IRDye® 680LT 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.

Part Number: 928-38066

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Published August 2011. The most recent version of this pack insert is posted at http://biosupport.licor.com/support

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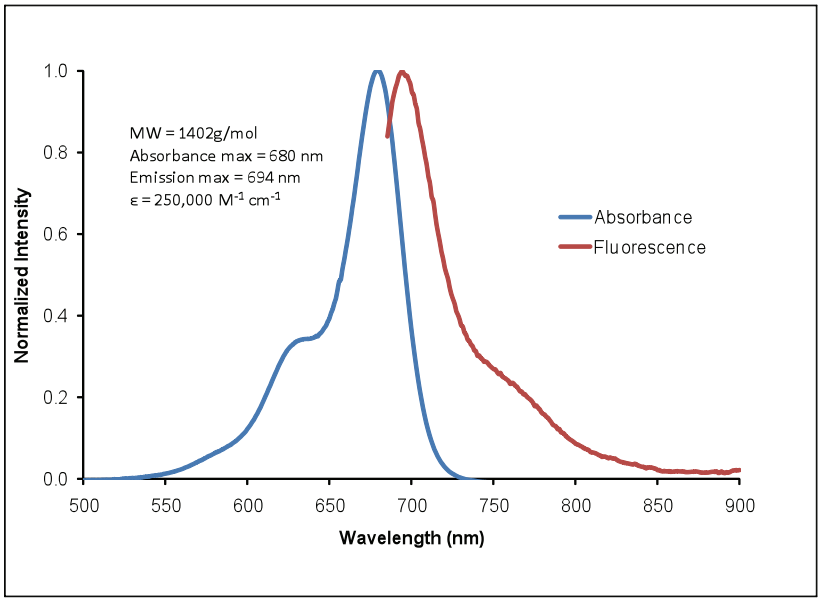
I. Introduction

The IRDye 680LT Protein Labeling Kit – High MW is optimized to label proteins for use with the Odyssey® Infrared Imaging System, Aerius Automated Imaging System, or other imaging systems with near-infrared detection. Labeled proteins may be used for Western blots, In-Cell Western™ (ICW) assays, immunofluorescent assays, and other applications. IRDye 680LT is not intended for use with *in vivo* applications.

The kit is optimized for labeling 1 mg of protein with molecular weight 45 - 200 kDa at a concentration of 1 mg/mL. For proteins of lower molecular weight, use IRDye 680LT Protein Labeling Kit – Low MW (P/N 928-38068). For small amounts of protein (100 µg) with molecular weight 14 - 200 kDa, use IRDye 680LT Protein Labeling Kit – Microscale (P/N 928-38070).

IRDye 680LT bears an NHS ester reactive group that will couple to proteins and form a stable conjugate. IRDye 680LT displays an absorption maximum of 680 nm and an emission maximum of 694 nm in methanol (Figure 1). These spectral characteristics match the 700 nm channel on the Odyssey and Aerius Systems.

Figure 1. IRDye 680LT in Methanol



II. Kit Components

- 3 x IRDye 680LT Reactive Dye vials (0.125 mg each) (store at -20°C)
- 1 x 0.5 mL 1 M Potassium Phosphate (K_2HPO_4), pH 9 (store at 4°C)
- 1 x 25 mL 1X PBS (store at 4°C)
- 1 x 0.5 mL ultra pure water (store at 4°C)
- 3 x Zeba™ Spin Desalting Columns, Product 89891 (store at 4°C).

  Zeba Spin Desalting Columns are manufactured by Thermo Fisher Scientific, Rockford, IL USA.

  Note: The minimum recommended protein molecular weight for these columns is 7 kDa.

- Protocol for IRDye 680LT 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 (e.g., 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 some of 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 commercially available kits (e.g. Thermo Fisher Scientific Pierce® BCA™ Protein Assay). If using a colorimetric analysis, the standards must be prepared in the same protein as the samples to be measured. 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 proteins and protein solutions 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 8.5, as necessary, with the 1 M Potassium Phosphate buffer (K_2HPO_4), pH 9, provided in the kit. 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.

