

## Alexa Fluor® 555 Microscale Protein Labeling Kit (A30007)

### Quick Facts

**Storage upon receipt:**

## Component A

- 2–6°C or ≤–20°C, if desired
- Protect from light
- Protect from moisture

## Component B

- Protect from moisture

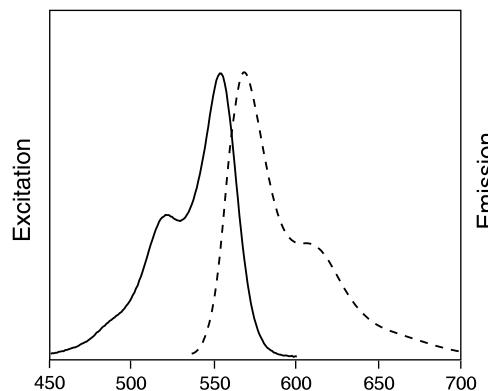
## Components C and D

- Store at room temperature

## Component E

- 2–6°C only; DO NOT FREEZE

**Ex/Em of conjugate:** 555/565 nm



**Figure 1.** Fluorescence excitation (—) and emission (---) spectra of Alexa Fluor® 555 goat anti–mouse IgG antibody in pH 7.2 buffer.

Alexa Fluor® 555 dye is resistant to quenching at high degrees of labeling, and conjugates labeled with this dye are typically brighter than similar proteins labeled with Cy3 dye.<sup>1</sup> Each of the vials of Alexa Fluor® 555 succinimidyl ester supplied in the kit is sufficient for labeling one sample (20–100 µg at a concentration of 1 mg/mL) of protein that has a molecular weight between ~12 and ~150 kDa.

### Introduction

The Alexa Fluor® 555 Microscale Protein Labeling Kit provides a convenient means for labeling small amounts (20–100 µg) of purified protein with our superior Alexa Fluor® 555 dye. This kit has been optimized for labeling proteins with molecular weights between 12 and 150 kDa, and contains everything needed to perform three labeling reactions and to separate the resulting conjugates from excess dye. Convenient spin filters are used to purify the labeled protein with yields between 60 and 90%, depending primarily on the molecular weight of the starting material. Labeling and purification can be completed in as little as 30 minutes. For labeling larger amounts of protein, we recommend either the easy-to-use Alexa Fluor® 555 Protein Labeling Kit (A20174), which is optimized for 1 mg samples of >40 kDa proteins, or the Alexa Fluor® 555 Monoclonal Antibody Labeling Kit (A20187), which is optimized for 100 µg samples of mono- or polyclonal antibodies.

The Alexa Fluor® 555 reactive dye has a succinimidyl ester moiety that reacts efficiently with primary amines of proteins to form stable dye-protein conjugates. Alexa Fluor® 555 dye-labeled proteins have fluorescence excitation and emission maxima of approximately 555 and 565 nm, respectively (Figure 1). The

### Materials

**Kit Contents**

- Alexa Fluor® 555 carboxylic acid, succinimidyl ester (Component A) 3 vials
- Sodium bicarbonate (Component B) 84 mg
- Reaction tubes (Component C) 3
- Spin filters (Component D) Nanosep MF 0.2 µm centrifugation devices, 3
- Purification resin (Component E) Bio-Gel P-6 fine resin suspended in PBS,\* ~3 mL settled

**Storage and Handling**

Upon receipt, all kit components can be stored refrigerated at 2–6°C until required for use. The reactive dye (Component A) may be stored at ≤–20°C, if desired, and should be protected from light. Components A and B should be protected from moisture. DO NOT FREEZE COMPONENT E. When stored appropriately, the kit components should be stable for approximately 6 months.

## Protein Preparation

**IMPORTANT:** The purified protein should be at a concentration of 1 mg/mL in a buffer that does not contain primary amines (e.g., ammonium ions, Tris, glycine, ethanolamine, triethylamine, glutathione), or imidazole. All of these substances will significantly inhibit protein labeling. Also, partially purified protein samples, or protein samples containing carriers like BSA (e.g., antibodies) will not be labeled well and should not be used. The presence of low concentrations (<0.1% (w/v)) of biocides, including sodium azide and thimerosal, will not significantly affect the labeling reaction.

To aid in removing low molecular weight components from the protein sample (desalting) prior to labeling, it is possible to use dialysis or small-scale gel filtration. There are a number of easy-to-use, low-volume dialysis options available, including Tube-O-DIALYZER micro-dialysis cartridges from Genotech ([www.gbiosciences.com](http://www.gbiosciences.com)).

