SAIVI™ Alexa Fluor™ 647 Antibody/Protein Labeling Kit

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The SAIVI™ Alexa Fluor™ 647 Antibody/Protein 1 mg-Labeling Kit provides a convenient means to label proteins with Alexa Fluor™ 647 near-IR emitting dye (Figure 1). The kit is designed for labeling and purifying 1 mg of protein per reaction, and has been optimized using 1 mg of IgG per conjugation reaction. Comparable amounts of other proteins (>20 kDa) can also be labeled. For labeling smaller amounts of proteins (20–100 µg), we recommend the Alexa Fluor™ 647 Microscale Protein Labeling Kit (Cat. No. A30009).

To conveniently control the average number of fluorescent dye molecules that become covalently attached to each protein molecule (the degree of labeling, or DOL), this kit includes a DOL modulating reagent and instructions for decreasing the DOL from its intrinsic highest value by adding specific amounts of DOL modulating reagent to the labeling reaction. Using this method, you can quickly and reproducibly obtain protein preparations with varying ratios of dye to protein without significantly altering the labeling conditions or the purification procedure (Figure 1), allowing more efficient optimization in applications such as *in vivo* imaging, where the DOL of a protein can have significant effects on factors such as signal-to-background, biodistribution, and blood clearance.

Contents and storage

Material	Amount	Storage[1]	Stability
Alexa Fluor™ 647 Reactive Dye (Component A)	3 vials (each containing a magnetic stir bar)	• Store at 2–6°C protected from light. • Do not freeze.	When stored properly, kit components are stable for at least 3 months.
Sodium bicarbonate (MW=84) (Component B)	84 mg		
Purification columns (Component C)[2]	3 each		
DOL modulating reagent, lyophilized solid (Component D)	1 vial		
Collection tubes	6 tubes		
Number of labelings: Each vial of reactive dye contains the appropriate amount of dye to label approximately 1 mg of IgG (MW ~145,000) as 0.5 mL of IgG solution at 2 mg/mL.			

[1] The kit can be stored under the conditions listed. For optimal storage conditions of individual components, refer to the labels on the vials or bags. Note that the reactive dye (Component A) may be stored frozen at ≤–20°C or at 2–8°C. Do not freeze the purification columns (Component C).

[2] The resin in each column is supplied in a 0.1 N NaCl/0.05% sodium azide solution.

Equipment required but not supplied

- Benchtop centrifuge capable of 1,000 × *g*

Labeling protocol

Prepare the proteins

- For optimal labeling efficiency, the purified protein must be in a buffer free of ammonium ions or primary amines.
- The presence of significant concentrations of proteins other than the intended labeling target in the solution (e.g., BSA or gelatin carrier in antibody preparations) will likely result in poor labeling, due to competition effects.
- If the protein is in or has been lyophilized from an unsuitable buffer (e.g., Tris or glycine), the buffer should be replaced with phosphate-buffered saline (PBS) by dialysis or another method. Impure proteins (e.g., antibodies in crude serum or proteins stabilized with bovine serum albumin (BSA) or gelatin) will not label well.
- The presence of low concentrations of sodium azide (≤3 mM) or thimerosal (≤1 mM) will not interfere with the conjugation reaction.

For tips on optimizing the procedure for other proteins or for antibody solutions at lower concentrations, see “Optimize the kit for use with other proteins and/or concentrations” on page 3 or “Optimization and troubleshooting” on page 3.

Labeling reaction

1. Prepare a 1 M solution of sodium bicarbonate by adding 1 mL of deionized water (dH_2O) to the provided vial of sodium bicarbonate (Component B). Vortex or pipet up and down until fully dissolved. The bicarbonate solution, which will have a pH ~8.3, can be stored at 4°C for up to 2 weeks.

2. Prepare a solution of DOL modulating reagent. Briefly centrifuge the DOL modulating reagent (Component D) to collect any solid at the bottom of the vial. Add 330 µL of sterile deionized water, cap the tube tightly, and vortex.

3. Prepare 0.5 mL of a ~2 mg/mL protein solution by diluting it with an appropriate buffer and add sodium bicarbonates as directed here, depending on the nature of the starting protein sample.
   - For protein samples already in a volume of appropriate buffer, add 1/10 volume of 1 M sodium bicarbonate to the ~500 µL of protein sample (at 2 mg/mL).
   - For protein samples lyophilized from an appropriate buffer, prepare a 2 mg/mL solution of the protein by adding a sufficient volume of 0.1 M sodium bicarbonate buffer. (Prepare 0.1 M sodium bicarbonate buffer by diluting the 1 M solution from step 1 10-fold with deionized water.)

4. If desired, add an appropriate volume of DOL modulating reagent solution to the protein vial and mix gently but thoroughly. To decrease the DOL by ~40%, add 15 µL of the DOL modulating reagent solution prepared in step 2. To decrease the DOL by ~70%, add 50 µL of the DOL modulating reagent solution.

