Quasar® 670WS Protein Labeling Kit Instructions

Catalog No. PLK-Q670WS-1

For Research Use Only. Not for use in diagnostic procedures. See MSDS for specific handling instructions.

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1. Storage

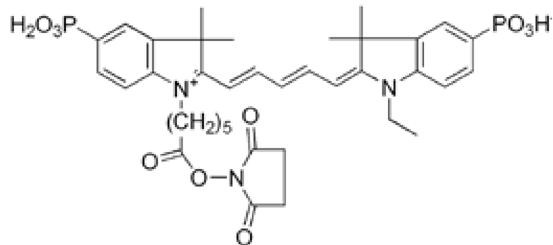
Keep the reactive dye protected from light. Store unopened Dye Packs dry, at -15 to -30 °C in the dark. The remaining kit components should be stored at 2 to 8 °C. When stored properly, the kit components should be stable for more than six months from the shipping date.

2. Introduction

Quasar® water-soluble (WS) Protein Labeling Kits are designed for the labeling of proteins of molecular weights greater than 10 kD using the succinimidyl-ester of Biosearch Technologies' Quasar WS dyes. These dyes are formulated to permit high labeling densities without fluorescence quenching and to avoid non-specific binding with proteins. The Quasar WS active ester reacts with amines on the protein to form stable covalent amide linkages. The protein-dye conjugates have absorption maxima at 655 nm and fluorescence emission maxima at 668 nm.

The Quasar WS Protein Labeling Kit contains all materials and reagents required for three separate labeling reactions and purification of the resulting labeled proteins. Each of the three vials contains approximately 60 nmol of Quasar 670WS active ester, which is sufficient for the labeling of 1 mg of an IgG antibody with 3-5 dyes per protein molecule.

The absorption and emission spectra of the Quasar 570WS and Quasar 670WS dyes are well separated from each other so these dyes can be used together in multicolor applications.



Quasar 670WS N-Succinimidyl Ester

The Quasar 670WS dye is a preferred replacement for the Cy™ 5, Alexa Fluor® 647, DyLight® 649, and HiLyte Fluor® 647 dyes.

NOTE: The following materials and protocols have been optimized for IgG antibodies. Other antibodies and proteins may also be labeled but separation media and labeling conditions may vary in order to produce optimal results.

Altering the protein concentration or the reaction pH will change the labeling efficiency of the reaction. Successful labeling can be achieved in the pH range 7.3 - 9.3, however, longer labeling reaction times are required at lower pH. Higher protein concentrations usually increase labeling efficiency. Solutions of up to 10 mg/mL protein have produced good conjugation reactions.

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3. Contents

Component 1: Three foil Dye Packs containing dried dye in reaction vials (blue caps)

Component 2: One vial of Reaction Buffer Salt (sodium bicarbonate) for making coupling buffer (colorless cap)

Component 3: Gel filtration column (Econo-Pac® 10DG Desalting Column from Bio-Rad Laboratories) with yellow stopper for the separation of labeled protein from non-conjugated dyes

Component 4: Elution buffer (60 mL, phosphate buffered saline (PBS), pH 7.3 containing 0.05% sodium azide)

Component 5: Transfer pipettes (6)

Component 6: Sample collection tubes (6)

Component 7: Product specification booklet with directions for using the labeling kit

4. Preliminary Work

A 0.5 mL volume of protein solution is used for standard labeling reactions. Purified protein should be prepared at a concentration of 2 mg/mL in buffer (not in serum). PBS is recommended as the buffer. The buffer cannot contain ammonium ions, or primary or secondary amines. If the protein is in an unsuitable buffer (e.g. Tris or glycine), the buffer must be replaced, preferably with PBS, by dialysis or by using the provided gravity column. The presence of low concentrations (< 2%) of biocides such as azide or thimerosal does not affect protein labeling.

