USER GUIDE

AQuora® Maleimide Dyes

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AQuora® Maleimide dyes are thiol-reactive fluorophores engineered with SuperHydrophilic™ technology designed to improve solubility during labeling and of the dye-labeled conjugate. As a result, dye-labeled conjugates made with AQuora® Maleimide dyes yield enhanced signal and signal-to-noise ratios in fluorescence-based applications, including fluorescent western blotting, fluorescence-based microscopy, flow cytometry, and cell-based assays.

Example Conjugation Reaction

- AQuora® Maleimide dyes can be conjugated to many proteins or peptides through cysteine thiol groups. The maleimide group reacts with thiols to form thioether bonds. Maleimide reagents are moisture-sensitive, and the dyes are light- and oxygen-sensitive. Therefore, protect the dye from light and keep it blanketed with dry, inert gas, such as nitrogen or argon, whenever it is open or in use.

General Protocol for Dye Labeling of Antibody

Prepare the antibody for labeling

- The optimal antibody concentration for labeling is 5-10 mg/mL. When using lower antibody concentrations, the amount of dye added to the labeling reaction may have to be increased to obtain an optimal degree of labeling (DOL) or fluor-to-protein (F/P) labeling ratio.

1. Prepare the thiol-containing protein in a neutral pH buffer, such as 10-100 mM phosphate or Tris buffer, pH 7-7.5, at a 5-10 mg/mL concentration. Optionally, the buffers may also be made to contain 0.15 M NaCl. Buffers should be degassed under vacuum to remove excess dissolved oxygen because sulfhydryl groups can rapidly oxidize to disulfides upon oxidation. Proteins containing disulfides but no native free thiol groups may be reduced with a limiting amount of a reducing agent such as DTT or TCEP. Alternatively, thiols may be created on proteins using a thiolation reagent (such as a dPEG®-SPDP compound or a dPEG®-SATA reagent).

2. If any precipitate (cloudiness or aggregate material) is visibly present, filter the protein stock solution through a 0.2 µm syringe filter.

3. Determine the protein or antibody concentration using the following formula. To measure the antibody's absorbance at 280 nm (A_{280}), a small aliquot of the protein solution may have to be diluted with buffer to keep the absorbance within the spectrophotometer's linear range.

   Antibody concentration (mg/mL) = [(A_{280})(dilution factor)] / (extinction coefficient of the antibody)

   Where the dilution factor is the amount that a small sample of the antibody solution had to be diluted to measure its absorbance. For instance, if 10 µL of the antibody solution was diluted with 90 µL of buffer to measure the A_{280} nm, then the dilution factor is 10 (because 10 µL was diluted up to 100 µL).

   And where the extinction coefficient (ε_{IgG} in $cm^{-1}M^{-1}$) is the absorbance at 280 nm of a 1 mg/mL concentration of the antibody (using a 1 cm path length). For many polyclonal antibodies, the extinction coefficient is approximately 1.4. If your antibody gives a different extinction coefficient, then replace this value with your determined value.

4. Determine amount of antibody to label (mg) using the follow formula:

   mg of antibody = (volume of antibody solution, in mL)(antibody concentration, in mg/mL)

5. Adjust the antibody concentration to approximately 10 mg/mL by adding reaction buffer or concentrating the solution as needed.

Prepare the dye stock solution

1. Before opening, bring the vial containing the dye to room temperature to prevent moisture condensation. Protect from light. While working with the dye, keep it blanketed under an inert atmosphere such as nitrogen or argon.

2. Most users will want to work with the dye as a stock solution in a dry, water-miscible organic solvent. Nearly all AQuora® dyes are soluble in dimethyl sulfoxide (DMSO), N,N'-dimethylacetamide (DMAC), or N,N'-dimethylformamide (DMF). Dye solutions with a 10 mg/mL concentration may be conveniently prepared. To prepare the dye solution, add 100 µL of anhydrous solvent to the vial containing 1 mg of reactive dye.

