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A DIVISION OF QUANTA BIODESIGN LIMITED

Product Number: 71-0001-0309

AQuora® Fluorescein-Maleimide

AQuora® Fluorescein - Maleimide is a thiol reactive fluorochome engineered to incoprorate our SuperHydrophilic technology designed to improve solubility during labeling and of the final dye labeled conjugate. As a result, AQuora® Fluorescein labeled conjugates result in 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® Fluorescein maintains photophysical properties of spectrally comparable dyes (FITC and carboxyfluorescein).
This AQuora® Fluorescein is activated with a maleimide (MAL) functional group which reacts with thiols to form thioethers.

Product Number:	**71-0001-0309**
Unit Size:	1 mg
Format:	Lyophilized
Storage and Handling:	Store at -20C. Protect from light. May ship at ambient temperatures.
Instructions for Use:	Equilibrate dye to room temperature before opening. Dissolve the reactive dye in an anhydrous organic solvent such as dimethylsulfoxide (DMSO), dimethylacetamide (DMAC), or dimethylformamide (DMF) at a concentration of 10 mg/ml. Reactive dyes prepared in anhydrous solvent, protected from light, and sealed for storage at -20° C, are stable for up to 1 year upon receipt or 3 months after opening. Do not store reactive dyes prepared in anhydrous solvent for longer periods as stability cannot be assured.

Label Information	**AQuora® Fluorescein**
Reactive Group	Maleimide
Molecular Weight	1060
Extinction Coefficient (-M-cm)	76000 M-1cm-1 at 495 nm
A280 Correction Factor	0.19
Excitation / Emission Maximum (nm):	495 nm/520 nm ± 4nm
Spectrally Similar Dyes:	FITC and Carboxyfluorescein

Example Reaction Considerations

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

Prepare the protein or antibody for labeling.

1. Prepare the thiol-containing protein in a neutral pH buffer, such as 10-100 mM phosphate or Tris buffer, pH 7-7.5. The buffers may also be made to optionally contain 0.15 M NaCl. Buffers should be degassed to remove excess dissolved oxygen because sulfhydryls can rapidly oxidized 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

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a dPEG-SATA reagent).

2. If any precipitate is visibly present, filter the protein stock solution through a 0.2 µM syringe filter.
3. Determine the protein concentration and the moles of protein to be labeled. For antibodies, the concentration may be determined by measuring the absorbance of the solution at 280 nm and using the following calculation:
   Concentration = _____(A280) x _____(dilution factor) / 1.4 = _____mg/ml, where 1.4 is the absorbance at 280 nm of a 1 mg/ml concentration of the average antibody (using a 1 cm path length). If your antibody or protein gives a different extinction coefficient than 1.4, then replace this value with your determined value.
4. Determine mg of antibody to label:
   _____ml of antibody recovered x _____(mg/ml) = _____mg of antibody
5. Adjust the antibody concentration to 10mg/ml by adding reaction buffer or concentrating the solution as needed.

Prepare the dye stock solution.

1. Bring the vial containing the lyophilized dye to room temperature to prevent moisture condensation upon opening. Protect from light.
2. Add anhydrous solvent [dimethylsulfoxide (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.
3. Determine molar concentration of dye stock.
   Molar concentration of dye: 1mg dye /_____ml solvent / MW of the dye (g/mol) = _____M

Determine the volume of dye to be used for labeling. The molar excess of dye over the antibody or protein used in the reaction for the optimal final degree of labeling should be empirically determined and is dependent upon the concentration of the protein, the pH of the reaction conditions, 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 dye over the protein may be less than that used for amine-reactive dyes.

- Calculate the moles of dye needed based upon molar excess desired.
  (moles protein)(molar fold excess of dye) = moles of dye needed
- Calculate volume of dye.
  Volume of label = (_____mol dye) / (_____ M dye stock)(10^6 µl/L) = _____ul dye

Reaction conditions:

- Add the calculated volume of AQ Fluor to the thiol-containing antibody mixture *while* gently vortexing for even distribution. Close tube and vortex again for ~30 seconds.
- Allow the conjugation reaction to gently rock for 1-2 hour at room temperature. A 25° C constant temperature water bath may be used with periodic mixing to enhance the reproducibility of results.

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

- Prepare 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.
- Ensure that the packed column has a volume capacity 20 times larger than the reaction sample volume. Equilibrate the packed column with 3 column volumes of purification buffer.
- Load the sample onto the column gently and start the UV monitoring system, if available.
- Elute the dye-labeled protein by connecting the solvent reservoir for continuous flow of purification buffer through the column.
- Collect the early-eluting colored volume corresponding to first peak which is the dye-labeled protein. The

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broad, second peak is the excess dye.

Alternative Method: For small quantities, purify excess dye from labeled antibodies using an Amicon Ultracel 50K MWCO 0.5ml spin concentrator and wash with ~6 washes of purification buffer.

Quantification: The non-reacted excess dye must be removed for accurate quantitation of protein concentration and the degree of labeling (DOL or the dye-to-antibody ratio).

1. Dilute a small amount of labeled protein in the purification buffer in triplicate.
2. Measure the absorbance at 280 nm using a 1 cm quartz cuvette and at the A_{max} of the dye.
3. Calculate the concentration of protein using the following formulas and example based upon antibody labeling:

Molar Concentration of Diluted Stock = $(A_{280}-(A_{max})(CF)) / \varepsilon_{IgG}$ at 280nm
CF = A_{280} correction factor (see above under Label Information)
ε_{IgG} at 280 nm = 205800

Diluted Stock IgG (mg/ml) = (Molar Concentration, mmoles/ml)(MW_{IgG}, mg/mmole)
MW_{IgG} = 150000 mg/mmole

Stock Concentration = [Diluted Stock IgG (mg/ml)](DF) = mg/ml
DF = Dilution Factor used to measure absorbance

Example: An antibody was labeled with AQ550. The absorbance was measured at 280 nm and 554 nm from a sample diluted 100x (DF = 100).
A_{280} = 0.105
A_{554} = 0.366
Diluted sample concentration (M) = $(0.105-(0.366)(0.08))/205800 = 3.68 \times 10^{-7}$ M IgG
Diluted sample concentration (mg/ml) = $(3.68 \times 10^{-7}$ moles IgG)(150000) = 0.055 mg IgG/ml
Stock Concentration = (0.055 mg/ml)(100) = 5.5 mg IgG/ml

4. Calculate the degree of labeling (moles dye per moles of protein) using the following formulas:

Degree of Labeling (DOL) = $A_{max} / (\varepsilon_{fluor})$(Diluted sample concentration (M))

Example Continued:
Diluted sample concentration (M) = 3.68×10^{-7} M
A_{554} = 0.366
ε_{fluor} = 150000 $M^{-1}cm^{-1}$
Degree of Labeling (DOL) = $0.366 / [(150000\ M^{-1}cm^{-1})(3.68 \times 10^{-7}\ M)]$ = 6.6 AQ550 per antibody

Target Degree of Labeling
The target Degree of Labeling should be empirically determined based on the antibody or protein being labeled and intended application. For many antibodies used in immunoassays, a final degree of labeling 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 sulfhydryl groups that interfere with the reaction.	Maleimide groups react rapidly with free sulfhydryls (thiols). Remove all free thiols (e.g., cysteine) from the conjugation buffer.
	The conjugation buffer contained free amines (e.g., glycine, imidazole).	Maleimides react with free amines at ≥pH 7.5. 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.

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	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