We suggest PBS, pH 7.2–7.5, as a suitable prelabeling dialysis buffer, although 100 mM sodium bicarbonate buffer can also be used. If bicarbonate buffer is used, you may omit step 1.1 of the labeling reaction as well as the addition of 1/10 volume of bicarbonate in step 1.2.

## Labeling Reaction

Table 1 shows the recommended amount in nanomoles of reactive dye that should be added for each nanomole of protein to be labeled. This is the dye: protein molar ratio (MR). The MR values are based on two parameters: 1) the molecular weight of seven representative proteins ranging from 12 to 150 kDa, and 2) the optimal Alexa Fluor® 555 degree of labeling (DOL) for these proteins, as determined in our laboratories. Because your proteins may differ from those listed, the recommended MR for both a lower and a higher DOL are also included in Table 1. For your initial labeling attempt, choose the optimal MR for the protein listed in Table 1 that is closest in molecular weight to the one you are labeling. Use the lower and higher MR as a guide for relabeling if your protein is under- or overlabeled (see Notes). Table 1 also shows typical % yields for the indicated Alexa Fluor® 555 conjugates.

Use equation 1 to calculate the appropriate volume of reactive dye stock solution to use:

### Equation 1

$$\frac{[(\mu\text{g protein/protein MW}) \times 1,000] \times \text{MR}}{8.11} = \mu\text{L reactive dye to add to sample}$$

where  $\mu\text{g}$  protein is the mass of protein you want to label, protein MW is the molecular weight of your protein in Da, MR is the dye: protein molar ratio from Table 1, and 8.11 is the concentration of the reactive dye stock solution (see step 1.3 below). For example, to label 60  $\mu\text{g}$  of IgG (MW 150,000):

$$\frac{[(60/150,000) \times 1,000] \times 16}{8.11} = 0.8 \mu\text{L of dye}$$

Do NOT prepare the reactive dye stock solution (step 1.3) until you are ready to start the labeling reactions. This reactive dye hydrolyzes in water and therefore should be used immediately.

**Table 1.** Recommended Alexa Fluor® 555 dye: protein molar ratios (MR) and typical yields for labeling 12–150 kDa proteins.

Protein (MW in kDa)	For Lower DOL	For Optimal DOL	For Higher DOL	% Yield
parvalbumin (12)	≤2	3	≥6	60
soybean trypsin inhibitor (20)	≤6	15	≥20	56–78
thrombin (40)	≤9	17	≥22	79–89
streptavidin (60)	≤9	17	≥22	85–97
transferrin (80)	≤8	16	≥22	89–93
F(ab) <sub>2</sub> (100)	≤8	13	≥18	77–101
IgG (150)	≤12	16	≥20	82–92

**1.1** Prepare a 1 M sodium bicarbonate solution by adding 1 mL deionized water to the vial of sodium bicarbonate (Component B). Vortex or pipet up and down until the reagent is fully dissolved. The bicarbonate solution will have a pH of ~8.3 and can be stored at 2–6°C for up to two weeks. It can also be frozen for long-term storage.

**1.2** Transfer 20–100  $\mu\text{L}$  of a 1 mg/mL solution of protein (20–100  $\mu\text{g}$ ) to a reaction tube (Component C). Add 1/10 volume (2–10  $\mu\text{L}$ ) of 1 M sodium bicarbonate, and mix by pipetting up and down several times.

**1.3** Add 10  $\mu\text{L}$  dH<sub>2</sub>O to one vial of Alexa Fluor® 555 succinimidyl ester (Component A). Completely dissolve the contents of the vial by pipetting up and down. The concentration of this reactive dye stock solution is 8.11 nmol/ $\mu\text{L}$ . As noted above, this solution should be prepared immediately before use, and any leftover solution should be discarded.

**1.4** Add the appropriate volume of reactive dye solution, based on equation 1, to the reaction tube containing the pH-adjusted protein and mix thoroughly by pipetting up and down several times.

