

ATTO 594 maleimide

Catalog number: 2861
Unit size: 1 mg

Component	Storage	Amount (Cat No. 2861)
ATTO 594 maleimide	Freeze (< -15 °C), Minimize light exposure	1 mg

OVERVIEW

ATTO 594 is a bright, red fluorescent dye characterized by a strong absorption, high fluorescence quantum yield, and exceptional thermal and photostability. The dye exhibits superior water solubility and hydrophilicity, facilitating its use in various aqueous environments. ATTO 594 is optimally excited within the 560-615 nm range, making it compatible with both 561 nm and 594 nm laser lines commonly used in advanced fluorescence imaging systems. Upon conjugation to biomolecules, ATTO 594 becomes anionic, carrying a net charge of -1, which may influence its binding characteristics and performance in assays. Its photostability and brightness make it particularly suited for high-resolution techniques like single-molecule detection and super-resolution microscopy, including PALM, dSTORM, and STED. Additionally, ATTO 594 is highly compatible with flow cytometry (FACS), fluorescence *in situ* hybridization (FISH), and various other fluorescence-based assays, supporting its broad utility in complex biological studies.

The maleimide derivative of ATTO 594 is widely used for labeling biomolecules with free thiol (SH) groups, including antibodies, proteins, thiol-modified oligonucleotides, and low molecular weight ligands. Maleimides react readily with sulfhydryl groups, forming stable thio-ether bonds between the dye and the biomolecule, facilitating robust and reliable labeling for diverse experimental applications.

PREPARATION OF STOCK SOLUTIONS

Unless otherwise noted, all unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles

ATTO 594 maleimide stock solution (Solution B)

Add anhydrous DMSO into the vial of ATTO 594 maleimide to make a 10 mM stock solution. Mix well by pipetting or vortex.

Note: Prepare the dye stock solution (Solution B) before starting the conjugation. Use promptly. Extended storage of the dye stock solution may reduce the dye activity. Solution B can be stored in the freezer for up to 4 weeks when kept from light and moisture. Avoid freeze-thaw cycles.

Protein stock solution (Solution A)

Mix 100 µL of a reaction buffer (e.g., 100 mM MES buffer with pH ~6.0) with 900 µL of the target protein solution (e.g. antibody, protein concentration >2 mg/mL if possible) to give 1 mL protein labeling stock solution.

Note: The pH of the protein solution (Solution A) should be 6.5 ± 0.5.

Note: Impure antibodies or antibodies stabilized with bovine serum albumin (BSA) or other proteins will not be labeled well.

Note: The conjugation efficiency is significantly reduced if the protein concentration is less than 2 mg/mL. For optimal labeling efficiency, the

final protein concentration range of 2-10 mg/mL is recommended.

Optional: if your protein does not contain a free cysteine, you must treat your protein with DTT or TCEP to generate a thiol group. DTT or TCEP are used for converting a disulfide bond to two free thiol groups. If DTT is used you must remove free DTT by dialysis or gel filtration before conjugating a dye maleimide to your protein. Following is a sample protocol for generating a free thiol group:

1. Prepare a fresh solution of 1 M DTT (15.4 mg/100 µL) in distilled water.
2. Make IgG solution in 20 mM DTT: add 20 µL of DTT stock per ml of IgG solution while mixing. Let stand at room temp for 30 minutes without additional mixing (to minimize reoxidation of cysteines to cystines).
3. Pass the reduced IgG over a filtration column pre-equilibrated with "Exchange Buffer". Collect 0.25 mL fractions off the column.
4. Determine the protein concentrations and pool the fractions with the majority of the IgG. This can be done either spectrophotometrically or colorimetrically.
5. Carry out the conjugation as soon as possible after this step (see Sample Experiment Protocol).

Note: IgG solutions should be >4 mg/mL for the best results. The antibody should be concentrated if less than 2 mg/mL. Include an extra 10% for losses on the buffer exchange column.

Note: The reduction can be carried out in almost any buffers from pH 7-7.5, e.g., MES, phosphate, or TRIS buffers.

Note: Steps 3 and 4 can be replaced by dialysis.