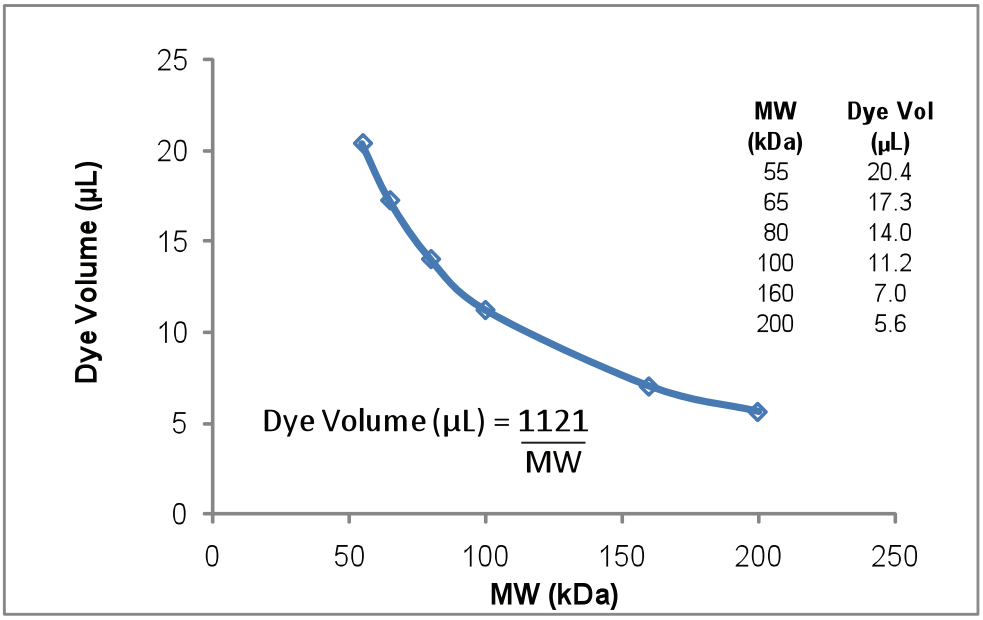
3. Cool/warm the protein to 20-25°C before reaction with the dye.

   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. IRDye 680LT High MW Required Dye Amount



Notes:

- **For secondary antibodies for Western blot applications, use 4 µL of dye.**
- Using the dye amounts shown in Figure 2 typically results in a dye-to-protein (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.
- 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.

2. Warm dye to ambient temperature, protected from light. Dissolve 1 tube of dye with 25 µL of ultra pure water provided in the kit; mix ***thoroughly*** by vortexing until completely dissolved.

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

3. Add the appropriate amount of dye to 1.0 mg of protein; mix thoroughly but gently by inversion. React for 2 hours at 20°C, protecting the vial from light.

   Note: The kit is optimized for labeling proteins at 20°C. Little difference in labeling efficiency was noted from 20-25°C. Proteins which are temperature sensitive can be labeled at 4°C; however, the labeling reaction should be incubated overnight to achieve equivalent levels of labeling. Any remaining reactive dye must be removed from the sample immediately after 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 Zeba™ Spin Desalting Columns can be downloaded from www.piercenet.com. The Zeba Spin Desalting 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. Remove the bottom closure from the column and loosen but do not remove cap.

2. Place column in a 15 mL collection tube.

3. Centrifuge column at 1,000 x *g* for 2 minutes to remove storage solution which contains azide.

   Note: If using a fixed angle rotor, place a mark on the side of the column where the compacted resin is slanted upward. Place column in centrifuge with the mark facing outward in all subsequent centrifugation steps.

4. Add 2.5 mL of 1X PBS to the column.

5. Centrifuge at 1000 x *g* for 2 minutes to remove buffer.

6. Repeat wash step two or three additional times, discarding buffer from the collection tube.

   Note: Resin will appear compacted and dry after centrifugation.

7. Place column in a new collection tube, remove cap and slowly apply the entire sample (approximately 1 mL) to the center of the compact resin bed.

8. Centrifuge at 1000 x *g* for 2 minutes to collect the sample. Discard the column after use.

9. Dye-labeled conjugates obtained following the Zeba™ Spin Desalting Column protocol typically contain less than 5% unreactive dye.

   Note: Never reuse the Zeba Spin Desalting Columns.

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.

1. Dilute the labeled conjugate 1:10 to 1:50 in a mixture of 1:1 PBS:Methanol so that the maximum absorbance reading at A_{680} 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 680 nm (A_{280} and A_{680}).