**1.5** Incubate the reaction mixture for 15 minutes at room temperature.

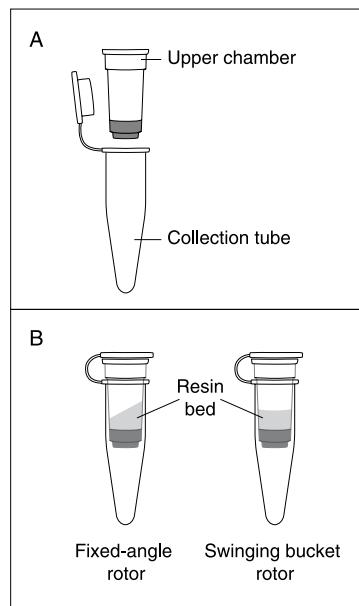
## Conjugate Purification

**2.1** To prepare for separating the labeled protein from unreacted dye, take the container of gel resin (Component E) and one spin filter (Component D; Figure 2) out of the kit. Fully resuspend the gel resin by gently rocking the container; do not vortex or use a magnetic stir bar to agitate the material. Fill the upper chamber of the spin filter up to the lip with the suspended gel resin; approximately 800  $\mu\text{L}$  of resin will be needed. Centrifuge the spin filter at 16,000  $\times$  g in a microcentrifuge for a total of 15 seconds (including run-up time). Using a fixed-angle rotor will cause the resin to pack down with a low side and a high side. The edge of the resin bed on the low side should be about 2–3 mm above the green collar, and the edge of the high side should not be above the top lip of the spin filter. If a swinging bucket rotor is used, the resin bed should fill about half of the upper chamber (~5 mm).

Figure 2 illustrates the two parts of the spin filter and what the filled spin filter should look like after centrifugation. If the bed is too small, add more suspended resin and centrifuge again. If there is too much resin, resuspend the resin in the upper chamber in buffer, remove the necessary amount, and centrifuge again to repack the bed.

**2.2** Occasionally, some resin will get into the collection tube during filter preparation. When the resin bed is at the correct level, rinse out the collection tube under the spin filter several times with buffer to remove any resin particles that may be found there. Replace the resin-containing insert.

If you wish to purify the conjugate in a buffer other than the PBS, pH 7.2, in which the resin is suspended, there are two ways to exchange the buffer. While the resin in the bottle is completely



**Figure 2.** Spin filter. (A) An empty filter showing the separate parts. (B) Appearance of the resin bed after centrifugation in a fixed-angle rotor or swinging bucket rotor.

settled, you can decant or aspirate the buffer provided and replace it with another buffer of your choice. Add your buffer to the bottle, mix gently to resuspend the resin, and let it settle completely. Carefully remove the buffer again, and repeat this washing process several times. You can also exchange the buffer after the resin bed is prepared in the spin filter, by washing your chosen buffer through the bed several times by brief low-speed centrifugation. The Bio-Gel P-6 fine resin provided is stable between pH 2 and 10.

**2.3** After the spin filter is prepared, pipet no more than 50 µL of the conjugate reaction mixture onto the center of the resin bed surface. If the volume of conjugate reaction is 51–100 µL, divide it into two aliquots and purify them on separate spin filters. Place the spin filter(s) in the microcentrifuge with the high side of the resin bed on the outside. Centrifuge at 16,000 × g for a total of 1 minute.

Note: We have not carefully evaluated reusing a spin filter for two aliquots of a larger ( $\geq 50 \mu\text{L}$ ) sample, and we do not recommend it.

**2.4** Each collection tube now contains purified dye-labeled protein in approximately 60–100 µL of buffer. The unreacted dye is retained in the filter and the resin will have a red color (see Notes). The procedure described in steps 1.1–2.3 can be performed in as little as 30 minutes.

## Determination of Degree of Labeling (DOL)

Several spectrophotometric methods are available for determining the DOL of the Alexa Fluor® 555 dye-labeled protein conjugate. They are based on obtaining the protein concentration by absorbance at 280 nm ( $A_{280}$ ) and at 555 nm ( $A_{555}$ ).

**3.1** The easiest way to analyze the conjugates spectrophotometrically is using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, Delaware, USA). This instrument requires only 2 µL of sample and is a full-featured UV/Vis instrument. A variety of cuvettes are available for use with small sample volumes if you would prefer to not dilute your labeled protein in order to use standard 0.5 or 1.5 mL cuvettes. Quartz cuvettes from Starna Cells, Inc. (Atascadero, California, USA) that hold 15–115 µL of sample are ideal for this purpose.

**3.2** The conjugate samples (appropriately diluted) can also be placed in wells of a microplate and read at 280 and 555 nm in a microplate reader that permits the user to specify the desired detection wavelengths, such as the Tecan Safire (Tecan US, Research Triangle Park, North Carolina, USA).

**3.3** The conjugate samples can be diluted as necessary prior to measurement of  $A_{280}$  and  $A_{555}$  using cuvettes and spectrophotometers of your choice. However, excessive dilution of some proteins with low intrinsic  $A_{280}$  may prevent you from deriving accurate  $A_{280}$  values for your samples. The entire conjugate sample, or a portion of it, should be diluted only to the minimum volume necessary for your cuvettes and spectrophotometer to avoid relying on readings below the optimal range for your instrument.