   Anhydrous solvents are prepared by allowing the solvent to stand over activated 3 Å molecular sieves at room temperature for a minimum of 72 hours before use. To activate 3 Å molecular sieves, bake them for 16 hours under vacuum at 250°C. Once the vacuum is released, transfer the sieves at once to an inert atmosphere (nitrogen or argon) and allow them to cool to room temperature. While under an inert atmosphere, transfer the sieves to suitable containers for solvent treatment or storage. The solvent to be dried should be added to the room-temperature sieves under an inert atmosphere once they are in suitable containers. Please note that the long-term stability of dyes in solvent is not guaranteed. Furthermore, reactive dyes stored in wet organic solvents will likely lose activity quickly due to hydrolysis.

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3. Determine the molar concentration of the dye stock solution according to the following equation:

   Molar concentration of dye (M) = [(mg dye) / (mL solvent)] / (MW of dye in g/mol)

Determine the volume of dye to be used for labeling

- The mole excess of dye over the antibody used in the reaction for the optimal final degree of labeling (DOL) should be analytically determined. In general, the mole excess depends upon the concentration of the protein, the pH of the reaction conditions, the reaction time, and the number of available thiols. Thiols are usually present in a limited amount compared to the number of amines on proteins, so the optimal mole excess of a thiol-reactive dye over the protein may be different than that used for amine-reactive dyes. The optimal mole excess of dye should be experimentally determined for labeling antibodies prepared at a concentration of 5-10 mg/mL. For efficient labeling of all the thiols present, the mole excess of dye should be in the range of 3-6 times more than the number of thiols per protein or antibody. More dilute antibody solutions may require greater amounts of dye to obtain the same DOL.

1. Calculate the appropriate volume of dye solution to add per mg of antibody according to the following equations:

   Mole IgG = (g IgG) / (150,000 g/mol)

   where the MW of an intact polyclonal IgG antibody is approximately 150,000 g/mol. When labeling monoclonal or recombinant antibodies, antibody fragments, or other proteins, adjust the MW to match that of the protein being labeled.

   Moles of thiols present = (moles IgG)(number of thiols/IgG)

   Mole excess of dye desired in reaction per mole of thiols: _____

   Moles of label required = (moles thiols)(mol excess of dye desired)

   Volume of label needed (µL) = (mol label required) / [(M of dye solution)(10^6 µL/L)]

Reaction conditions

1. Add the calculated volume of the AQuora® dye to the antibody or protein solution *while* gently vortexing for rapid dissolution. Cap the tube and gently vortex again for ~30 seconds.

2. Allow the conjugation reaction to gently rock for at least 2 hours at room temperature. Longer reaction times may increase the DOL and yield of the reaction; however, the time of the reaction should be controlled to obtain similar DOL's from batch to batch. A 25°C constant temperature water bath may be used with periodic mixing to enhance the reproducibility of labeling results.

Purification

- The purification method should be adjusted based upon the quantity of antibody to be labeled and the reaction volume.

1. Prepare a Sephadex® G-50 (or equivalent) size exclusion support (trademark: Cytiva Bioprocess R&D AB) in a purification buffer appropriate for the final application or use 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2.

2. Pack the hydrated G-50 support into a suitable column, ensuring that the packed gel has a volume 20 times larger than the reaction sample volume to be purified. Equilibrate the packed gel with 3 bed volumes of purification buffer.

3. Gently load the sample onto the top of the gel bed and start the UV monitoring system, if available.

4. Elute the dye-labeled protein using the purification buffer by connecting the column to a solvent reservoir to maintain continuous flow through the column. Collect appropriately sized fractions as the buffer flows through the column.

5. Collect and pool together the early-eluting, colored fractions corresponding to the first peak, which is the dye-labeled protein. The broad second peak is the excess dye, which may be discarded as aqueous waste.

   Alternative Method: For small volumes of labeling reactions, remove excess dye from labeled antibodies using an Amicon® Ultra 50K MWCO 0.5 mL spin concentrator (trademark: Merck KGaA, Darmstadt, Germany) and wash with ~6 washes of purification buffer by repeatedly diluting and concentrating the labeled antibody.

Analysis of Labeled Antibody

- The non-reacted excess dye must be completely removed for accurate quantitation of labeled antibody concentration and DOL (or the fluor-to-protein (F/P) ratio).

