Fluorescein One-Shot Antibody-Labeling Kit

Technical Manual

Catalog # F-9001-009K

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Safety Information

WARNING – CHEMICAL HAZARD. Some chemicals used can be potentially hazardous, and can cause injury or illness.

- Read and understand the Material Safety Data Sheets (MSDS) available at Solulink.com before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (e.g. safety glasses, gloves, or clothing). For additional safety guidelines consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's clean-up procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling and disposal.

Table of Contents

I. Introduction

a. Product Description

The Fluorescein Antibody Labeling Kit is designed to label a microscale quantity of antibody (100 µg) with 3 to 5 fluorescein molecules per antibody. The kit contains sufficient reagents to perform two labeling reactions, 100 µg of antibody per reaction. Fluorescein-labeled antibodies can be used for standard immunofluorescent staining and imaging of cells or tissues. This kit contains all the necessary components to label and purify an antibody in about 90 minutes.

b. Features and Benefits

The Fluorescein Antibody Labeling Kit uses an isomeric mixture of the 5- and 6-carboxyfluorescein N-hydroxysuccinimidyl ester **(Figure 1)** to modify antibody lysine residues. Reaction conditions have been optimized to provide a consistent level of fluorescein incorporation and maximal immunofluorescent signal output. The kit includes simple step-by-step instructions for labeling and quantification of incorporated fluorescein molecules. The **Fluorescein Protein MSR Calculator** is recommended that automatically determines antibody concentrations and fluorescein incorporation levels without the user having to perform complicated calculations. The kit can be used to attach fluorescein groups to all types of mammalian antibodies, including mouse, rat, rabbit, goat, sheep, horse, cow, hamster, or human IgG.

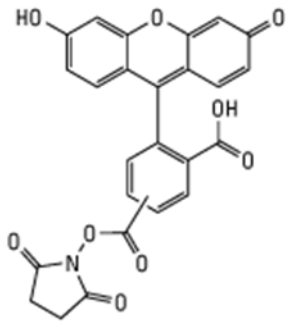


Figure 1. 5-(and 6-) carboxyfluorescein succinimidyl ester. Structure of 5-(and 6-) carboxyfluorescein succinimidyl ester used for labeling antibodies in the kit.

c. Fluorescein Procedure Diagram

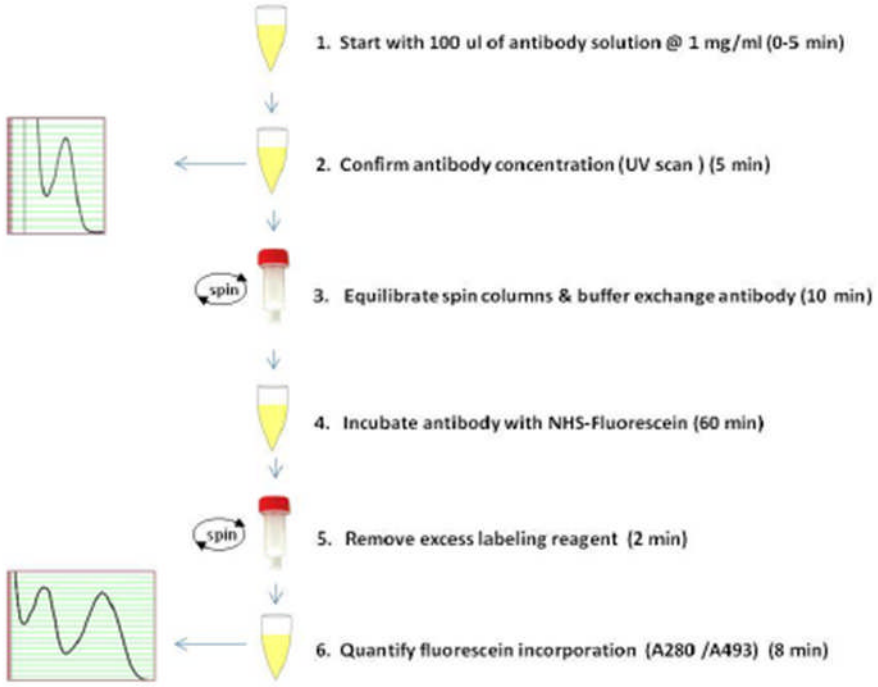


Figure 2. Summary of Fluorescein labeling procecure.

d. Process Summary

1. ***Sample Preparation***: Adjust antibody to 1 mg/mL in 100 µL buffer
2. ***Sample Analysis:*** Confirm initial antibody concentration with a spectrophotometer
3. ***First Buffer Exchange:*** Equilibrate spin columns & buffer exchange antibody
4. ***Label Antibody***: Incubate antibody with NHS-Fluorescein
5. ***Second Buffer Exchange:*** Remove excess labeling reagent
6. ***Quantify Fluorescein Incorporation:*** Measure A_{280}/A_{494} and use provided **Fluorescein Protein MSR Calculator**

e. Important Labeling Parameters to Consider

The Fluorescein Antibody Labeling Kit is specifically designed to add fluorescein groups to a single 100 microgram quantity of antibody. The kit provides consistent and reliable fluorescein incorporation by controlling the following reaction variables:

- Initial antibody mass (100 µg) and volume (100 µL)
- Reaction buffer ionic composition and pH
- Reaction time (60 min)
- Reaction stoichiometry

A factor critical to labeling consistency is the accurate confirmation of initial antibody concentration. The procedure recommends the use of a spectral scan (220–350 nm)

rather than a single wavelength scan at 280 nm to measure antibody concentration. A spectral scan provides additional information that often reveals the presence of interfering additives or preservatives that will alter the sample's A_{280}. Common additives present in commercially obtained antibody preparations include preservatives such as sodium azide, thimerosal, protein stabilizers such as *BSA or gelatin, or other small-molecule additives such as glycine or trehalose. If a commercial source of antibody contains any of these additives, please refer to the Troubleshooting Guide section in the Appendix to determine how to proceed.

