VRDye™ Protein Labeling Kit – High MW

Part Numbers:

928-65040 IRDye® 650 Protein Labeling Kit - High MW

928-54040 VRDye 549 Protein Labeling Kit - High MW

928-49040 VRDye 490 Protein Labeling Kit - High MW

LI-COR®

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I. Introduction

The VRDye Protein Labeling Kit – High MW is optimized to label primary or secondary antibodies for use with microscopy, flow cytometry, immunohistochemistry (IHC), or other applications where fluorophore-conjugated antibodies are required. The kit may also be used to label proteins spanning the molecular weight range of 50 kDa to 200 kDa.

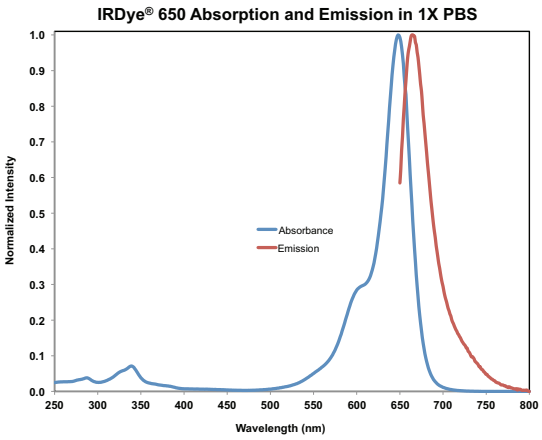
The kit is optimized for labeling 1 mg of an IgG antibody. For smaller amounts of antibody (100 µg) use IRDye® 650 Protein Labeling Kit – High MW-Microscale (P/N 928-65044); VRDye 549 Protein Labeling Kit – High MW-Microscale (P/N 928-54044), or VRDye 490 Protein Labeling Kit – High MW-Microscale (P/N 928-49044).

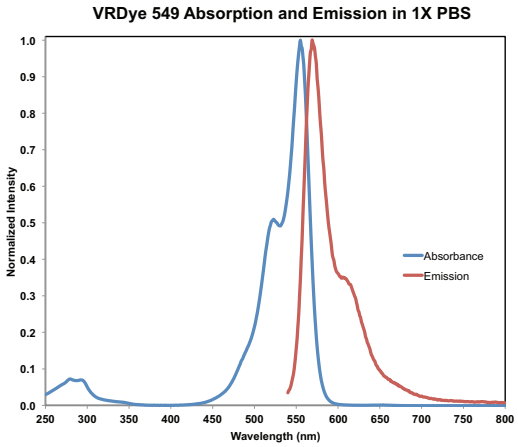
The visible dyes included in these kits are activated with N-hydroxysuccinimide (NHS) esters, which is the most commonly used reactive group for labeling proteins. NHS esters react with primary amines, forming stable, covalent bonds. The absorption maximum and minimum wavelengths are shown in Table 1.

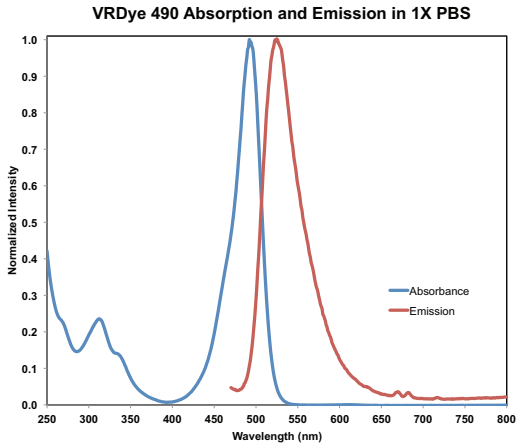
Table 1. Properties of Visible NHS Ester Dyes.

Dye	Abs_{max}	Em_{max}	$\varepsilon(M^{-1}cm^{-1})$	MW (g/mole)	CF*
IRDye 650	648	655	230,000	1097	0.03
VRDye 549	550	575	150,000	1040	0.07
VRDye 490	491	515	73,000	1011	0.11

**CF is a correction factor for the absorbance of the dye at Abs_{max} to the absorbance of the protein at 280 nm.*







II. Kit Components

- 3 Reactive Dye vials (0.15 mg) (store at -20 °C)
- 0.5 mL 1 M Potassium Phosphate (K_2HPO_4), pH 9 (store at 4 °C)
- 25 mL PBS (store at 4 °C)
- 0.5 mL ultra pure water (store at 4 °C)
- 3 Zeba™ Desalting Spin Columns, P/N 89881 (store at 4 °C)
  NOTE: The minimum recommended protein molecular weight for these columns is 7 kDa.
- Pierce Zeba Desalting Spin Column instructions
- Protocol for VRDye Protein Labeling Kit – High MW
- Quick Reference Guide

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 commercially-available kits (e.g. Pierce® BCA™ Protein Assay Kit), which are commercially available. 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 pH 8.5 with the 1 M Potassium Phosphate (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, pH 9. For example, for 1.0 mL of protein solution, add 0.1 mL of 1 M Potassium Phosphate, 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 (see IV. 3).

