

IVIS | XenoLight™ CF750

Fluorescent Dye Kits for *In Vivo* Imaging

Material	Amount
XenoLight CF750, SE (Component A)	3 x 0.1 μmole
DMSO, anhydrous (Component B)	150 μL
Sodium bicarbonate solution, 1M, pH 8.3 (Component C)	1 mL
1X PBS, pH 7.4 (Component D)	50 mL
NanoSep Ultrafiltration Vial 10 MWCO (Component E)	3 x 1
Reaction Vial (Component F)	3 x 1
Storage Vial, Sterile (Component G)	3 x 1
Syringe, 1 mL (Component H)	3 x 1
Syringe filter 4mm, 0.2 μm (Component I)	3 x 1

Table 1. XenoLight CF750 nm. Rapid Antibody Labeling Kit for Small Animal *In Vivo* Imaging
If stored as directed, stable for at least 3 months from time of reception.

STORE AT 4 °C,
DO NOT FREEZE.
DESICCATE,
PROTECT FROM LIGHT

CAUTION: NOT INTENDED
FOR HUMAN OR ANIMAL
DIAGNOSTIC OR
THERAPEUTIC USES

XenoLight CF750 nm
Part Number: 125674

Spectral Property

$\lambda_{abs}/\lambda_{em} = 755/775$ nm (protein conjugate in pH 7.4 buffer; see Figure 1 for spectra);
 $\epsilon = \sim 250,000$;

A_{280}/A_{max} , or CF = 0.03 (correction factor for estimating degree of protein labeling)

Introduction

Caliper's XenoLight CF Rapid Antibody Labeling kits are designed for preparing fluorescently labeled antibodies for *in vivo* imaging in small animals. Each kit contains one of our superior near-IR XenoLight CF dyes and everything else you need for carrying out the labeling reaction and purifying the labeled product.

The reactive dye has a succinimidyl ester group, which reacts with an amine group of the protein (i.e., lysine side-chain amine) to form a stable amide linkage. The dye is supplied in three separate vials, each containing sufficient dye for labeling 1-2 mg of IgG antibody. Following the labeling reaction, unconjugated dye is conveniently and rapidly removed by ultrafiltration using the provided NanoSep membrane filtration vials.

Protocol for Labeling IgG antibodies

The protocol below is for labeling 1 mg of an IgG antibody. The procedure may be scaled up or down for any amount of protein as long as the ratios of the reagents are maintained.

Note: Warm all reagents to room temperature before use.

1. Prepare the Antibody for Labeling

If the antibody is already in solution at ≥ 1 mg/mL in PBS or a similar buffer free of any amine-containing chemicals or preservatives, such as Tris, ammonium or amino acids, it is ready for the labeling reaction (See next step). However, if any amine-containing chemical is present, it must be removed as it will interfere with the labeling reaction. This can be accomplished by dialysis against 1X PBS (Component D) using a micro-dialysis device (not provided), or by ultrafiltration using a NanoSep® vial provided in the kit (see procedure below). Presence of sodium azide does not affect the labeling.

To remove amine contaminants by ultrafiltration:

- Load the antibody solution (0.3 mL max each time- do not overfill or you may experience some loss of antibody) in the upper chamber of a NanoSep vial (Component E) and centrifuge at 14,000 rpm until nearly all the liquid is in the lower collection tube;
- Empty the collection tube and add any additional antibody solution to the upper chamber. Repeat steps (a) and (b) until all the antibody solution has been centrifuged.
- Dilute the concentrated antibody in the upper chamber to ~0.3 mL with 1X PBS (Component D) and centrifuge to complete the second round of ultrafiltration;
- Repeat Step (c).
- Transfer the ultrafiltered antibody to the provided reaction vial (Component F) with a total volume of ~0.9 mL 1X PBS.

Important: Save the used NanoSep vial for dye removal below.

Caution! Avoid touching the membrane of the NanoSep chamber during liquid transfer using a pipette. Any damage to the membrane may result in serious product loss.

Note: The NanoSep vial contains a membrane that is permeable only to molecules with a molecular weight less than ~10,000 Daltons. Thus, small molecules freely pass through the membrane into the collection tube while the antibody, which has a molecular weight of ~150,000 Daltons, stays above the membrane.

2. Carry out the Labeling Reaction

- Add 0.1 mL 1M pH 8.3 sodium bicarbonate (Component C) to the antibody solution. If necessary, add 1X PBS so that the antibody concentration is ~1 mg/mL.