f. Materials Provided and Storage Conditions

Components	Unit	Storage Conditions
NHS-Fluorescein	2 x 10.0 µg	Room temp.
10X Modification Buffer	1.5 mL	Room temp.
Collection Tubes	8 x 1.5 mL	Room temp.
Zeba™ Spin Columns	4 x 0.5 mL	Room temp.
Fluorescein-IgG Control	100 µg	Room temp.
Anhydrous DMF	500 µL	Room temp.

g. Additional Materials Required but Not Provided

UV-VIS or NanoDrop™ Spectrophotometer

Semi-micro quartz cuvette (50–100 µL capacity) (not required w/NanoDrop™) Variable speed microcentrifuge (e.g., Eppendorf 5415D, IEC MicroMax, or similar) 1.5 mL microfuge tubes, molecular grade water, P-10, P-200, P-1000 pipettes

II. Fluorescein Antibody Labeling Protocol

a. Sample Preparation (0–5 min)

Antibodies are packaged in a variety of different physical forms, including solids and liquids. Individual antibody samples vary greatly from vendor to vendor and are often sold in a variety of different sizes and/or concentrations. Proceed as follows to prepare your particular sample for labeling.

Antibody Sample (Solid Form) Initial

Sample: 100 µg/vial

Resuspend the sample in 100 µL 1X Modification Buffer (1:10 Diluted from 10X Modification Buffer; add 1 mL Modification Buffer to 9 mL molecular grade water) to yield a 1 mg/mL solution and proceed directly to Sample Analysis (Section II-b).

Initial Sample: >120 µg/vial

Resuspend the sample in a sufficient volume of 1X Modification Buffer to yield a 1 mg/mL solution. Transfer a 100 µL volume to a new 1.5 mL tube and refrigerate the unused portion of the sample. Proceed directly to Sample Analysis (Section II-b).

Initial Sample: <90 µg/vial

We do not recommend using the Fluorescein Antibody Labeling Kit to label antibody quantities smaller than 90 µg. Obtain additional material before proceeding to Sample Analysis (Section II-b).

Antibody Sample (Liquid Form) Initial

Sample: 1 mg/mL

If the initial antibody sample is already resuspended in a suitable buffer such as PBS or TBS at 1 mg/mL and 100 µL, proceed directly to Sample Analysis (Section II-b).

Initial Sample: >1 mg/mL

If the initial antibody sample is resuspended at a concentration greater than 1 mg/mL, transfer a volume equal to 100 µg to a new tube and dilute the sample to a final volume of 100 µL using 1X Modification Buffer. Proceed directly to Sample Analysis (Section II-b).

Initial Sample: <1 mg/mL

If the initial antibody sample is resuspended at a concentration significantly less than 1 mg/mL, the antibody sample will require a concentration step before proceeding. The sample must first be concentrated to 1 mg/mL and 100 µL using a suitable ultrafiltration spin filter. Spin filters are available from various vendors (e.g., Amicon or Sartorius). An ultrafiltration spin filter is not provided with this kit.

b. Sample Analysis (5–10 min)

Measure and confirm antibody concentration by scanning (220–350 nm) the sample on a UV-VIS or NanoDrop™ spectrophotometer as outlined below for your specific type of instrument.

Important: Do not attempt to label or quantify antibody samples containing protein-based carriers such as BSA or gelatin (see Troubleshooting Guide).

b1. UV-VIS Spectrophotometer

1. Program a spectrophotometer to scan from 220–-350 nm. Follow the manufacturer's instructions for each instrument.
2. Using a clean semi-micro quartz cuvette, blank the instrument with the appropriate sample buffer (e.g., 1X Modification Buffer).
3. Discard the blank solution.
4. Transfer the antibody sample (e.g., 100 µL @ 1 mg/mL) to the clean cuvette and scan.
5. Record the A_{280} from the scan.
6. Recover the sample volume from the cuvette and transfer to a clean 1.5 mL microfuge tube.

b2. NanoDrop™ Spectrophotometer

1. Blank the NanoDrop™ spectrophotometer by placing a 2 µL drop of the appropriate sample buffer (e.g., PBS or 1X Modification Buffer) on the sample pedestal and click the 'Blank' icon.
2. After blanking, immediately reclick the 'Measure' icon to validate a flat baseline. Clean the pedestal and repeat (if necessary) until a flat baseline is obtained.

   Note: Sometimes air bubbles become trapped on the pedestal during sample measurement causing baseline offsets. Remove trapped air bubbles and rescan to ensure that a proper baseline is obtained.
3. Transfer a 2 µL volume of antibody solution @ 1 mg/mL to the pedestal; click the 'Measure' icon. Wait until the spectrum (220–350 nm) appears in the window. **Note:** There is no need to recover the 2 µL aliquot after a scan.
4. Record the antibody concentration directly from the NanoDrop™ display window in mg/mL.
5. After confirming antibody concentration and volume (1.0 + 0.1 mg/mL and 100 + 10 µL), proceed directly to the buffer exchange procedure (Section II-c).

   Note: Antibody concentration as well as sample volume is important because an accurate mass and volume estimation is critical to maintaining the specified ratio of labeling reagent to antibody during the modification reaction.

