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Quasar® 670WS Protein Labeling Kit, Microscale Instructions

Catalog No. PLKM-Q670WS-100

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

Contents

1. Storage	4
2. Introduction	4
3. Contents	6
4. Preliminary Work.....	6
5. Conjugation Reaction	7
6. Purification	8
7. Determination of Degree of Labeling	9
8. Absorption and Fluorescence Properties of Conjugates	10
9. Storage of Conjugates	12
10. Troubleshooting.....	12
11. References	14
12. Related Products.....	14

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.

3. Contents

Component 1: Five 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: Five Spin Columns (Pro-Spin Columns from Princeton Separations) for the separation of labeled protein from non-conjugated dyes

Component 4: Elution buffer (10 mL, phosphate buffered saline, pH 7.3 containing 0.05% sodium azide)

Component 5: Column waste collection tubes (5)

Component 6: Sample collection tubes (5)

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

4. Preliminary Work

A 50 μL volume of protein solution is used for standard labeling reactions. Purified protein should be pre-prepared at a concentration of about 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 such as Tris, glycine, ethanolamine, triethylamine or glutathione. If the protein is in an unsuitable buffer (e.g. Tris or glycine), the buffer must be replaced, preferably with PBS. The presence of low concentrations (< 2%) of biocides such as azide or thimerosal does not affect protein labeling.

5. Conjugation Reaction

Protein to be conjugated should be dissolved at 2 mg/mL in 50 mM phosphate buffer (pH 7.0-7.3) such as phosphate-buffered saline (PBS). Acceptable labeling may be obtained with 0.05 mg to 0.15 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 antibodies 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 label it. 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 0.1 mg total protein that is labeled by this procedure.

5.1. Gently tap the spin column to ensure that the dry gel has settled in the bottom of the spin column.

5.2. Remove the top column cap and reconstitute the column

by adding 0.65 mL of the supplied elution buffer. Replace the column cap and vortex for approximately 5-10 seconds or until all of the dry gel is suspended in the buffer. Remove any air bubbles by sharply tapping the bottom of the column. Allow the column to hydrate for the duration of the labeling reaction, at least 30 minutes. Proceed directly to the protein labeling reaction.

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

5.4. Centrifuge or tap the vial of reactive dye 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.

5.5. Add the 50 µL of protein solution to the reactive dye vial and mix thoroughly by gentle vortexing.

5.6. Add 5 μ L of the coupling buffer to the reactive dye vial with protein and mix thoroughly by gentle vortexing. 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

6.1. Two minutes before the labeling reaction is finished, remove the caps on both ends of the reconstituted spin column, place the column into the 2 mL waste collection tube and centrifuge for two minutes at 750 x g. If using a fixed-angle microcentrifuge, keep track of the position of the column using the orientation mark molded into the column.

6.2. If there is a drop at the end of the column, blot it dry. Discard the waste collection tube and fluid. Do not allow the gel material to dry excessively. Process the sample within the next few minutes.

6.3. Briefly centrifuge the reaction vial. Carefully transfer the protein-labeling mixture to the center of the column without disturbing the gel surface. Do not contact the sides of the column.

6.4. Place the column into the sample collection tube (1.5 mL) and place both into the centrifuge. Take care to maintain proper column orientation. The highest point of the gel media should always point towards the outside of the rotor. Spin the column and collection tube at 750 x g for 2 minutes. The purified sample will collect in the bottom of the collection tube.

6.5. Discard the spin column, cap the sample tube and continue to determination of degree of labeling.

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 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 μ L-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.

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

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

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

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

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

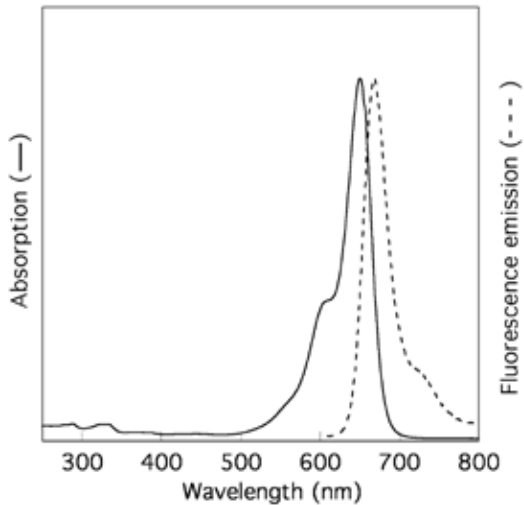
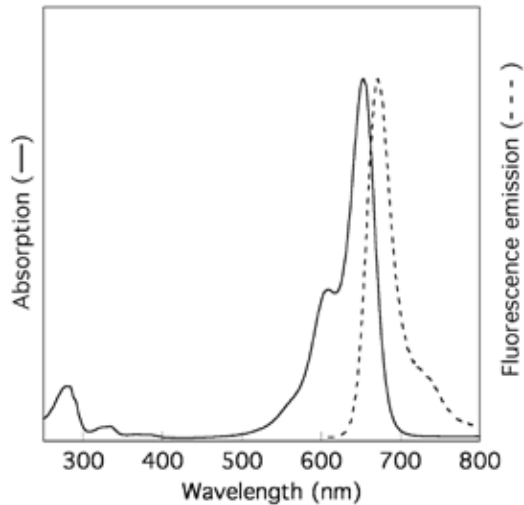


Figure 2. Quasar 670WS-IgG Conjugate



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 it or add bovine serum albumin (BSA) or other stabilizing proteins at 1-10 mg/mL. In the presence of 0.05% 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 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, the 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.
- Dilute solutions of proteins (≤ 1 mg/mL) will not label efficiently. If the protein solution cannot be concentrated, then less than 0.1 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 spin columns, 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 670WS Protein Labeling Kit, Catalog No. PLK-Q670WS-1

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

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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