SAMPLE EXPERIMENTAL PROTOCOL

This labeling protocol was developed for the conjugate of Goat anti-mouse IgG with ATTO 594 maleimide. You might need further optimization for your particular proteins.

Note: Each protein requires a distinct dye/protein ratio, which also depends on the properties of dyes. Over-labeling of a protein could detrimentally affect its binding affinity while the protein conjugates of low dye/protein ratio give reduced sensitivity.

Run conjugation reaction

1. Use a 10:1 molar ratio of Solution B (dye)/Solution A (protein) as the starting point: Add 5 µL of the dye stock solution (Solution B, assuming the dye stock solution is 10 mM) into the vial of the protein solution (95 µL of Solution A) with effective shaking. The concentration of the protein is ~0.05 mM assuming the protein concentration is 10 mg/mL, and the molecular weight of the protein is ~200KD.

Note: We recommend using 10:1 molar ratio of Solution B (dye)/Solution A (protein). If it is too less or too high, determine the optimal dye/protein ratio at 5:1, 15:1, and 20:1, respectively.

2. Continue to rotate or shake the reaction mixture at room

temperature for 30-60 minutes.

Purify the conjugation

The following protocol is an example of dye-protein conjugate purification by using a Sephadex G-25 column.

1. Prepare Sephadex G-25 column according to the manufacture instruction.
2. Load the reaction mixture (From "Run conjugation reaction") to the top of the Sephadex G-25 column.
3. Add PBS (pH 7.2-7.4) as soon as the sample runs just below the top resin surface.
4. Add more PBS (pH 7.2-7.4) to the desired sample to complete the column purification. Combine the fractions that contain the desired dye-protein conjugate.

Note: For immediate use, the dye-protein conjugate needs to be diluted with staining buffer, and aliquoted for multiple uses.

Note: For longer-term storage, the dye-protein conjugate solution needs to be concentrated or freeze-dried.

Characterize the Desired Dye-Protein Conjugate

The Degree of Substitution (DOS) is the most important factor for characterizing dye-labeled protein. Proteins of lower DOS usually have weaker fluorescence intensity, but proteins of higher DOS tend to have reduced fluorescence too. The optimal DOS for most antibodies is recommended between 2 and 10, depending on the properties of dye and protein. For effective labeling, the degree of substitution should be controlled to have 5-8 moles of ATTO 594 maleimide to one mole of antibody. The following steps are used to determine the DOS of ATTO 594 maleimide labeled proteins.

Measure absorption

To measure the absorption spectrum of a dye-protein conjugate, it is recommended to keep the sample concentration in the range of 1-10 μM depending on the extinction coefficient of the dye.

Read OD (absorbance) at 280 nm and dye maximum absorption ($\lambda_{\text{max}} = 602 \text{ nm}$ for ATTO 594 dyes)

For most spectrophotometers, the sample (from the column fractions) needs to be diluted with de-ionized water so that the OD values are in the range of 0.1 to 0.9. The O.D. (absorbance) at 280 nm is the maximum absorption of protein, while 602 nm is the maximum absorption of ATTO 594 maleimide. To obtain accurate DOS, make sure that the conjugate is free of the non-conjugated dye.

Calculate DOS

You can calculate DOS using our tool by following this link: <https://www.aatbio.com/tools/degree-of-labeling-calculator>

EXAMPLE DATA ANALYSIS AND FIGURES

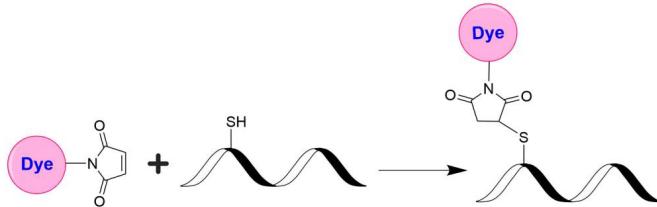


Figure 1. Fluorescent dye maleimides are the most popular tool for conjugating dyes to a peptide, protein, antibody, thiol-modified oligonucleotide, or nucleic acid through their SH group. Maleimides

react readily with the thiol group of proteins, thiol-modified oligonucleotides, and other thiol-containing molecules under neutral conditions. The resulting dye conjugates are quite stable.