   Important: If the antibody spectrum appears altered, distorted, or contains a large baseline offset, please refer to the Troubleshooting Guide found in the Appendix.

c. First Buffer Exchange Procedure (10 min)

1. Prepare the two spin columns provided by twisting off the bottom closures and loosening the red caps (do not remove caps).
2. Place each spin column into separate collection tubes (provided).
3. Place spin columns opposite each other in the centrifuge and spin @ 1,500 x g for 1 min to remove the storage solution from the resin. After centrifugation, the column matrix will appear dry and white in color.
4. Remove each column from the centrifuge and discard the eluate from each collection tube.
   Note: Do not discard the collection tubes.
5. Using a marker pen, place a mark on the outside of each spin column where the compacted resin is slanted upward.
6. Using the same marker pen, mark the top of one cap with the letter **A** and the other with the letter **B**.
7. Add 300 µL **1X Modification Buffer** to the top of resin beds **A** and **B.** Loosely recap the lids.
   Note: When loading buffer or sample, do not disturb the resin bed with the end of the pipette tip.
8. Place the spin columns back into their collection tubes, centrifuge at 1,500 x g for 1 min.
   Important: Always orient the spin column with the pen mark aiming outward and away from the center of the rotor.
9. Repeat steps 7 and 8 two additional times, discarding the flow-through buffer each time.

10. After the final spin, transfer spin column A to **a new collection tube (provided).** **Note**: Do not transfer the **B** spin column to a new 1.5 mL collection tube at this time.
11. Load the antibody sample (100 µL @ 1mg/mL) onto the top of spin column **A** resin.
12. Add 100 µL **1X Modification Buffer** to the top of spin column **B** resin. Recap the lid loosely.
    Note: The **B** assembly now only serves as a balance tube.
13. Centrifuge the oriented **A** and **B** spin columns @ **1500 x g for 2 min.**
14. Set the eluate from collection tube **A** aside and transfer later. **Note**: Do not discard the collection tube.
15. Remove the **B** spin column assembly from the centrifuge and discard the eluate from the collection tube. Add 300 µL **1X Modification Buffer** to the **B** resin, cap the spin column, and set it aside in the same collection tube for later use.
    Important: 300 µL of **1X Modification Buffer** is necessary to keep the resin bed hydrated for the next hour.

d. Fluorescein Labeling Procedure (60 min)

1. Open a vial of Fluorescein-NHS labeling reagent and add 5 µL DMF (provided) directly to the bottom of the vial.
   Important: Do not add more than 5 µL DMF.
2. With the aid of a P-10 pipette, carefully pipette the DMF solution up and down, rinsing several times to completely dissolve the yellow Fluorescein-NHS pellet on the bottom. The solution will appear clear but slightly yellow in color.
3. Add the buffer-exchanged antibody solution from step c-14 above to the Fluorescein-NHS/DMF solution and gently pipette to mix. The solution should be clear and yellow in appearance.
4. Allow the reaction to proceed **for 60 min** at room temperature **in the dark.**
5. Place the still hydrated **B** spin column assembly (step c-15) containing 300 µL **1X Modification Buffer** from section into the centrifuge. Add 300 µL water to the previously used **A** spin column assembly and place it opposite the **B** spin column in the centrifuge as a balance tube.
6. Centrifuge the two columns at **1,500 x g for 1 min**. Discard the flow-through from each spin column assembly.
7. Transfer the **B** spin column to **a new collection tube (provided)** and proceed to the next section.

e. Second Buffer Exchange Procedure (3 min)

1. Add the contents of the fluorescein labeling reaction (from step d-4 above) to the center of the compacted **B** resin spin column. Recap the column loosely.
2. Apply 100 µL of water to the center of the **A** spin column. Recap loosely.
   Note: This column will serve as a balance tube.
3. Orient the two columns in the centrifuge and spin at **1,500 x g for 2 min**.
   Note: Approximately 100 µL should be recovered from the bottom of each collection tube.
4. Transfer the fluorescein-labeled antibody from the bottom of the **B** collection tube to a new amber 1.5 mL tube (or **covered by foil wrap.)**

f. Determination of Fluorescein Incorporation (10 min)

The degree of fluorescein incorporation is determined by scanning the fluorescein-labeled antibody on a UV-VIS or NanoDrop™ spectrophotometer. However, some alterations to each instrument's scanning procedure are required.

f1. UV-VIS Spectrophotometer

1. Program a spectrophotometer to scan from 220–-600 nm. Follow the manufacturer's instructions for each instrument.
2. Using a clean semi-micro quartz cuvette (50–100 µL), blank the instrument using an appropriate volume of 1X Modification Buffer (provided).
3. Discard the blank solution.
4. Transfer the fluorescein-labeled antibody (e.g., 100 µL @ 1 mg/mL) to the clean cuvette and scan.
5. Obtain and record the A_{280} and A_{494} from the scan.
6. Recover the sample from the cuvette and transfer to a clean 1.5 mL tube. Label appropriately.
7. Input the sample volume and absorbance values into the calculator available at Solulink.com to determine the fluorescein molar substitution ratio (MSR).

f2. NanoDrop™ Spectrophotometer

1. Turn on the NanoDrop™ spectrophotometer and click on the NanoDrop™ icon to launch the software.
2. Place a 2 µL drop of molecular grade water on the clean pedestal; click OK.
3. When the main menu appears, select the UV-VIS menu option. **Note:** Do not use the Protein A_{280} menu option on the NanoDrop™.

4. After the UV-VIS menu appears, blank the NanoDrop™ spectrophotometer by placing a 2 µL drop of 1X Modificaton Buffer on the sample pedestal and click the 'Blank' icon.

5. After blanking, immediately reclick the 'Measure' icon to validate a flat baseline. Clean the pedestal and repeat (if necessary) until a flat baseline is obtained.
   Note: Sometimes air bubbles become trapped on the pedestal during sample measurement, causing baseline offsets. Remove trapped air bubbles and rescan to ensure a proper baseline is obtained.

6. Transfer a 2 µL volume of the fluorescein-labeled antibody solution to the pedestal; click the 'Measure' icon. Wait until the spectrum (220–750 nm) appears in the window. **Note:** There is no need to recover the 2 µL aliquot after a scan.

7. Obtain and record the A_{280} and A_{494} values from the scan. **Multiply the values by ten** and record each value.
   Note: Absorbance values need to be converted from a 1 mm path length to a 10 mm path length.

