IRDye® 800CW Protein Labeling Kit – High MW

Developed for:

Aerius, and Odyssey® Family of Imagers

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your Odyssey Imager model.

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I. Introduction

The IRDye 800CW Protein Labeling Kit – High MW is optimized to label proteins for use with the Odyssey® Infrared Imaging System, Aerius Automated Imaging System, or other in vivo imaging systems with near-infrared detection. Labeled proteins may be used for Western blots, In-Cell Western™ (ICW) immunofluorescent assays, in vivo imaging, and other applications.

The kit is optimized for labeling 1 mg of protein with molecular weight 45 - 200 kDa. For proteins of lower molecular weight, use IRDye 800CW Protein Labeling Kit – Low MW (P/N 928-38042). For small amounts of protein (100 µg) with molecular weight 14 - 200 kDa, use IRDye 800CW Protein Labeling Kit – Microscale (P/N 928-38044).

IRDye 800CW infrared dye bears an NHS ester reactive group that will couple to proteins and form a stable conjugate. Fluorescent conjugates labeled with IRDye 800CW display an absorption maximum of 774 nm, and an emission maximum of 789 nm in 1X PBS (Figure 1). These spectral characteristics match the 800 nm channel on the Odyssey and Aerius Systems.

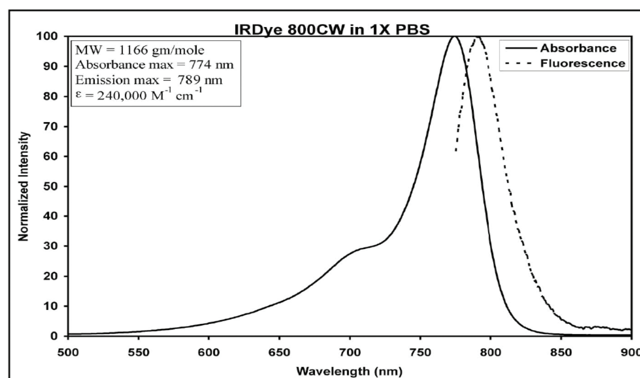

Figure 1. Absorption and emission spectra of IRDye 800CW in 1X PBS.

II. Kit Components

- 3X IRDye 800CW Reactive Dye vials (0.1 mg). (store at -20 °C)
- 1X 0.5 mL 1 M Potassium Phosphate (K_2HPO_4), pH 9 (store at 4 °C)
- 1X 25 mL 1X PBS (store at 4 °C)
- 1X 0.5 mL ultra pure water (store at 4 °C)
- 3X Pierce® Zeba™ Desalting Spin Columns, P/N 89891 (store at 4 °C)

  NOTE: The minimum recommended protein molecular weight for these columns is 7 kDa.
- Pierce Zeba Desalting Spin Columns instructions
- Protocol for IRDye 800CW Protein Labeling Kit – High MW

III. Preparation of Protein Solution for Conjugation

To perform a labeling reaction, it is critical for the protein to be in a preservative-free phosphate buffer at pH 8.5. Preservative (i.e. sodium azide) removal and buffer exchange can be accomplished by passing the protein through a desalting column similar to those provided in the kit (additional columns not included). For buffer exchange, the column should be equilibrated with 50 mM phosphate buffer, pH 8.5. Alternatively, the protein solution can be dialyzed (cassette not included) against 50 mM phosphate buffer, pH 8.5. If the protein solution is free of preservatives and in a buffer with a pH lower than 8.5, the pH of the solution can be raised by adding the concentrated phosphate buffer (1 M potassium phosphate, pH 9), included in the kit, to the protein solution.

1. Prepare 1.0 mg of protein in a phosphate buffer without sodium azide, at a concentration of 1 (± 0.1) mg/mL.

   NOTES:

   - *Protein concentration can be determined spectrophotometrically using the extinction coefficient of the protein or colorimetrically using several kits (e.g. Pierce® BCA) which are commercially available. If using a colorimetric analysis, the standards must be prepared using the same protein as the samples to be measured. Bovine Serum Albumin (BSA) does not provide a representative standard curve for all proteins.*
   - *Use of a more dilute or more concentrated protein solution will result in decreased or increased labeling of the protein, respectively.*
   - *The protein to be labeled must be in a buffer that is free of primary amines and ammonium ions. Tris or glycine buffers cannot be used for conjugation. Even trace amounts of components containing primary amines will decrease labeling efficiency.*
   - *Unpurified antibodies (such as ascites fluid and crude serum), cell lysates, and proteins that contain BSA or other proteinaceous stabilizers, will not label well and have not been characterized for use with this kit.*

2. Raise the pH of the preservative-free protein solution to pH 8.5 with the 1 M potassium phosphate buffer (K_2HPO_4), pH 9, provided in the kit, as necessary. If the protein solution is in 1X PBS, adjust the pH by adding 1/10th volume of 1 M potassium phosphate buffer, pH 9. For example, for 1.0 mL of protein solution, add 0.1 mL of 1 M potassium phosphate buffer, pH 9.