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1. Dilute a small amount of the labeled protein in the purification buffer to measure its absorbance properties. It is important to dilute the sample enough to get the measurement of the A_{max} of the dye within the linear range of the spectrophotometer.

   Recommended dilution factor: 1:49 (which is a 1→50 dilution) to 1:99 (or 1→100)

2. Measure the absorbance at 280 nm (A_{280}) and at the A_{max} of the dye using a 1 cm quartz cuvette.

3. Calculate the concentration of antibody using the following formulas and the values determined above:

   Concentration of diluted antibody (M) = $[A_{280}-(A_{max})(CF)] / \varepsilon$IgG at 280 nm

   Where CF is the A_{280} correction factor of the dye ($CF = A_{280}/A_{max}$) (see above under Label Information for the specific dye CF value), and

   ε_{IgG} at 280 nm = 205,800 $cm^{-1}M^{-1}$

   This value represents the extinction coefficient for a typical polyclonal antibody. If the sample is not a polyclonal antibody, then replace this value with the extinction coefficient appropriate for the antibody or protein being labeled.

   Diluted antibody concentration (mg/mL) = (Molarity, mmol/mL) (MW_{IgG}, mg/mmol)

   Where MW_{IgG} = 150,000 mg/mmol

   Original antibody sample concentration (mg/mL) = (diluted antibody conc., in mg/mL)(DF)

   Where DF is the dilution factor used to measure the absorbance

4. Calculate the degree of labeling (in moles of dye per moles of antibody) using the following formula:

   Degree of Labeling (DOL) = $[(A_{max})(DF)] / [(\varepsilon_{dye}\ cm^{-1}M^{-1})$(antibody conc., M)]

Target Degree of Labeling

- The optimal DOL should be determined based on the antibody or protein being labeled and its intended application. For many antibodies used in immunoassays, a final DOL of 4-7 dyes per antibody yields a maximal signal and signal-to-noise ratio.

Problem	Cause	Solution
The protein was not labeled or was insufficiently labeled	The maleimide has hydrolyzed and is non-reactive.	Solutions containing maleimide reactive groups can be prepared in aqueous buffers at or slightly below pH 7, but they should not be stored for long periods in such buffers.
	The conjugation buffer contained free thiol groups or reducing agents (i.e., TCEP) that interfere with the reaction.	Maleimide groups react rapidly with free thiols and TCEP. Remove all interfering substances from the conjugation buffer.
	The conjugation buffer contained free amines (e.g., glycine, imidazole).	Maleimides react with free amines at higher pH. Therefore, remove free amines from your buffer.
	Too low of molar excess in the reaction and/or protein concentration was too dilute.	Increase the molar excess of reactive dye and prepare the protein at a concentration of ~5-10 mg/mL. The more dilute the protein, the higher the molar excess of dye necessary to reach the target degree of labeling.
	The protein is not sufficiently reduced to expose all thiols.	Try reducing the protein more extensively.
	The protein does not have many surface-accessible free thiols.	Try modifying the protein with a thiolation reagent to create thiols or use a different reactive group on the dye (*e.g.*, NHS) to label the protein.
	The protein was not modified sufficiently with a thiolation reagent to create free thiols.	Use a higher mole excess of thiolation reagent to the protein to be modified.
	The thiol groups on the protein oxidized to disulfides before the labeling reaction was complete.	Degas all buffers to remove excess dissolved oxygen and include at least 10 mM EDTA in all buffers to prevent metal-catalyzed oxidation.
The degree of labeling is higher than anticipated	Insufficient removal of excess dye	Separate labeled proteins by size exclusion chromatography. Passive dialysis is not recommended for the removal of excess reactive dyes.
	Conjugation buffer pH ≥7.5.	At pH at or above 7.5, maleimides will react competitively with free amines and thiols. In proteins with lots of surface amines, this leads to too much labeling. Keep the conjugation buffer at or slightly below pH 7.
Detection of dye-labeled protein was low or absent	Instrument settings are not optimal or set for specific fluor	Ensure instrument specifications for excitation and emission are appropriate for the fluor.
	Protein was insufficiently labeled	See solutions above
	Protein over-labeled causing fluorophore quenching and/or inhibition of binding capacity of the protein	Optimize reaction conditions for a degree of labeling appropriate for the assay

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