8. Input the total sample volume and both absorbance values into the **Fluorescein Protein MSR Calculator** to determine the fluorescein molar substitution ratio (MSR).

III. Labeling Antibodies with Fluorescein: Some Examples

Fluorescein Labeling of Bovine IgG (Example 1)

A bovine IgG (100 µg lyophilized solid) was dissolved in 100 µL 1X Modification Buffer to yield a 1 mg/mL solution. The sample was then scanned on a NanoDrop™ spectrophotometer (Figure 4) in the software's UV-VIS menu. The spectrum of the antibody was ideal as expected for a pure antibody, free of additives or preservatives. The initial concentration was determined to be 1.03 mg/mL (E1% value of 12.40) . After a 1st buffer exchange, the antibody was labeled using NHS-Fluorescein reagent for 60 minutes as described. The buffer-exchanged antibody was then rescanned (220–750 nm) on the NanoDrop™ in the UV-VIS menu as illustrated in Figure 6. The fluorescein molar substitution ratio (MSR) was then obtained using **Fluorescein Protein MSR Calculator**. The resulting fluorescein MSR was 4.3 with a 87 µg of recovery of antibody.

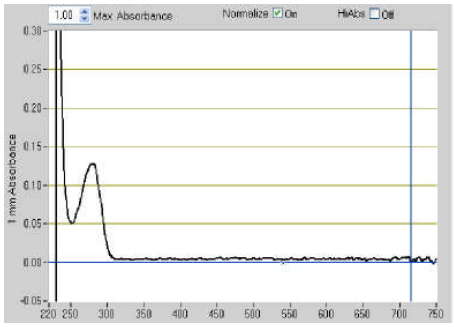


Figure 4. Antibody after 1st buffer exchange.

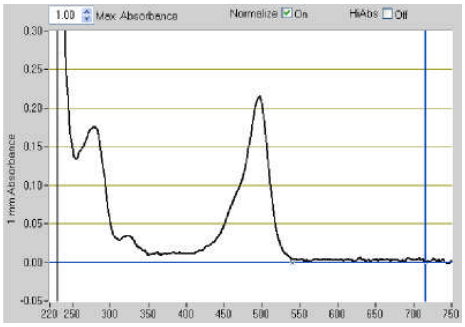


Figure 5. Antibody after labeling with fluorescein.

The same bovine IgG sample was scanned on a Beckman spectrophotometer:

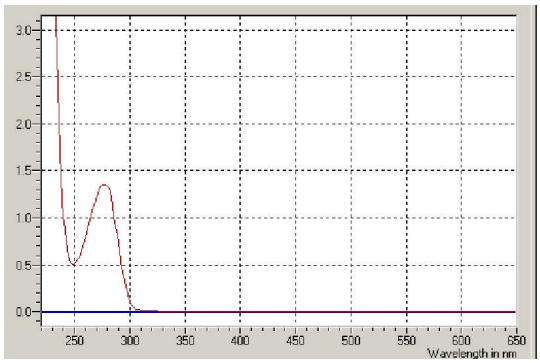


Figure 6. Antibody after 1st buffer exchange.

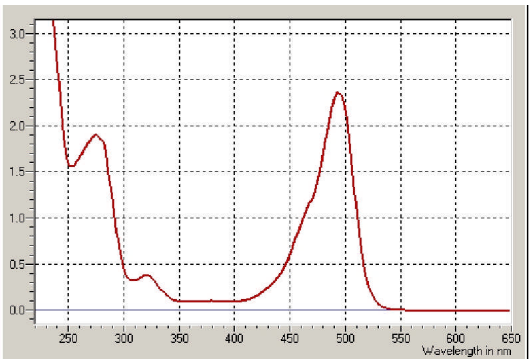


Figure 7. Antibody after labeling with fluorescein.

Fluorescein Labeling of Affinity-Purified Anti-HER2 (Example 2)

A commercial anti- her2 (100 µg lyophilized solid) was dissolved in 100 µL 1X Modification Buffer to obtain a 1 mg/mL solution. An aliquot (2 µL) of the sample was first scanned on a NanoDrop™ spectrophotometer with the protein A_{280} menu to confirm the initial antibody concentration **(Figure 8).** A buffer exchange into 1X Modification Buffer was then carried out as described using a spin column **(Figure 9).** The sample was then labeled with NHS-Fluorescein reagent for 60 minutes. The labeled sample was then rescanned (220–750 nm) as shown in **Figure 10.** The fluorescein molar substitution ratio (MSR) was then obtained using **Fluorescein Protein MSR Calculator**. The resulting fluorescein MSR was 4.7 with a 92 µg of recovery of antibody.

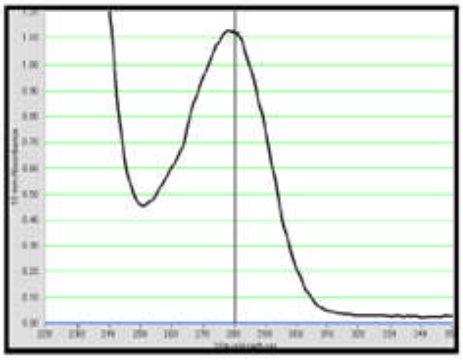


Figure 8. Antibody before buffer exchange. (protein A_{280} menu)

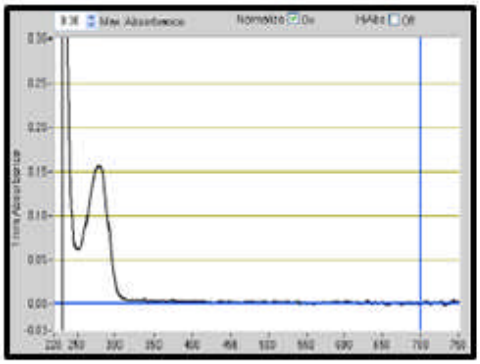


Figure 9. Antibody after buffer exchange (UV-VIS menu)

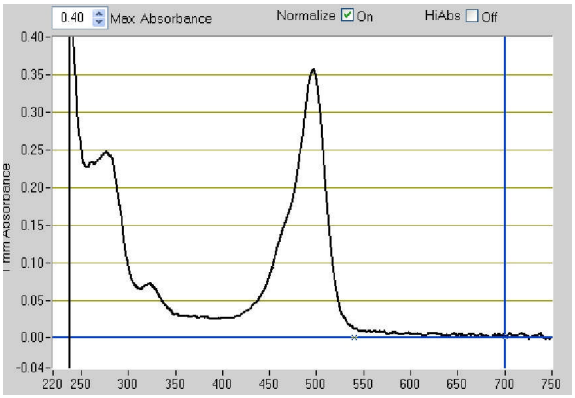


Figure 10. Antibody after fluorescein labeling (UV-VIS menu).