3. Cool/warm the protein to 20 - 25 °C before reaction with the dye.

   NOTE: If the protein is temperature-sensitive, the labeling reaction may be carried out at a lower temperature.

IV. Protein Labeling Reaction

1. Use Figure 2 to determine the appropriate amount of dye to add based on the molecular weight of the protein.

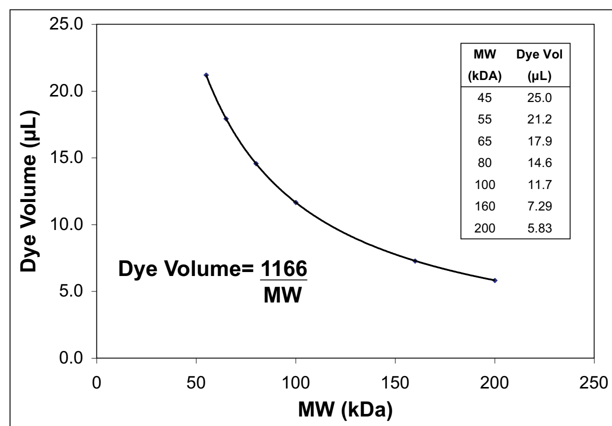

Figure 2. Suggested dye amounts based on protein molecular weight.

NOTES:

- *Using the dye amounts shown in Figure 2 typically results in a D/P ratio of 1:1 to 3:1.*
- *The optimal degree of labeling will vary for different applications. The effect of labeling on the biological activity of the protein will depend on factors including size and amino acid composition. Over-labeling may cause high background or self-quenching of the dye.*
- *Due to variation in amino acid composition, different proteins will react with the dye at different rates. It may be necessary to adjust the standard protocol to achieve optimal labeling.*
- *A D/P ratio of 1:1 - 2:1 for an IgG antibody is suitable for both Western and In-Cell Western™ applications. Higher D/P ratios (3:1 - 4:1) for an IgG antibody may still be usable for Western blot detection, but may exhibit increased background and therefore not perform optimally for other applications.*
- *For in vivo imaging applications, the dye/protein ratio of the conjugate may affect biological or biochemical activity of the protein, signal-to-noise ratio, blood clearance, and biodistribution (for example, Schellenberger et al., 2004).*

2. Dissolve 1 tube of dye with 25 µL of ultra pure water provided in the kit; mix thoroughly by vortexing.

   NOTE: Work quickly, as dye reactivity decreases over time.

3. Mix the appropriate amount of dye to 1.0 mg of protein. React for 2 hours at 20 °C, protecting the vial from light.

   NOTE: Little difference in labeling efficiency was noted from 4 - 25 °C; however, the kit is optimized for reactions at 20 °C. If the labeling reaction is performed at temperatures less than 20 °C, any remaining reactive dye must be removed from the sample immediately after the 2-hour incubation (see Section V). At 20 - 25 °C, there is little or no reactive dye remaining in the reaction mix after the 2-hour incubation.

4. Separate the free dye from the protein conjugate as outlined in Section V.

V. Separation of Conjugate from Free Dye

Detailed instructions for use of the Pierce® Zeba™ Desalting Spin Columns are included with this kit. Instructions can also be downloaded from www.piercenet.com.

The Pierce Zeba Desalting Spin Columns (5 mL) are suitable for 0.5 - 2.0 mL of reaction volume. Other sizes are available from the manufacturer. The recommended protein molecular weight for the spin columns is > 7 kDa.

1. Read and follow instructions for use of Pierce Zeba Desalting Spin Columns before proceeding.

   NOTE: Never reuse the Pierce Zeba Desalting Spin Columns.

2. Remove the 0.05% azide preservative from the column by following the Pierce Zeba Desalting Spin Column “Procedure for Buffer Exchange” using the 1X PBS supplied in the kit.

3. Use the Pierce Zeba Desalting Spin Column to purify the dye-labeled conjugate.

   NOTE: Dye-labeled conjugates obtained following the Pierce Zeba Desalting Spin Column protocol typically contain 5% or less free dye.