3. Calculate the dye/protein ratio using this formula: $$D/P = \left[\frac{A_{680}}{\varepsilon_{Dye}}\right] \div \left[\frac{A_{280} - (0.10 \times A_{680})}{\varepsilon_{Protein}}\right]$$

   In which:

   - 0.10 is a correction factor for the absorbance of IRDye 680LT at 280 nm (equal to 10% of its absorbance at 680 nm).

   - ε_{Dye} and $\varepsilon_{Protein}$ are molar extinction coefficients for the dye and protein, respectively.

   - ε_{Dye} is 250,000 M^{-1} cm^{-1} and $\varepsilon_{Protein}$ is 203,000 M^{-1} cm^{-1} (for a typical IgG; 150,000 *g*/mol) in a 1:1 mixture of PBS:Methanol.

   - $\varepsilon_{Protein}$ can be determined spectrophotometrically or found in the literature. (Use of the correct extinction coefficient for the protein is essential for accurate determination of the D/P ratio.)

4. Calculate the final protein concentration using this formula:

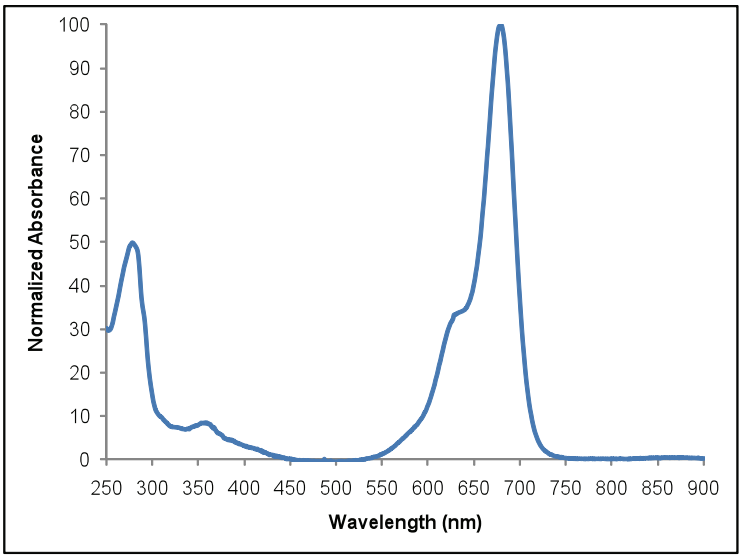
$$Protein\ Conc.\left(\frac{mg}{mL}\right) = \frac{A_{280} - (0.10 \times A_{680})}{\varepsilon_{Protein}} \times MW_{Protein} \times Dilution\ Factor$$

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 (45 - 200 kDa) purified by Zeba™ Spin Desalting Columns was found to be greater than 80%.

Figure 3. IRDye 680LT Labeled Goat anti-Mouse in 1:1 PBS:Methanol with D/P 2.3.



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). 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. Storage conditions and buffers may vary depending on the protein; however, for optimum stability, IRDye 680LT labeled proteins should be stored near pH 7.0 and 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.
- To avoid over-labeling the protein due to unreacted NHS ester, purify the labeling reaction immediately after the suggested 2 hour reaction time.

- ***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 aggregation and non-specific staining (which increases assay background). 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 for different proteins may vary widely, so we recommend that you prepare 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 is not optimal.	Use less dye or more protein in the labeling reaction.
	Labeling reaction carried out too long.	Incubate reaction for 2 hours. Purify by spin column immediately.
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.

Troubleshooting *(Continued)*

Problem	Possible Cause	Solution/Prevention
High background in Westerns (continued).	Primary antibody detects non-specific bands.	Test alternative primary antibodies with one labeled secondary antibody.
	Over-labeling of protein.	Re-check D/P ratio and possibly repeat labeling reaction using a lower amount of dye. For secondary antibodies, try more dilute solutions.
	Poor blocking.	Try a different blocking buffer.
		Try a different membrane.
Low signal in Westerns	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.
	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.

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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4647 Superior St. • P.O. Box 4000 • Lincoln, Nebraska 68504
North America: 800-645-4267 • International: 402-467-0700
LI-COR GmbH, Germany, Serving Europe, Middle East and Africa: +49 (0) 6172 17 17 771
LI-COR Ltd, UK, Serving UK, Ireland and Scandinavia: +44 (0) 1223 422104
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Doc # 988-12347
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