5. Conjugation Reaction

The protein to be conjugated should be dissolved at 2 mg/mL in a 50 mM phosphate buffer (pH 7.0-7.3) such as PBS. Acceptable labeling may be obtained with 0.5 mg to 1.5 mg total protein. A smaller amount of protein is likely to result in over-labeling (possibly inactivated antibodies, appearance of non-specific binding, and reduced fluorescence quantum yield). A larger amount of protein will result in under-labeled proteins that are not optimally bright. If the protein is mixed with carrier protein or is in hybridoma supernatant or ascites fluid, it is still possible to for it to be labeled however, any other protein in the solution will also be labeled. It is essential that all non-target protein present in the sample be counted in the 1 mg total protein that is labeled by this procedure.

5.1. Add 1.0 mL of the elution buffer to the vial of Reaction Buffer Salt (colorless cap) and shake or vortex until all solid has dissolved. The bicarbonate solution can be stored at 2 to 8 °C for 2 weeks or in a freezer indefinitely.

5.2. Centrifuge or tap the vial of reactive dye for one minute to ensure that all of the dye is in the bottom of the vial.

CAUTION: This dye is intensely colored and very reactive. Care should be exercised when handling the dye vial to avoid staining clothing, skin and other items.

5.3. Add the 0.5 mL of protein solution to the reactive dye vial, which will serve as the reaction vial.

5.4. Add 50 µL of the sodium bicarbonate coupling buffer to the reactive dye vial with protein and mix thoroughly by gentle vortexing or by manually inverting the capped tube several times. Do not vortex vigorously as this will cause the protein solution to foam. The reaction is incubated at room temperature for 30 minutes with additional mixing approximately every 10 minutes. Extending the reaction time may lead to over labeling of the protein.

6. Purification

When used appropriately, the purification column is effective at separating labeled proteins from the unconjugated dye and from the reaction buffer salts. The column is designed for loading volumes of 0.7 mL or less. Loading larger volumes of reaction mixture may lead to incomplete separation and the presence of unconjugated dye with the labeled protein. If your reaction volume is larger than 0.7 mL then it is best to concentrate it prior to loading or to divide it into portions and purify each portion separately.

6.1. While the labeling reaction is incubating, decant the buffer from the top of the column. Mount the column on a ring stand.

6.2. Add 12 mL fresh elution buffer. Remove the tip from the column to start the outflow of the column and allow the buffer to run through the column into a collection tube or small beaker. Flow will stop when the meniscus reaches the disk at the top of the column packing. The column will not run dry.

6.3. Centrifuge the reaction vial for one minute.

6.4. Carefully transfer the protein-labeling mixture to the center of the top of the column and allow the solution to enter the packing. If necessary the reaction vial and pipette may be washed with 0.1 mL of the elution buffer and the wash can be added to the top of the column.

6.5. Add 0.5 mL of elution buffer to the top of the column and let this enter the column.

6.6. Add 1.5 mL of elution buffer to the top of the column. As this volume of buffer moves through the column, a faster moving blue band of labeled protein will separate from the unconjugated dye. When the 1.5 mL of elution buffer has completely run into the column packing, the leading edge of the faster moving blue band should be about two thirds of the way down the column.

6.7 Add an additional 2.0 mL of elution buffer to the top of the column and collect the faster moving blue band in a clean tube as it elutes from the column. The labeled protein should

be entirely eluted by the 2.0 mL of buffer and collected in a single tube.

Notes

1. The elution buffer contains 0.05% sodium azide as a preservative. It should be protected from excess light and stored at 2 to 8 °C.

2. The remaining free dye can be removed from the column (prior to additional separations) with 12 mL of the elution buffer solution. To store the column for future use, place the yellow stopper on the bottom of the column, add a few mL of elution buffer to cover the frit on the top of the column and then replace the cap on the top of the column. Store at 2 to 8 °C.

3. Unconjugated dye can also be separated from the labeled protein by dialysis or with spin columns. Dialysis does not give as efficient and rapid separation as gel filtration. Gel filtration should be used whenever possible.