VI. Calculation of Dye/Protein Ratio and Protein Concentration

Once the free dye has been sufficiently removed, the dye-to-protein ratio of the conjugate can be determined. Calculate the number of dye molecules per protein molecule (dye/protein or D/P ratio) by measuring absorbance with a UV-Vis spectrophotometer.

IMPORTANT: When the protein is highly labeled with IRDye 800CW, the absorption spectrum in 1X PBS shows a strong "blue" shoulder (~705 nm) near the normal dye peak. This distortion will cause the calculated D/P ratio to be lower than the true ratio. To correct for this, always dilute the conjugate in a solvent mixture of 1X PBS and methanol (1:1) to determine the dye/protein ratio.

1. Dilute the labeled conjugate 1:10 to 1:50 in a mixture of 1X PBS and methanol (1:1) such that the maximum absorbance reading at A_{780} is less than 2.0 and the A_{280} is greater than 0.12.

2. Measure the absorbance of the conjugate at 280 nm and 780 nm (A_{280} and A_{780}).

3. Calculate the dye/protein ratio using this formula: $$D/P = \left[\frac{A_{780}}{\varepsilon_{Dye}}\right] \div \left[\frac{A_{280} - (0.03 \times A_{780})}{\varepsilon_{Protein}}\right]$$

   In which:
   - 0.03 is a correction factor for the absorbance of IRDye 800CW at 280 nm (equal to 3.0% of its absorbance at 780 nm).
   - ε_{Dye} and $\varepsilon_{Protein}$ are molar extinction coefficients for the dye and protein, respectively.
   - ε_{Dye} is 270,000 M^{-1} cm^{-1} and $\varepsilon_{Protein}$ is 203,000 M^{-1} cm^{-1} (for a typical IgG) in a 1:1 mixture of PBS:methanol. *Proteins other than IgG may have very different molar extinction coefficients; use of the correct extinction coefficient for your protein is essential for accurate determination of the D/P ratio.*

4. Calculate the final protein concentration using this formula:

$$\text{Protein Conc.}\left(\frac{mg}{mL}\right) = \frac{A_{280} - (0.03 \times A_{780})}{\varepsilon_{Protein}} \times MW_{Protein} \times \text{Dilution Factor}$$

   In which:
   - $MW_{Protein}$ is the molecular weight of the protein.
   - Dilution factor is the dilution of the labeled conjugate prior to measurement by spectrophotometer.

   NOTES:
   - *The protein concentration may also be determined colorimetrically; however, the dye quantification must be performed using a UV-Vis spectrophotometer.*
   - *The typical recovery of dye-labeled proteins (14 - 190 kDa) purified by Pierce Zeba™ Desalting Spin Columns was found to be greater than 80%.*

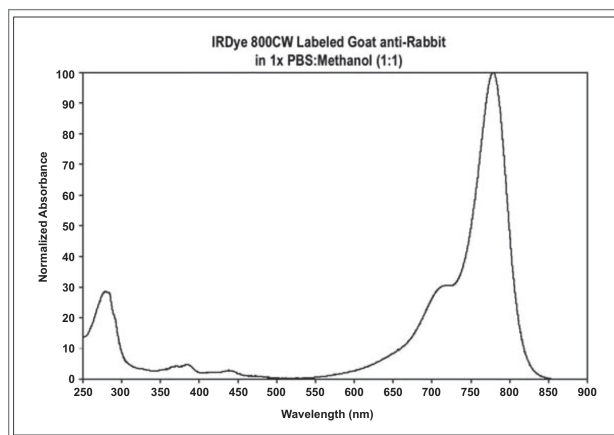

Figure 3. The spectrum of a typical IgG protein labeled with IRDye 800CW is provided for reference.

VII. Handling of Labeled Conjugates

To improve shelf life and stability of labeled conjugates, add sodium azide (0.01%) and bovine serum albumin (1 - 10 mg/mL). Higher amounts of sodium azide will cause degradation of the dye and should be avoided. BSA should only be added after the D/P ratio is determined, as BSA absorbs at 280 nm and will interfere with D/P ratio calculation. Do not add BSA or sodium azide to samples for in vivo imaging applications. IgG conjugates should be stored at 4 °C and protected from light. They are stable at 4°C for up to six months, or they can be frozen in small aliquots for longer-term storage. For conjugated proteins other than IgGs, optimal storage conditions and buffers may vary; however, all dye conjugates should be protected from light. Avoid freeze-thaw cycles of conjugates, as this will greatly reduce performance.

VIII. Troubleshooting Guide

For best results, read and follow the protocol carefully.