**3.4** Calculate the concentration of the protein in the sample:

### Equation 2

$$\text{Protein concentration (mg/mL)} = \frac{[A_{280} - 0.08(A_{555})] \times \text{dilution factor}}{A_{280} \text{ of protein at } 1 \text{ mg/mL}}$$

In this equation, 0.08 is a correction factor for the fluorophore's contribution to the  $A_{280}$ .

### Equation 3

$$\text{Protein concentration (M)} = \frac{\text{answer from Eq. 2}}{\text{protein molecular weight (Da)}}$$

If you know the molar extinction coefficient ( $\epsilon$ , in  $\text{cm}^{-1}\text{M}^{-1}$ ) of your protein at 280 nm, use this value as the divisor in equation 2 to directly calculate the molarity of the protein.

### **Example:**

You have labeled IgG (MW 150,000;  $A_{280}$  at 1 mg/mL = 1.4;  $\epsilon = 210,000 \text{ cm}^{-1}\text{M}^{-1}$ ) and diluted the sample 1:10 to make the measurements. The readings obtained are  $A_{280} = 0.15$  and  $A_{555} = 0.45$ .

$$\text{Protein concentration (mg/mL)} = \frac{[0.15 - 0.08(0.45)] \times 10}{1.4} = 0.81 \text{ mg/mL}$$

$$\text{Protein concentration (M)} = \frac{0.81}{150,000} = 5.4 \times 10^{-6} \text{ M}$$

$$\text{OR } \frac{[0.15 - 0.08(0.45)] \times 10}{210,000} = 5.4 \times 10^{-6} \text{ M}$$

### **3.5 Calculate the degree of labeling:**

#### **Equation 4**

$$\text{DOL} = (\text{moles dye})/(\text{mole protein}) = \frac{A_{555} \times \text{dilution factor}}{150,000 \times \text{protein concentration (M)}}$$

where  $150,000 \text{ cm}^{-1}\text{M}^{-1}$  is the approximate molar extinction coefficient of the Alexa Fluor® 555 dye.

### **Example:**

$$\text{DOL} = \frac{0.45 \times 10}{150,000 \times 5.4 \times 10^{-6} \text{ M}} = 5.6$$

**3.6** If the  $A_{280}$  of your protein is too low to measure accurately, you can get an approximate protein concentration by estimating the % yield of the conjugate. The % yield from the spin filters is related to the molecular weight of the protein, and typical yields are provided in Table 1. Once you have a % yield, the approximate protein concentration can be calculated using equation 5:

#### **Equation 5**

$$\text{Concentration (mg/mL) labeled protein} = \frac{(\text{mass (mg) starting protein} \times \% \text{ yield})}{\text{volume (mL) recovered}}$$

**3.7** Divide the concentration (in mg/mL) by the protein's molecular weight (in Da) to calculate the approximate molar concentration of the protein. To determine the approximate DOL, you still must spectrophotometrically determine the  $A_{555}$  of the conjugate. However, this value is usually significantly higher than the  $A_{280}$  even if the conjugate has been diluted significantly. The  $A_{555}$  is used in equation 4 (step 3.5) to determine the DOL.

## **Storage of Conjugates**

We typically store labeled protein at 2–6°C, protected from light. It may be necessary to add a stabilizer like BSA (1–10 mg/mL) or glycerol to your conjugate to improve stability. In the presence of 2 mM sodium azide or other biocides, a typical antibody conjugate should be stable at 2–6°C for several months. Your proteins may have special storage requirements. If it is appropriate to do so with your proteins, you can divide the conjugate into small aliquots and freeze them at ≤–20°C for longer storage. AVOID REPEATED FREEZING AND THAWING, AND PROTECT FROM LIGHT.

## **Notes**

Many protein- and dye-specific properties determine how efficiently a protein can be labeled with an amine-reactive dye. Important factors include the number of solvent-accessible primary amines in the protein, the protein's pI, and its solubility and stability at pH 8–8.3. Reactive labels vary in amine reactivity, often in a protein-specific way, and their behavior can be predicted with confidence for only a few proteins such as antibodies and streptavidin. Thus, the recommended dye: protein molar ratios in Table 1 are given as guidelines only, and we cannot guarantee that they will yield optimal labeling with your particular protein(s).