5. Transfer the protein solution to a vial of Alexa Fluor™ 647 reactive dye. This vial contains a magnetic stir bar. Gently pipette the solution up and down to fully dissolve the dye (excess agitation of the solution can result in protein denaturation.

6. Stir the solution for ~1 hour at room temperature, protected from light. You can perform labeling at lower temperatures, if necessary; labeling overnight on ice usually yields results similar to those achieved by labeling for 1 hour at room temperature. DOL control using the DOL modulating reagent works similarly at either temperature.

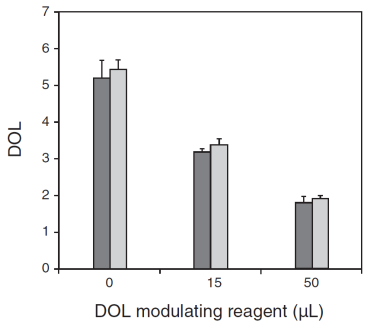

Figure 1 Modulation of the degree of labeling achieved using the DOL modulating reagent. Observed labeling is 60% and 30% of the unmodulated value using 15 µL or 50 µL DOL modulating reagent, respectively. Labeling results obtained at room temperature for 60 minutes or on ice for 20 hours are essentially identical.

Purify the labeled proteins

Thermo Scientific™ Zeba™ Dye and Biotin Removal Spin Columns contain a ready-to-use resin that is uniquely designed for rapid removal of non-conjugated fluorescent dyes with exceptional protein recovery. The purification resin is designed to separate free dye from proteins with MW >20 kDa. For smaller proteins, gel filtration media of a suitable molecular weight cutoff should be selected. Labeled peptides may be separated from free dye by TLC or HPLC. Removal of free dye after a labeling reaction is essential for the accurate determination of dye to protein ratios. For optimal protein recovery and dye removal, ensure that the appropriate amount of sample and buffer conditions are used.

IMPORTANT! Protein conjugates that are between 20–50 kDa require a more alkaline buffer system to elute and will retain on the column if the buffer system is not changed. See procedure below for purifying 20–50 kDa conjugates.

Procedural guidelines

- Do not reuse the purification resin.
- Limit DMF and other organic solvents to ≤10% of solvent volume loaded onto the column.
- If labeling a 20-50 kDa protein, refer to "Purify 20-50 kDa conjugates" on page 2 to ensure conjugate recovery.

Prepare the spin column

1. Twist to remove the bottom plug of the column, then loosen the cap. Do not remove the cap.

2. Place the column in a collection tube, then centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes to remove the storage buffer. Discard the flowthrough.

3. If using a fixed-angle rotor, place a mark facing away from the rotor center. For all subsequent centrifugation steps, place the column in the centrifuge with the mark facing away from the rotor center.

   IMPORTANT! Improper orientation of the column during centrifugation can result in reduced small molecule removal.

4. If desired, the resin storage buffer can be exchanged using a buffer of choice. To exchange, add 2 mL of equilibration buffer to the column, then centrifuge at 1,000 × *g* for 2 minutes. Discard the flowthrough.

Purify 20-50 kDa conjugates

If purifying a 20–50 kDa protein, a buffer exchange is required to ensure conjugate recovery.

1. Following storage buffer removal, apply 500 µL of 0.2 M, pH 9.4 bicarbonate buffer to the column (Cat. No. 28382).

2. Centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes.

3. For optimal conjugate recovery, repeat steps 1 and 2 two more times for a total of 3 column washes to ensure equilibration.

Process the sample

1. Place the prepared column into a new collection tube, then remove the cap.
2. Slowly apply the reaction mixture (~0.5 mL) of sample to the center of the settled resin.
3. Centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes to collect the sample. The sample will be in the collection tube, and the column can now be discarded.
4. *(Optional)* The column may be washed with an additional ~0.5 mL of suitable buffer (e.g., PBS) to maximize the recovered sample, applied as in steps 2 and 3. Note that this extra wash step will dilute the recovered conjugate and may be omitted if higher concentration is desired.

Determine the Degree of Labeling *(Optional)*

1. Dilute the absorbance of the purified conjugate at both 280 nm (A_{280}) and 650 nm (A_{650}) using a diluted sample from the conjugation.
2. Calculate the concentration of protein in the sample:

$$\text{Protein concentration (M)} = \frac{[A_{280} - (A_{650} \times 0.03)] \times \text{dilution factor}}{203{,}000}$$

Where 203,000 is the molar extinction coefficient (ε) in $cm^{-1}M^{-1}$ of a typical IgG at 280 nm and 0.03 is a correction factor for the fluorophore's contribution to the absorbance at 280 nm. If you know the molar extinction coefficient (in $cm^{-1}M^{-1}$) of your protein at 280 nm, use this value as the divisor in the equation above to calculate the molarity of your protein.