NOTES AND TIPS:

- *The protocol is optimized for a protein concentration of 1 mg/mL. Deviations from this amount will affect the D/P ratio.*
- *The pH of the reaction is critical. The reaction should be run using phosphate buffer at pH 8.5.*
- *It is important to note that the labeling reaction will continue even after the suggested 2-hour reaction time, if NHS ester is still present.*
- *Under-labeling: Different proteins and antibodies will react with the fluorophore at different rates. For this reason, the standard protocol provided here may not always produce optimal labeling. To label with a higher D/P ratio, try re-labeling the same protein sample, or perform a new reaction with fresh protein sample using either less protein at the same concentration, or more of the reactive dye to increase the molar ratio of dye-to-protein in the reaction.*
- *Over-labeling: If a protein is too heavily labeled, it may not function well in the intended assay. Over-labeling can cause fluorophore quenching (which reduces desired signal), aggregation, and non-specific staining (which increases assay background). Over-labeling may also affect biological/biochemical activity, biodistribution, or clearance of conjugates in vivo. If over-labeling occurs, reduce the degree of labeling in subsequent reactions by adding more protein at the same concentration, or reducing the amount of reactive dye added. Either approach will decrease the molar ratio of dye-to-protein in the reaction.*
- *Preparation of protein conjugates for other applications: The optimal degree of labeling may vary for different applications. For in vivo imaging applications, the dye/protein ratio of the conjugate may affect biological or biochemical activity of the protein, signal-to-noise ratio, blood clearance, and biodistribution (for example, Schellenberger et al., 2004). The optimal degree of labeling for different proteins may vary widely, so we recommend preparing several conjugates with different D/P ratios for evaluation in your desired application.*
- *Labeling of proteins: It is recommended that you perform a pilot labeling reaction using the conditions described in this protocol and evaluate the resulting D/P ratio. If necessary, the labeling conditions can then be altered to change the D/P ratio. As a general rule, lower MW proteins should be labeled with fewer dye molecules, while higher MW proteins can tolerate a higher degree of labeling. Please note that when calculating the labeling ratio of the conjugate (Section VI) you must use the correct extinction coefficient for the protein.*

Troubleshooting

Problem	Possible Cause	Solution / Prevention
Protein is over-labeled (D/P ratio is too high)	Free dye present (i.e. the real amount of labeling may be less than it appears)	Process conjugates through a second spin column to remove free dye.
	Reactive dye and/or protein concentration in labeling reaction not optimal	Use less dye or more protein in labeling reaction.
	Temperature of labeling reaction too high	Perform reaction at 20 - 25 °C.
	Labeling reaction carried out too long	Incubate reaction for 2 hrs. Purify immediately by spin column.
Protein is under-labeled (D/P ratio is too low)	Reactive dye and/or protein concentration in the labeling reaction not optimal	Re-label the conjugate to increase the D/P ratio.
	Protein with inherently low labeling efficiency	Use more dye.
	Reactive contaminant(s) or preservatives present in original protein solution	Dialyze or desalt unlabeled protein prior to labeling reaction.
	NHS ester content too low	Use a fresh vial of dye for labeling reaction. Do not allow dye solution to stand for more than a few minutes before use.
	Protein concentration too low	Concentrate protein before labeling reaction.
High background in Westerns	Excessive free dye	Process conjugates through a second spin column to remove free dye.
	Over-labeling of protein	Re-check D/P ratio and possibly repeat labeling reaction.
	Poor blocking	Try a different blocking buffer.
		Try a different membrane.
Low signal in Westerns	Protein over-labeled	Over-labeling can cause self-quenching of the dye, leading to reduced signal and higher background.
	Protein under-labeled	Re-label conjugate to increase D/P ratio.
	Inappropriate blocker used	Try a different blocking buffer. Primary antibody performance is highly dependent on choice of blocker.
High background in In-Cell Western assays	Excessive free dye	In-Cell Western assays are very sensitive to free dye; process conjugates through a second spin column to remove free dye.

Troubleshooting (continued)

Problem	Possible Cause	Solution / Prevention
High background in In-Cell Western assays *(continued)*	Over-labeling	D/P ratios higher than 2:1 will cause excess background in this assay. Label new antibody with a lower D/P ratio.
Distorted absorption spectrum	Some distortion in 1X PBS is normal, especially at high D/P ratio	Record UV/Vis spectrum in 1:1 methanol:PBS.
	Distortion observed when protein is over-labeled	Use less dye or more protein in labeling reaction.

IX. Reference

Schellenberger, E.A., R. Weissleder, and L. Josephson. 2004. Optimal modification of annexin V with fluorescent dyes. Chembiochem. 5:271-274

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