The number of reactive fluorescent dyes that can be attached to a protein before fluorescence quenching or protein inactivation or precipitation occurs is roughly proportional to the protein's molecular weight. For example, the optimal DOL would usually be 1–2 for a ~20 kDa protein, while the optimal DOL for a ~150 kDa protein, e.g., an IgG, would usually be 5–8. The DOL that you obtain with a protein using the Alexa Fluor® 555 Microscale Protein Labeling Kit may be higher or lower than the generally accepted optimum. We highly recommend that you evaluate your protein conjugate in its intended application before you conclude that it is under- or overlabeled. A number of conditions can cause under- or overlabeling.

### **Underlabeling**

- Even trace amounts of primary amine-containing components (e.g., Tris, glycine, ammonium ions, ethanolamine, triethylamine, or glutathione) or imidazole in the starting protein sample will decrease labeling efficiency. The ElutaTube microdialysis vials provided can be used to remove these low molecular weight substances from the protein sample prior to labeling.
- Efficient labeling will probably not occur if the concentration of protein starting material is <1 mg/mL.
- The addition of sodium bicarbonate (step 1.2) is designed to raise the pH of the reaction mixture to ~8, as 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 1/10 volume of bicarbonate solution will not raise the pH to the optimal level. Either more bicarbonate can be added, or the buffer can be exchanged with PBS, pH 7.2 (and bicarbonate solution added again), or with 100 mM sodium bicarbonate buffer, pH 8.3, by dialysis or another method prior to starting the labeling reaction.
- Because proteins react with fluorophores at different rates and retain biological activity at different degrees of dye labeling, the recommendations shown in Table 1 may not always result in optimal labeling. To increase the DOL, the same protein sample can be relabeled, or a new protein sample can be labeled using more reactive dye. Three vials of reactive dye are provided to allow three labeling reactions. Although this kit was designed for optimal labeling in 15 minutes at room temperature, higher DOL may be obtained with longer incubation times. We have not evaluated incubation times >15 minutes.

- Underlabeling may be the reason for the fluorescent signal being lower than expected in your application. Should this occur, relabel the sample, or label another sample with more reactive dye.
- We have observed that dye-labeling of some proteins to any degree can destroy their biological activity.

### **Overlabeling**

- Overlabeling may be indicated by the formation of a red precipitate in the reaction mixture or deposition of red particles on the upper surface of the resin bed after centrifugation of the conjugate. Precipitation will usually result in a decreased yield of conjugate. If your % yield is <50%, it is likely that the protein is overlabeled. Repeat the labeling reaction with less reactive dye. Some proteins cannot be labeled with amine-reactive dyes under any circumstances and may irreversibly precipitate.
- If no visible precipitate forms during labeling but the fluorescent signal in your application is lower than expected, the fluo-

rescence of the conjugate may be quenched due to overlabeling. To reduce the DOL, use a smaller amount of reactive dye, or try labeling the protein at a concentration of >1 mg/mL. (Note: We have not evaluated labeling efficiency with this kit on proteins at concentrations >1 mg/mL.)

- One cause of apparent overlabeling is inefficient removal of unreacted dye. Although using the spin filters in this kit exactly as described removed all traces of free dye from all of the proteins we tested, it is possible that some free dye may be present in your sample after the purification step. The presence of free dye, which can be determined by thin layer chromatography, will result in erroneously high calculated DOL values. Free dye remaining after use of the spin filter can be removed by applying the conjugate to another spin filter or by extensive dialysis. Applying no more than 50 µL of conjugate to each spin filter is the best way to avoid contamination with free dye.
- We have observed that dye-labeling of some proteins to any degree can destroy their biological activity.

\* PBS = phosphate-buffered saline (10 mM potassium phosphate, pH 7.2, 150 mM NaCl)

† MWCO = molecular weight cutoff

### **Reference**

1. J Histochem Cytochem 51, 1699 (2003).

### **Product List** *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
A30007	Alexa Fluor® 555 Microscale Protein Labeling Kit *for 20-100 µg protein* *3 labelings*	1 kit
A20187	Alexa Fluor® 555 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20174	Alexa Fluor® 555 Protein Labeling Kit *3 labelings*	1 kit

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**Invitrogen European Headquarters**

Invitrogen, Ltd.  
3 Fountain Drive  
Inchinnan Business Park  
Paisley PA4 9RF, UK  
Phone: +44 (0) 141 814 6100 • Fax: +44 (0) 141 814 6260  
Email: [euroinfo@invitrogen.com](mailto:euroinfo@invitrogen.com)  
Technical Services: [eurotech@invitrogen.com](mailto:eurotech@invitrogen.com)

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