IV. Appendix

a. Fluorescein IgG Control

The Fluorescein Antibody Labeling Kit provides a lyophilized fluorescein- labeled bovine IgG at a known fluorescein molar substitution ratio (refer to lot-specific certificate of analysis). This control can be used to validate the accuracy of your measurements on a spectrophotometer or NanoDrop™. To use the control, proceed as follows:

1. In a semi-micro quartz cuvette (50–100 µL), record a "blank" buffer spectrum using 1X Modification Buffer (220–700 nm). Discard the blank buffer solution from the cuvette. **Note:** A NanoDrop™ (UV-VIS) can also be used to scan the sample.
2. Resuspend the control (lyophilized fluorescein-IgG, 100 µg) using **100 µL molecular grade water** at 1.0 mg/mL and close the lid.
3. Centrifuge the control very briefly at low speed (30 sec at 1,500 x g).
4. Remove an aliquot and scan the fluorescein-labeled antibody.
5. Record the A_{280} and A_{494} values generated from the spectrum.
6. Input these values along with the E1% (12.40) and MW (150 kDa) into the **Fluorescein Protein MSR Calculator** to determine the fluorescein molar substitution ratio.
7. Confirm the value obtained with the lot-specific MSR found on the product data sheet.

b. Troubleshooting Guide

Many of the problems associated with labeling antibodies using the procedure arise from inaccuracies in measuring initial antibody concentration. For this reason, the protocol uses a scan (220–400 nm) to estimate antibody concentration rather than a single wavelength measurement @ 280 nm.

A scan provides greater assurance that a sample's concentration is accurate, since a spectrum will often reveal the presence of A_{280}-altering additives. These spectral aberrations are often revealed when scanning a sample, but not from a single point, A_{280} measurement. Distortions to an antibody's intrinsic spectrum or other spectral aberrations such as baseline offset errors will lead to problems when estimating sample concentration. Errors of this type are likely to lead to poor labeling results because the protocol requires precise control of the stoichiometry between antibody mass and labeling reagent (NHS-Fluorescein).

Spectral errors can also be associated with antibody preparations that contain additives or preservatives. A host of factors can affect the accuracy of the expected concentration, including:

Antibody sample contains preservatives (e.g., sodium azide or thimerosal)
Antibody sample contains protein-based additives (e.g., BSA or gelatin) Antibody sample contains an unknown concentration of some additive Antibody (protein) becomes degraded during storage
Buffer blank is unknown or cannot be reproduced (baseline offset errors)
Antibody sample is under-filled by vendor
Antibody sample is over-filled by vendor
Improperly calibrated spectrophotometer (e.g., lamp output may be low)

In the examples that follow, we provide various spectra for reference and comparison purposes. These are intended to aid the troubleshooting process. Suggested corrective actions are also included for each type of sample spectrum problem.

Example 1. High-purity mouse IgG (lyophilized solid, no additives or preservatives).

A commercial mouse IgG (100 µg solid) was resuspended in 100 µL 1X Modification Buffer @ 1 mg/mL solution; the sample was scanned as illustrated in **Figure 11**. This sample was free of all preservatives, protein-stabilizers, and any other interfering additives. Note the ideal shape of the spectrum, confirming both purity and concentration of this 'ideal' sample.

Corrective action: None. The theoretically expected A_{280} value for this mouse IgG is 1.35 vs. the experimentally measured value of 1.34 **(see Table 4**). The measured value is well within the acceptable labeling range (1 $\pm$ 0.1 mg/mL).

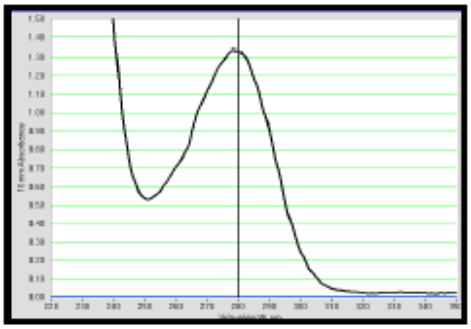


Figure 11. Antibody without preservatives or additives. UV -spectrum (220–350 nm) of a highly purified mouse IgG antibody @ 1 mg/mL without any additives or preservatives. Note the uniform shape and flat baseline @ 350 nm.

Antibody Source	Antibody A280 (1-cm path)
Human IgG	1.36 ± 0.136
Human IgE	1.53 ± 0.153
Rabbit IgG	1.35 ± 0.135
Donkey IgG	1.50 ± 0.150
Horse IgG	1.50 ± 0.150
Mouse IgG	1.40 ± 0.140
Rat IgG	1.40 ± 0.140
Bovine IgG	1.24 ± 0.124
Goat IgG	1.36 ± 0.136
Avian IgY	1.27 ± 0.127

Table 4. Expected A_{280} value of various antibody solutions (1 ± 0.1 mg/ml) for the listed antibodies (1 cm pathlength).

Example 2 Bovine IgG containing sodium azide preservative.

