Quanta BioDesign, Ltd
7500 Montgomery Drive,
Plain City, Ohio 43064

Office 614 792.2958
Fax 614 760.9781

QuantaBioDesign.com

AQ LIFE SCIENCES
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Product Number: AQ-12019LF

AQuora® PB-Maleimide

AQuora® PB-Maleimide is a thiol-reactive fluorophore engineered with SuperHydrophilic™ technology designed to improve solubility during labeling and of the dye labeled conjugate. As a result, the dye labeled conjugates made with AQuora® PB-Maleimide yields enhanced signal and signal-to-noise ratios in fluorescence-based applications including fluorescent western blotting, fluorescence-based microscopy, flow cytometry, and cell-based assays. AQuora® PB-Maleimide is a coumarin-based dye with photophysical properties comparable to Pacific Blue.

This AQuora® PB-Maleimide dye is activated with a maleimide (MAL) group which reacts with a thiol to form a thioether bond.

Alexa Fluor® and DyLight® are registered trademarks of Thermo Fisher Scientific.

Product Number:	**AQ-12019LF**
Unit Size:	1 mg
Format:	Solid
Concentration:	Refer to the lot-specific Certificate of Analysis for the dye concentration.
Storage and Handling:	Store at -20°C. Protect from light. May ship at ambient temperatures.
Instructions for Use:	Equilibrate dye solution to room temperature before opening. For its use in labeling reactions, follow the guidelines in the example protocol below.

Label Information	**AQuora® PB**
Reactive Group:	Maleimide
Molecular Weight:	940 g/mol
Extinction Coefficient (-M-cm):	19,000 $cm^{-1}M^{-1}$
A_{280} Correction Factor:	0.400
Excitation / Emission Maximum (nm):	404 nm/458 nm ± 4 nm
Spectrally Similar Dyes:	Pacific Blue

Example Conjugation Reaction

AQuora® PB-Maleimide is a thiol-reactive dye that can be conjugated to many proteins or peptides through cysteine thiol groups. The maleimide reacts with thiols to form thioether bonds. Maleimide reagents are moisture sensitive, and the dyes are light and oxygen sensitive; therefore, store the dye solution protected from light and blanketed with inert gas, such as nitrogen or argon.

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.

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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 concentration of 5-10 mg/ml. The buffers may also be made to optionally contain 0.15 M NaCl. Buffers should be degassed under vacuum to remove excess dissolved oxygen because sulfhydryls 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 disulfide reducing agent (such as DTT or TCEP) or 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. A small aliquot of the protein solution may have to be diluted with buffer to measure the A_{280} nm in order to keep the absorbance within the linear range of the spectrophotometer.

   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. Bring the vial containing the dye (either supplied as a solid or as a DMAC solution) to room temperature to prevent moisture condensation upon opening. Protect from light. If the dye is supplied as a DMAC solution, use the concentration provided on the lot-specific certificate of analysis and skip to the next section.

2. If the dye is supplied as a solid, add anhydrous solvent [dimethyl sulfoxide (DMSO), dimethylacetamide (DMAC) or dimethylformamide (DMF)] and mix by vortexing. For a 10 mg/ml stock concentration, add 100 µl of anhydrous solvent to the vial containing 1 mg of reactive dye.

   To make the solvent anhydrous for this purpose, it should be treated with a molecular sieve which prior to use had been incubated at 250°C for 16 hours under vacuum to remove residual water and to fully activate the sieves, then cooled to room temperature. Reactive dyes prepared in an anhydrous solvent, protected from light, and sealed for storage at -20°C, are stable for at least 2 months. Do not store reactive dyes prepared in anhydrous solvent for long periods as stability

   cannot be assured. Reactive dyes dissolved in solvents that contain residual water will lose NHS

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ester activity due to hydrolysis.

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 is dependent upon the concentration of the protein, the pH of the reaction conditions, the time of the reaction, and 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 solutions of antibody 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 AQ® 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

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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 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 the appropriate 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 of the buffer to maintain continuous flow through the column. Collect appropriately sized fractions as the flow of buffer comes 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 Ultracel 50K MWCO 0.5 ml spin concentrator 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).

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 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 ($CF = A_{280nm}/A_{max}$) (see above under Label Information for the specific dye CF value), and

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

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

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.

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Troubleshooting

Problem	Cause	Solution
The protein was not labeled; 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 AQuora® 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 AQuora® 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 AQuora® 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