7. Determination of Degree of Labeling

7.1. Dilute an aliquot of the purified conjugate into PBS or other suitable buffer and measure the absorbance in a cuvette with a 1 cm path-length at both 280 nm (A_{280}) and 655 nm (A_{655}). For protein concentrations between 1 to 5 mg/mL a 5- to 20-fold dilution is recommended. The absorbance of the solution at 655 nm should be in the range 0.5-1.0. In order to avoid using large volumes of conjugate for absorption measurements, the use of 50 µL- Eppendorf® UVette® microcuvettes or a NanoDrop™ 1000 spectrophotometer is recommended.

7.2. Calculate the dye to protein ratio:

Molar extinction coefficients of 240,000 $M^{-1}cm^{-1}$ at 655 nm for the Quasar 670WS dye and 165,000 $M^{-1}cm^{-1}$ at 280 nm for the protein are used for the calculations.

$$[Q670WS] = \frac{A_{655}}{240{,}000} \quad mol/L$$

$$[Protein] = \frac{A_{280} - (0.026 \times A_{655})}{165{,}000} \quad mol/L$$

$$\frac{D}{P} = \frac{[Q670WS]}{[Protein]}$$

$$\frac{D}{P} = \frac{0.69 \times A_{655}}{A_{280} - (0.026 \times A_{655})}$$

8. Absorption and Fluorescence Properties of Conjugates

8.1. Absorption properties: The absorption maxima of Quasar 670WS dye-protein conjugates in PBS is between 650 – 656 nm. This is a bathochromic shift of about 2 to 3 nm from that of the free dye.

8.2. Fluorescence properties: The maxima of the fluorescence excitation and emission spectra of Quasar 670WS-protein conjugates in PBS (λ_{ex} 655 nm /λ_{em} 672 nm) are similar to those of the unconjugated dye.

Figure 1 shows the absorption and emission spectra of the Quasar 670WS dye and Figure 2 shows typical absorption and emission spectra of a Quasar 670WS-protein conjugate.

Figure 1. Quasar 670WS Dye

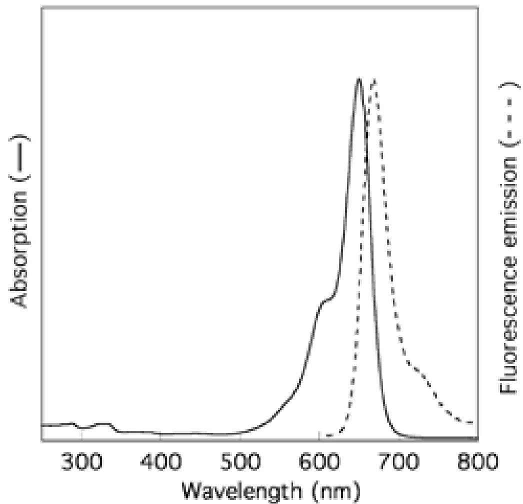
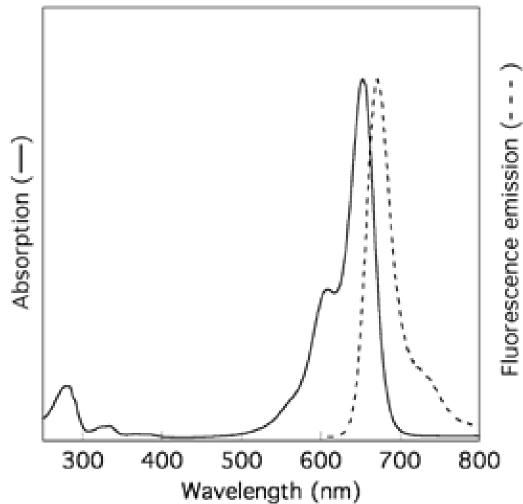


Figure 2. Quasar 670WS-IgG Conjugate



9. Storage of Conjugates

Store the labeled protein at 2 to 8 °C, protected from light. If the final concentration of purified protein conjugate is less than 1 mg/mL (see step 7.2.), either concentrate the solution or add bovine serum albumin (BSA) or other stabilizing proteins at 1-10 mg/mL. In the presence of sodium azide, the conjugate should be stable at 2 to 8 °C for several months. For longer storage, divide the conjugate into small aliquots and freeze at –15 to -30 °C. Avoid repeated freezing and thawing.