The presence of sodium azide @ 0.05 or 0.1% in a sample of bovine IgG @ 0.9 mg/mL is illustrated in **Figure 12.** The presence of this additive primarily alters the shape of the antibody's spectrum. As seen in the figure, the presence of this additive does not alter the measurement of the sample's true protein concentration. However, this is not always the case at high or unknown concentrations of additive. Uncertainties in the concentration of this additive may sometimes lead to large baseline offset errors when blanking the spectrophotometer, making it difficult to estimate protein concentration.

Corrective action: None if sample spectra are similar to those in **Figure 12**. These samples contain no significant baseline offset errors or other spectral distortions other than attenuation of the spectrum's valley and a general shift from 250 to 260 nm. Passage through the 1st spin column will remove all traces of azide

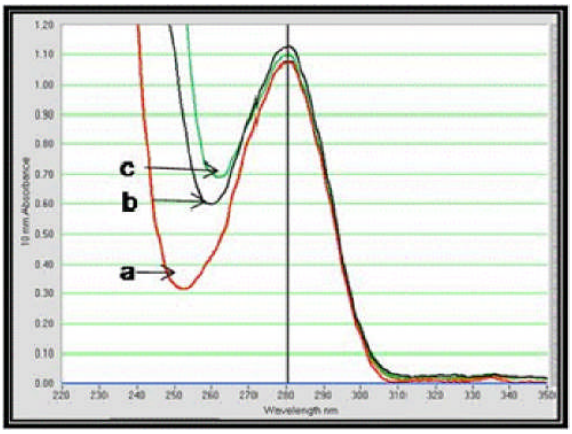


Figure 12. Antibody with known azide concentration. Superimposed spectra of purified bovine IgG @ 0.90 mg/mL with a) no sodium azide, b) spiked with 0.05% sodium azide, or c) spiked with 0.1% sodium azide.

Example 3. Rat IgG containing an unknown concentration of sodium azide.

Sometimes antibody samples contain high or unknown amounts of sodium azide. If the quantity of azide is not precisely known, it becomes difficult to properly blank a sample on the spectrophotometer. Unknown or high concentrations of this additive often contribute to large baseline offset errors that preclude accurate estimates of initial protein concentration. Two examples are illustrated in **Figure 13**.

Panel A illustrates the presence of high concentrations of sodium azide, leading to both a positive baseline offset error and a distorted antibody spectrum. **Panel B** illustrates a severe negative baseline offset error caused by the presence of an unknown quantity of the preservative. Both examples make an accurate estimate of initial protein concentration impossible.

Corrective action: In both **Panel A** and **Panel B**, we recommend passing each sample through the 1st spin column and rescanning to confirm antibody concentration. After the spin column exchanges the sample into 1X Modification Buffer, rescan the sample and recalculate the protein concentration. If the sample spectrum confirms a concentration of 1 $\pm$ 0.1 mg/mL, proceed to the fluorescein labeling procedure (II-e). If the sample contains significantly less or more than 1 mg/mL of antibody, we recommend contacting the antibody vendor and requesting additional information on how product concentration was determined.

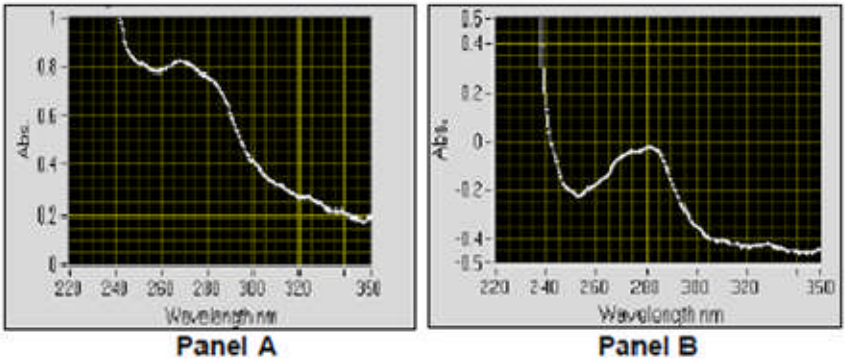


Figure 13. Antibody with unknown azide concentration. Two commercial rat monoclonal antibody preparations are illustrated in Panels A and B. The sample illustrated in Panel A contains a high concentration of sodium azide. The sample in Panel B contains an unknown quantity of sodium azide. Note the baseline offset errors and distortions to the spectra.

Example 4. Rabbit polyclonal IgG @ 1.0 mg/mL (improper blank solution).

At times, a scan of a commercial antibody preparation can generate a large baseline offset error. These offset errors generally occur when an improper buffer solution is used to blank the spectrophotometer. **Figure 14** is a scan of one such commercial preparation where the buffer blank could not be accurately matched. Although the spectrum may be normal in shape, it nonetheless contains a rather large 0.6 abs. unit offset @ 350 nm. Acceptable offsets typically range from 0 to 0.1 A units @ 350 nm. As a consequence, the estimated protein concentration from such a preparation is significantly higher (1.4 mg/mL) than the actual concentration (1.0 mg/mL) based on A_{280}. When using a NanoDrop™, offsets can also occur when air bubbles get trapped on the pedestal.