IV. Antibody Labeling Reaction

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

   NOTE: Dye should be added to the labeling reaction immediately as the dye reactivity decreases within 2 hours.

2. Immediately mix 8.7 µL of dye with 1.0 mg of antibody (MW 150,000 g/mole) at a concentration of 1 mg/mL.

   NOTES:
   The kit is optimized to produce a target Dye/Protein (D/P) Ratio of 5 for an IgG antibody with MW 150,000 g/mole. The labeled antibody should be suitable for microscopy, flow cytometry, and IHC applications.

   The optimal degree of labeling will vary for different applications. If a higher or lower degree of labeling (D/P) is desired for a particular application, scale the reaction accordingly.

   Over-labeling may block the antibody binding site; user optimization may be required for specific applications.

3. React for 2 hours at 20 °C, protecting the vial from light.

   NOTE: The kit is optimized for reactions at 20 - 25 °C. If the labeling reaction is performed at temperatures less than 20 °C, the reaction time should be increased. At 4 °C, the labeling reaction may need to be incubated overnight to achieve the same degree of labeling as

samples prepared at 20 - 25 °C. Any remaining dye should be removed from the sample immediately after the incubation (see Section VI).

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

V. Protein Labeling Reaction (50 – 200 kDa)

1. Use the appropriate equation to determine the volume of dye (in µL) to be added to 1 mg of protein at a concentration of 1 mg/mL.

$$Volume_{650}(\mu L) = \frac{1368}{MW_{Protein}}$$

$$Volume_{549}(\mu L) = \frac{1300}{MW_{Protein}}$$

$$Volume_{490}(\mu L) = \frac{1264}{MW_{Protein}}$$

In which :

- $Volume_{650}$ is the volume in µL of IRDye 650.
- $Volume_{549}$ is the volume in µL of VRDye 549.
- $Volume_{490}$ is the volume in µL of VRDye 490.
- $MW_{Protein}$ is the molecular weight in g/mol of the protein to be labeled.

NOTES:
The equations provide an 8-to-1 molar ratio of reactive dye to protein (1 mg of 1mg/mL) in the labeling reaction.

The molar ratio of dye to protein may need to be optimized for specific applications.

The actual D/P achieved for a given reaction ratio will vary with the characteristics of the protein.

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.

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

3. ***Immediately*** mix the appropriate volume of dye and protein.

NOTE: Dye should be added to the labeling reaction immediately as the dye reactivity decreases within 2 hours.

4. React for 2 hours at 20 °C, protecting the vial from light.

NOTE: The kit is optimized for reactions at 20 - 25 °C. If the labeling reaction is performed at temperatures less than 20 °C, the reaction time should be increased. At 4 °C, the labeling reaction may need to be incubated overnight to achieve the same degree of labeling as samples prepared at 20 - 25 °C. Any remaining dye should be removed from the sample immediately after the incubation (see Section VI).

5. Separate the free dye from the protein conjugate as outlined in Section VI.

VI. Separation of Labeled 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 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.

VII. 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 1X PBS such that the maximum absorbance reading for the dye (Abs_{max}) is less than 2.0 and the A_{280} is greater than 0.12.

2. Measure the absorbance of the conjugate at 280 nm (A_{280}) and the dye at the absorbance maximum (Abs_{max}) from Table 1.

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

In which:

- CF is a correction factor for the absorbance of the dye to the absorbance of the protein at 280 nm from Table 1.
- ε_{Dye} and $\varepsilon_{Protein}$ are molar extinction coefficients for the dye and protein, respectively.
- ε_{Dye} is the extinction coefficient for the dye (Table 1) and the protein, which is 203,000 M^{-1} cm^{-1} for a typical IgG.

  NOTE: 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:

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

In which:

- $MW_{Protein}$ is the molecular weight of the protein, which is 150,000 g/mole for a typical IgG.
- Dilution Factor is the dilution of the labeled conjugate prior to measurement by spectrophotometer.
- CF is a correction factor for the absorbance of the dye to the absorbance of the protein at 280 nm from Table 1.

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 protein purified by Pierce® Zeba™ Desalting Spin Columns was found to be greater than 80%.

VIII. Handling of Labeled Conjugates

To improve shelf life and stability of labeled antibody 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 be added only after the D/P ratio is determined, as BSA absorbs at 280 nm and will interfere with D/P ratio calculation. Antibody conjugates are stable for up to three months when stored at 4 °C and protected from light. For longer-term storage, antibody conjugates can be frozen in small aliquots. Always avoid freeze-thaw cycles of antibody conjugates, as this will greatly reduce performance.

For conjugated proteins other than IgGs, optimal storage conditions and buffers may vary; however, all dye conjugates should be protected from light.

IX. Hints and Tips

For best results, read and follow the protocol carefully.

- 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 can continue even after the suggested 2-hour reaction time, if NHS ester is still present.

X. Troubleshooting Guide

Under-labeling: Different proteins will react with the fluorophore at different rates. For this reason, the standard protocol provided here may not always produce optimal results for a specific application. Optimization may be required. 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 and biochemical activity. 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 VII) you must use the correct extinction coefficient for the protein.

XI. 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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