It is a good practice to centrifuge solutions of conjugates in a microcentrifuge before use; only the supernatant should then be used in the experiment. This step will remove any aggregates that may have formed during storage.

10. Troubleshooting

Under-labeling: If calculations indicate that the protein is labeled with significantly less than three moles of fluorophore per mole of a 165,000 Dalton protein, your protein may be under-labeled. A number of conditions can cause a protein to label inefficiently:

- Trace amounts of amine–containing components in the buffer will compete for dye and decrease the efficiency of protein labeling. If your protein has been in amine-containing buffers (e.g. Tris or glycine), dialysis of the protein against PBS will be necessary before labeling.

- Dilute solutions of proteins (≤1 mg/mL) will not label efficiently. If the protein solution cannot be concentrated, then less than 1 mg of protein should be used. This will increase the molar ratio of Quasar 670WS-succinimidyl ester to protein.

- The addition of sodium bicarbonate is designed to raise the pH of the reaction mixture to approximately 8.3, as succinimidyl esters react most efficiently with primary amines at slightly alkaline pH. If the protein solution is strongly buffered at a lower pH, the addition of bicarbonate will not raise the pH to the optimal level. Either more bicarbonate can be added, or the buffer can be exchanged with PBS, which is

only weakly buffered, or with 0.1 M sodium bicarbonate, pH 8.3, by dialysis or other method prior to starting the reaction.

- The standard protocol may not always result in optimal labeling because proteins, including antibodies, react with fluorophores at different rates and retain biological activity at different degrees of dye labeling. To increase the amount of labeling, re-label the same protein sample, or label a new protein sample using either less protein or more reactive dye per reaction. To increase the amount of dye in the reaction the contents of two vials may be combined: add the PBS solution of the protein to a dye reaction vial and dissolve the dye, transfer this solution to a second dye reaction vial, add the reaction buffer salt solution to begin the conjugation reaction.

Over-labeling: If calculations indicate that the protein conjugate is labeled with significantly more than five moles of fluorophore per mole of a 165,000 Dalton protein, the protein may be over-labeled. Although conjugates with a high number of attached dye molecules may be acceptable for use, over-labeling can cause aggregation of the protein conjugate and can also reduce the antibody's specificity for its antigen, both of which can lead to non-specific staining. Over-labeling can also cause fluorescence quenching of the attached dyes, which will decrease the fluorescence of the conjugate. To reduce the amount of labeling in future conjugation reactions, either add more protein to the reaction to decrease the molar ratio of dye to protein, or allow the reaction to proceed for a shorter time.

Inefficient removal of free dye: Although good success in removing free dye from protein conjugates is usually achieved with the provided column, it is possible that trace amounts of free dye will remain in the conjugate solution after purification, particularly if a low molecular weight protein is labeled. The presence of free dye, which can be determined by thin layer chromatography, will result in erroneously high calculated values for the degree of labeling (see Determination of Degree of Labeling). Remaining traces of free dye can be removed by applying the conjugate to another column or by extensive dialysis.

11. References

Mark V. Reddington, Bioconjugate Chemistry 2007, **18**, 2178- 2190.

12. Related Products

Quasar 570WS Protein Labeling Kit, Catalog No. PLK-Q570WS-1

Quasar 570WS Protein Labeling Kit, Microscale, Catalog No. PLKM-Q570-100

Quasar 670WS Protein Labeling Kit, Microscale, Catalog No. PLKM-Q670-100

Quasar 570WS Protein Labeling Dye, 5-Pack, Catalog No. PLD-Q570-5

Quasar 670WS Protein Labeling Dye, 5-Pack, Catalog No. PLD-Q670-5

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