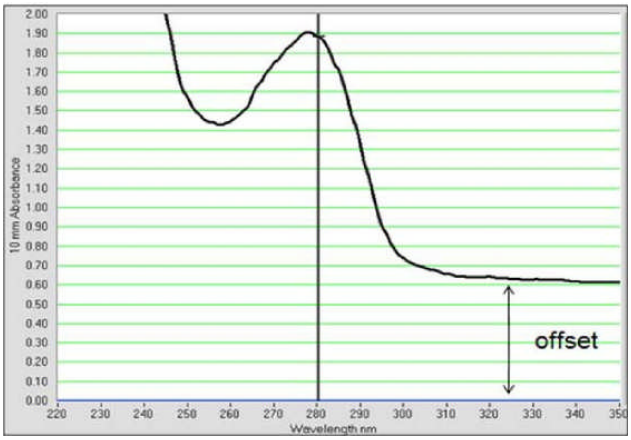


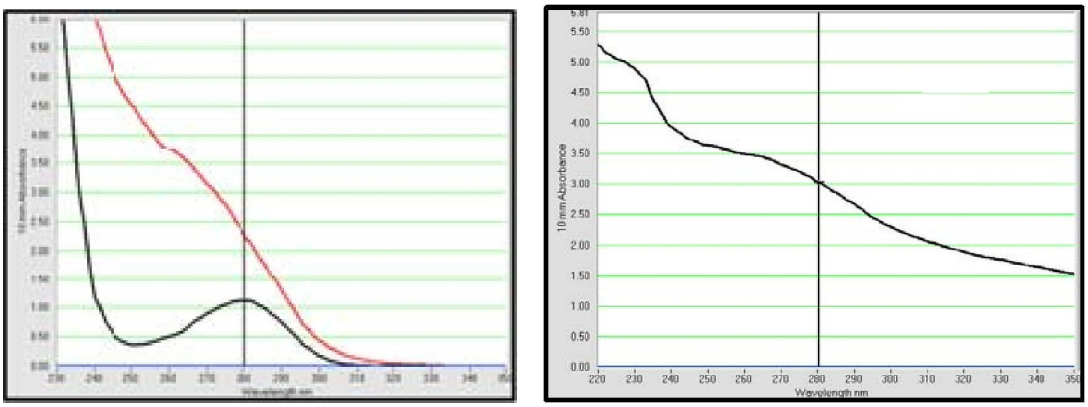
Figure 14. Antibody with improper blank. Baseline offset in a commercial rabbit polyclonal antibody preparation (5 mg/mL) diluted to 1 mg/mL in 1X Modification Buffer. Based on the A_{280} value, the estimated concentration was 1.40 mg/mL. The actual protein concentration after desalting the sample on a spin column to remove the buffer blank offset was 1 mg/mL.

Corrective action: For samples containing large baseline offset errors, we recommend passing the sample through the 1st spin column and rescanning to obtain a properly blanked sample spectrum. Since the spin column exchanges the antibody into a known buffer solution (1X Modification Buffer), it should readily remove any offset error originating from the buffer blank. After confirming sample concentration, proceed to the fluorescein labeling procedure (II-e). If after buffer exchange, it is determined that a sample contains significantly less or more than 1 mg/mL of antibody, we recommend contacting the antibody vendor and requesting additional information on how protein and package concentration/quality was determined. Always adjust sample concentration to 1 mg/mL and 100 µL before proceeding.

Example 5. Bovine IgG containing thimerosal preservative.

A bovine IgG sample was spiked with thimerosal preservative as illustrated in **Figure 15** (Panel A). In this example, thimerosal was introduced @ 0.01% into 100 µL of highly purified bovine IgG sample @ 0.9 mg/mL. Note the dramatic masking influence of this preservative over the intrinsic antibody spectrum. This preservative makes it impossible to properly blank a sample on the spectrophotometer. Small errors in this preservative's concentration create large baseline offset errors. A second thimerosal-containing sample is illustrated in Panel B. This sample contains a commercial monoclonal IgG solution @ 1mg/mL in PBS with an undetermined quantity of thimerosal. Note the large masking effect and baseline offset error created by this preservative.

Corrective action: We recommend passing the sample through the 1st spin column, and rescanning to confirm concentration. If the sample spectrum confirms a concentration of 1 $\pm$ 0.1 mg/mLl, proceed to the fluorescein labeling procedure (II-e). If the sample concentration is greater than 1 mg/mL, the sample must be adjusted with 1X Modification Buffer to 1 mg/mL and 100 µL before proceeding. If the sample contains significantly less or more than 1 mg/ml of antibody, we recommend that you contact your antibody vendor and request additional information on how product concentration was determined.



Panel A **Panel B**

Figure 15. **Antibody containing a preservative.** Superimposed spectra (Panel A) of purified bovine IgG @ 0.9 mg/mL spiked with a) no thimerosal or b) 0.01% thimerosal. Note the masking effect of the preservative. Panel B is a commercial antibody preparation at 1 mg/mL with an unknown concentration of the preservative.

Example 6. Low initial antibody concentration (bovine IgG).

In rare cases, the amount of antibody packaged by the vendor may be lower than expected. **Figure 16** illustrates an example of a commercial preparation of bovine IgG (100 µg solid) that was dissolved in 100 µL 1X Modification Buffer. The sample spectrum indicated a concentration of 0.68 mg/mL (A_{280} = 0.84, E1% = 12.40) which is significantly less than the expected concentration (1 mg/mL.).

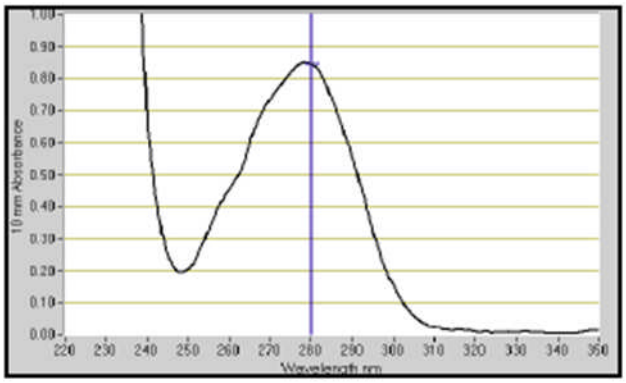


Figure 16. Low antibody concentration. A commercial bovine IgG sample containing significantly less antibody (68 µg) than expected from the original package label.

Corrective action: If a commercial antibody sample contains significantly less mass or concentration than expected, we recommend that you contact the vendor and request additional product information. Do not attempt to label less than 80 µg of buffer-exchanged antibody using this procedure.

Example 7. Protein-based carriers or additives (BSA or gelatin).