3. Calculate the degree of labeling:

$$\text{Moles dye per mole protein} = \frac{A_{650} \times \text{dilution factor}}{239{,}000 \times \text{protein concentration (M)}}$$

Where 239,000 is the molar extinction coefficient (ε) in $cm^{-1}M^{-1}$ of Alexa Fluor™ 647 dye at 650 nm.
For IgGs, we find that labeling with 3–7 moles of Alexa Fluor™ 647 dye per mole of antibody is optimal in many applications. The effects of DOL on factors such as signal-to-background, biodistribution, and blood clearance in *in vivo* applications may often be difficult to predict; therefore, preparation of conjugates at several dye:protein ratios may be necessary. Repeating the conjugation process with a new sample of the same protein (under otherwise identical conditions) in the presence of the DOL modulating reagent will provide conjugates with predictably lower DOL values.

4. If DOL modulation is carried out, calculate the % residual DOL in modulated preparations compared with the baseline (unmodulated) DOL as follows:

$$\frac{\text{DOL (with DOL modulating reagent)}}{\text{DOL (without DOL modulating reagent)}} \times 100 = \%\ \text{baseline DOL}$$

Storing and handling conjugates

Store the labeled protein at 2–8°C, protected from light. If the final concentration of purified protein conjugate is less than 1 mg/mL, add BSA or other stabilizing protein at 1–10 mg/mL. In the presence of 2 mM sodium azide, the conjugate should be stable at 2–8°C for several months. For longer storage, divide the conjugate into small aliquots and freeze at ≤–20°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.

Optimize the kit for use with other proteins and/or concentrations

- Proteins at less than 2 mg/mL
  - Proteins at concentrations less than 2 mg/mL will not label as efficiently. If the protein cannot be concentrated to ~2 mg/mL, you may wish to use less than 1 mg protein per reaction to increase the molar ratio of dye to protein. In addition, using a dilute protein solution, especially at <1 mg/mL will make it more difficult to efficiently remove the unconjugated dye from the dye-labeled protein with acceptable yields, since the provided purification columns are designed to purify conjugates from a total volume of less than 1 mL. For reaction volumes greater than 1 mL, you can divide the solution of the conjugate and apply it to multiple purification columns or, to avoid further dilution of the conjugate, you can remove free dye by extensive dialysis.
- Proteins with molecular weight (MW) other than ~145,000
  - Typically, lower MW proteins require fewer dye molecules and higher MW proteins require more dye molecules per protein for optimal labeling. For this reason, we recommend initially performing the reaction with 0.5 mL of 2 mg/mL protein solution, as described for IgGs. The labeling conditions can then be optimized based on the initial results, if desired.

Optimization and troubleshooting

Under-labeling

If calculations indicate that the protein is labeled with significantly less than 3 moles of fluorophore per mole of 145,000–150,000 MW protein, your protein may be underlabeled. A number of conditions can cause a protein to label inefficiently.

- Trace amounts of primary amine-containing components in the buffer react with the dye and decrease the efficiency of protein labeling. If your protein has been in amine-containing buffers (e.g., Tris or glycine), dialyze extensively versus PBS before labeling.
- Dilute solutions of protein (≤1 mg/mL) will not label efficiently.
- The addition of sodium bicarbonate (step 5) is designed to raise the pH of the reaction mixture to ~8, because TFP and

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.

- Because proteins, including different antibodies, react with fluorophores at different rates and retain biological activity at different degrees of dye labeling, the standard protocol may not always result in optimal labeling. To increase the amount of labeling, you can relabel the same protein sample, or you can label a new protein sample using less protein or more reactive dye per reaction. To increase the amount of dye in the reaction, you can combine the contents of two vials of reactive dye together. Some researchers obtain better labeling with overnight incubations at 2–8°C after an initial incubation of 1 hour at room temperature.
- If the protein did not elute during centrifugation, the conjugate may be eluted using a high salt concentration buffer (e.g., PBS, 0.3 M NaCl) or 0.2 M, pH 9.4 bicarbonate. Incubate the column with end-over-end mixing for 1 minute, then elute conjugate by spinning at 1,000 x *g* for 2 minutes. We recommend re-purifying the conjugate using size-exclusion resin, dialysis, or spin filtration.

Over-labeling

If calculations indicate that the protein conjugate is labeled with significantly higher DOL than recommended (moles of fluorophore per mole of 145,000-150,000 MW protein, your protein is probably 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 nonspecific staining. Over-labeling can also cause fluorescence quenching of the conjugate. To reduce the amount of labeling, add more protein to your 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

Despite removing most free dye from protein conjugates using the provided spin columns, it is possible that trace amounts of free dye will remain in the conjugate solution after purification. 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 (“Determine the Degree of Labeling (Optional)” on page 3). Remaining traces of free dye can be removed by applying the conjugate to another column or by extensive dialysis.

Limited product warranty

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Revision	Date	Description
A.0	21 January 2021	New manual.