and the hydrolyzed iodoacetamide must be removed from the labeled protein. We recommend using a Sephadex G-25 (or equivalent) gel filtration column (1 - 2 cm diameter and 10 - 20 cm length; for very hydrophilic dyes, e. g. ATTO 488, ATTO 514, ATTO 532, ATTO 594, a 30 cm column is preferable) for separation of dyeprotein conjugate from free dye.

• 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 iodoacetamide.
• To prevent denaturation of the conjugate after elution, add bovine serum albumin (BSA) or any other stabilizer of choice to a final concentration of 1 – 10 mg/ml.

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 from light. We recommend to centrifuge conjugate solutions in a micro-centrifuge before use. This step will remove any aggregates that may have formed during longterm storage.

Table 1: Properties of ATTO-dye labeled iodoacetamides:

Dye	MW	M^+	Δm	Δq	λabs	λem	εmax	CF_{260}	CF_{280}
ATTO 390	553	554	425.5	0	390	476	24000	0.46	0.09
ATTO 488	914	800	670.7	-1	500	520	90000	0.22	0.09
ATTO 514	1078	964	834.8	-1	511	532	115000	0.21	0.07
ATTO 532	970	856	726.8	-1	532	553	115000	0.22	0.11
ATTO 550	918	804	678.9	1	554	576	120000	0.23	0.1
ATTO 565	835	721	593.7	0	563	590	120000	0.27	0.12
ATTO 590	971	857	673.8	0	593	622	120000	0.39	0.43
ATTO 594	1129	1016	831.9	-1	603	626	120000	0.22	0.5
ATTO 633	876	762	634.8	1	630	651	130000	0.04	0.05
ATTO 647N	956	856	729	1	646	664	150000	0.04	0.03
ATTO 655	852	738	610.8	0	663	680	125000	0.24	0.08
ATTO 680	850	736	608.7	0	681	698	125000	0.3	0.17

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-Label; Δq: increase of electrical charge on conjugation with ATTO-Label; λabs: longest wavelength absorption maximum in nm; λem: fluorescence maximum in nm; εmax: molar decadic extinction coefficient at the longest-wavelength absorption maximum in $M^{-1}cm^{-1}$; $CF_{260} = \varepsilon 260/\varepsilon max$; $CF_{280} = \varepsilon 280/\varepsilon max$.