The Fluorescein antibody labeling procedure is not compatible with the presence of protein-based carriers such as BSA or gelatin. Their presence is generally detected when the A_{280} is much higher than anticipated, while the sample retains its normal protein spectrum or profile.

Corrective action: Contact the vendor to confirm the presence or absence of a protein additive. Do not attempt to label any product containing protein-based additives. Additives can be removed using affinity chromatography (e.g., Pierce's NAb™ Spin Columns) or other suitable methods. After affinity purification, ensure that the final protein-free antibody is desalted to remove any excess glycine buffer used for elution of the antibody from the affinity column.

Example 8. Saccharide-based carriers (5% trehalose).

The Fluorescein antibody labeling procedure is fully compatible with the presence of 5% trehalose. This additive does not interfere in any way with either a sample's spectrum or its concentration.

Corrective action: None. Proceed as directed in the procedure.

Example 9. Glycine-based buffers.

The Fluorescein antibody labeling procedure is not compatible with high concentrations of glycine buffer. This amino acid additive is sometimes found in high concentrations (e.g., 100 mM). Although its presence does not interfere with a sample's spectrum, it can overwhelm the exchange capacity of the spin column. Glycine is an amine contaminant that competes with the labeling reaction and must be removed.

Corrective action: Remove all traces of glycine and/or other amine-containing buffers by exhaustive dialysis or properly desalting the sample into a phosphate-based buffer.

Problem	Possible Cause	Recommended Action
Poor fluorescein modification of the antibody	-initial protein concentration was incorrect.	-follow the recommended procedures only. -concentrate or dilute the antibody sample into the required range (i.e., 1 mg/mL and 100 µL).
	-a large excess of non-protein amine contaminants are present in the antibody preparation (e.g., Tris or glycine buffer).	-before labeling, remove all amine contaminants. Some samples are so overly contaminated that exhaustive dialysis or two desalting steps may be required.
	-presence protein carrier (e.g., BSA or gelatin) contaminated the sample.	-remove and purify away all protein carriers such as BSA or gelatin by affinity or other chromatographic methods, re-adjust the initial antibody concen-tration to 1 mg/mL.
	-presence of preservative or other additive may be interfering with an accurate determination of the starting protein concentration.	-do not attempt to label an antibody containing any thimerosal. First, remove the preservative then re-measure and adjust the antibody concentration to 1 mg/mL and 100 µL.
	-presence of residual sodium azide interferes with the labeling reaction.	-refer to recommended actions in the Trouble-shooting Guide.
Complete failure of fluorescein labeling reaction	-improper mixing of reaction components. -improper operation of the spectrophotometer.	-make sure to mix the antibody-NHS-fluorescein reaction mixture completely. -use the fluorescein labeled IgG positive control provided to validate the spectro-

	-presence of amine contaminants.	photometer. -remove all amine contaminants such as glycine before labeling.
	-improper storage of the Fluoresceinreagent may have caused it to hydrolyze. -failure to follow the recommended protocol.	-store fluorescein dessicated at room temp. -follow the recommended labeling protocol.
Molar substitution ratio was out of recommended range (3–8 fluoresceins/antibody)	-initial antibody concentration used was too low or too high. -antibody may have precipitated due to over-modification of available lysine residues.	-make sure to properly estimate the initial antibody concentration. -concentrate or dilute the antibody sample into the recommend range (1 mg/mL in 100 µL) before proceeding. -follow the recommended protocol.
Low antibody recovery and/or sample precipitation	-antibody may have aggregated/precipitated during labeling. -incorrect antibody concentration. -antibody was over-modified. -Zeba column recovery problem.	-make sure to add 1 M Tris quench buffer to the labeled sample before final desalting. -follow the recommended guidelines. -always use a calibrated variable-speed centrifuge and spin at exactly 1.5 x g for the indicated time; spins at lower or higher speeds can compromise recovery.

c. Spin Column Antibody Recovery Yield

Table 5 summarizes typical antibody recovery yields from Zeba™ spin columns used in the procedure. Highly purified goat anti -mouse IgG samples (80–100 µg) were resuspended in 100 µL 1X Modification Buffer at 1 mg/mL. Each sample was processed on a Zeba™ column as described in the procedure. Protein concentrations were measured using a NanoDrop™ spectrophotometer (220–350 nm). Recovery yields average 86.7%

	Initial	After 1st Spin Column	After 2nd Spin Column	% Recovery
	Concentration (mg/ml) NanoDrop™	Concentration (mg/ml) NanoDrop™	Concentration (mg/ml) NanoDrop™	
Goat IgG #1	0.80	0.75	0.65	81.25
Goat IgG #2	0.82	0.89	0.74	90.20
Goat IgG #3	0.98	0.95	0.87	88.78

Table 5*. Typical* Zeba™ spin column antibody recovery yields.

d. Kit Disclaimer

Although Solulink has extensively optimized the Fluorescein Antibody Labeling Kit to label many different types of antibodies, it is nonetheless still possible that antibody binding affinity can be compromised or even lost during the labeling process.

Although rare, this phenomenon occurs because the antibody in question possesses one or more critical lysine amino acid residues directly at the antigen-binding site (ABS) and whose function in binding is compromised by labeling. Additionally, Solulink has observed that some monoclonal antibodies possess critical lysine residues (not necessarily at the binding site) that are absolutely critical for antibody stability/solubility. Once modified, these critical residues (usually one or two) cause complete loss of antibody recovery due to precipitation. This phenomenon is sometimes observed even at lowest fluorescein substitution levels and is not related to over-modification.

These situations are beyond the control of this kit and any other NHS-ester based labeling kit used to label antibodies in a non site-selective (stochastic) manner. Use of this labeling kit does not come with an implied guarantee or warranty for either loss of antibody binding affinity or precipitation in some cases. We feel our customers should be made aware that the fluorescein labeling process used in this kit can in some instances compromise the biological function of the antibody being modified.