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AQ LIFE SCIENCES
A DIVISION OF QUANTA BIODESIGN LIMITED

Product Number: 72-0002-0318

AQuora® 650-NHS Ester

AQuora® 650 - NHS Ester is an amine reactive fluorochrome engineered with SuperHydrophilic technology designed to improve solubility of the dye during labeling and of the dye labeled conjugate. As a result, the dye labeled conjugates made with AQuora® 650 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. AQuora® 650 is a cyanine-based dye with photophysical properties comparable to Alexa Fluor® 647, Cy5, and DyLight® 650.
This AQuora® 650 is activated with a N-hydroxysuccinimide (NHS) ester functional group which reacts with free primary amines, such as the amines on the side chain of lysines, forming a stable amide bond.

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

Product Number:	**72-0002-0318**
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 an anhydrous solvent, protected from light, and sealed for storage at -20° C, are stable for up to 2 months. Do not store reactive dyes prepared in anhydrous solvent for longer periods as stability cannot be assured.

Label Information	**AQuora® 650**
Reactive Group	NHS Ester
Molecular Weight	1710
Extinction Coefficient (-M-cm)	250000 M-1cm-1 at 654 nm
A280 Correction Factor	0.037
Excitation / Emission Maximum (nm):	654 nm/674 nm ± 4nm
Spectrally Similar Dyes:	Alexa Fluor® 647, Cy5, DyLight® 650, DyLight® 650 4x PEG

Example Conjugation Reaction

AQuora® 650-NHS Ester is an amine-reactive dye that can be conjugated to many proteins or peptides. The NHS ester reacts with primary amines to form a stable, covalent amide bond. The NHS Ester 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 antibody for labeling. The optimal antibody concentration for labeling is 5-10 mg/ml.

1. If any precipitate is visibly present, filter the antibody stock solution through a 0.2 µM syringe filter.
2. Dialyze the antibody against 50 mM sodium borate, 50 mM sucrose, pH 8.5 in a volume 200 times the sample volume using Slide-A-Lyzer® G2 50K MWCO dialysis membranes. Repeat dialysis once. Dialyze each time for at least 2 hr. For small volumes, an Amicon Ultracel 50K MWCO centrifugal filter can be used to buffer exchange and concentrate the antibody stock solution.

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3. Determine the Antibody Concentration:
   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 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 the molar concentration of the dye stock solution.
   Molar concentration of dye: 1mg dye /_____ml solvent / MW of dye (g/mol) = _____M

Determine the volume of dye to be used for labeling. The molar excess of dye over the antibody used in the reaction for the optimal final degree of labeling (DOL) should be empirically determined. In general, a 5-10-fold molar excess is appropriate for labeling antibodies prepared at a concentration of 5-10 mg/ml. 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:
   Mole IgG = (_____g IgG) / (150000 g/mol) = _____mol IgG, where the MW of an intact IgG antibody is approximately 150,000 g/mol. When using antibody fragments, adjust the MW to match that of the antibody being labeled.
   Mole excess desired in reaction: _____
   Moles of label = (_____mol IgG) x (mol excess) = _____mol label
   Volume of label = (_____mol label) / (_____M dye stock) ($10^{\wedge 6}$ µl/L) = _____µl label needed

Reaction conditions:

1. Add the calculated volume of AQ Fluor to the antibody mixture *while* gently vortexing for even distribution. Cap the tube and vortex again for ~30 seconds.
2. Allow the conjugation reaction to gently rock for 1 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 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 column has a volume capacity 20 times larger than the reaction sample volume. Equilibrate the packed column with 3 column volumes of purification buffer.
3. Load the sample onto the column gently and start the UV monitoring system, if available.
4. Elute the dye-labeled protein by connecting the solvent reservoir for continuous flow of purification buffer through the column.
5. Collect the early-eluting colored volume corresponding to the first peak which is the dye-labeled protein. The broad, second peak is the excess dye.

Alternative Method: For small quantities, purify excess dye from labeled antibodies using an Amicon Ultracel

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50K MWCO 0.5 ml spin concentrator and wash with ~6 washes of purification buffer.

Quantification: The non-reacted excess dye must be removed for accurate quantitation of antibody 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.
    a. Recommended dilution: 1:50 - 1:100
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 antibody using the following formulas and values above:

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 280nm = 205800

Diluted Stock IgG (mg/ml) = (Molar Concentration, mmol/ml)(MW_{IgG}, mg/mmol)
MW_{IgG} = 150,000 mg/mmolStock 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 and 554nm 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 of dye per moles of antibody) 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
Protein was not labeled; insufficiently labeled	The NHS Ester has hydrolyzed and is non-reactive	Prepare labeling reagent immediately before use in an anhydrous solvent. Do not prepare in an aqueous solution.
	Conjugation buffer contained primary amines that interfere with the reaction	Ensure reaction buffer is free of amines (i.e., tris or glycine)
	Too low of molar excess in the reaction and/or protein concentration too dilute	Increase the molar fold excess of reactive dye and prepare the protein at a concentration of ~5-10mg/ml. The more dilute the protein, the higher the molar excess of dye necessary to reach the target degree of labeling.
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.
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