Note: Labeling efficiency varies with antibody concentration. In general, the higher the antibody concentration, the higher the labeling efficiency of the dye. This protocol is optimized for 1 mg/mL antibody concentration, at which the labeling efficiency of the dye can be expected to be ~30%. Thus, the degree of labeling (DOL) can be predicted using $(n_d/n_p) \times 30\%$, where n_d/n_p is the molar ratio of dye to antibody used in the labeling reaction.

- Allow a vial of CF750 SE (0.1 μ mole) to warm up to room temperature, and then add 25 μ L anhydrous DMSO (Component B). Vortex to dissolve the dye and then centrifuge down the dye solution to the bottom of the vial.
- Add ~12 μ L of the dye stock from Step (2b) to the antibody solution prepared in Step (2a). Protect the antibody/dye solution from light by wrapping the vial in aluminum foil and incubate the reaction for 1 hr at room temperature on a slow rocker.

Note:

- At this dye-to-protein molar ratio (i.e., ~7.2), the degree of labeling (DOL) is expected to be 2~2.2 dye/protein. Animal studies showed that antibodies labeled with near-IR CF dyes at a DOL of ~2 produced excellent results. However, because our near-IR CF dyes are engineered to be biocompatible and less prone to fluorescence quenching, antibodies labeled with a higher DOL (i.e., 2-4) may produce even better in vivo results, although this has yet to be confirmed. If a higher DOL is desired, increase the dye-to-protein ratio. The optimal DOL will need to be determined empirically.
- Any left-over dye stock solution may be kept for later use. If stored at -20 °C, the dye solution should be stable for at least one month.

3. Purify the Labeled Antibody

- Transfer ~0.3 mL of the reaction solution from Step (2c) to the upper chamber of a NanoSep vial (Component E) and centrifuge.

Caution! Avoid touching the membrane of the NanoSep chamber during liquid transfer using a pipette. Any damage to the membrane may result in serious product loss.

- b) Centrifuge the vial at 14,000 rpm until nearly all of the liquid is in the collection tube below (5-10 min). A small amount of 1X PBS may be used to rinse the reaction vial and complete the solution transfer. Repeat until all of the antibody/dye solution has been centrifuged.
- c) Empty the collection tube, which contains the unconjugated free dye. Add ~0.3 mL 1X PBS (Component D) to the upper chamber of the NanoSep vial, which now contains the labeled antibody. Flick or shake the tube gently to fully dissolve the labeled protein. Centrifuge again to ultrafilter the antibody solution.

Note: The NanoSep vial contains a separation membrane that is permeable only to molecules with a molecular weight less than ~10,000 Daltons. Thus, small molecules, such as phosphate and the unconjugated free dye, freely pass through the membrane into the collection tube while the antibody, which has a molecular weight of ~150,000 Daltons, stays above the membrane. The process is opposite to gel filtration, where small molecules are trapped in the gel matrix while proteins pass through the gel.

- d) Repeat Step (3c) two more times. By the third ultrafiltration, the color of the solution in the collection tube should be almost clear, indicating complete removal of the free dye from the labeled antibody. If some free dye is still detected in the third wash, one or two more washes may be necessary to completely remove the unconjugated dye.
- e) Add an appropriate amount of 1X PBS to reach the desired antibody concentration.
- f) Filter the labeled antibody solution from Step (3e) into a sterile storage vial (Component G) using the provided syringe (Component H) and syringe filter (Component I). Firmly attach the filter to the end of the syringe and filter the antibody through in a slow, dropwise fashion.

Caution! Do not plunge the antibody through the filter too quickly or with too much force as the filter may disconnect from the syringe.

Note: Typical yield for preparing CF XenoLight dye-labeled antibody using this protocol is 80-90%.

4. Determine the Degree of Labeling (DOL)

- a) The final concentration of the antibody conjugate can be calculated from the formula:

$$[\text{conjugate}] \text{ (mg/mL)} = \frac{A_{280} - (A_{\text{max}} \times \text{CF})}{1.4} \times \text{dilution factor}$$

In the above formula:

- [conjugate] is the concentration (in mg/mL) of the antibody conjugate solution prepared in Step 3f.
- "dilution factor" is the fold of dilution used for spectral measurement (See the following Note).

- A_{280} and A_{max} are the absorbance readings of the conjugate at 280 nm and the absorption maximum (~755 nm for XenoLight CF750), respectively; CF is the absorbance correction factor (0.03 for XenoLight CF750) and the value 1.4 is the extinction coefficient of whole (H+L) IgG. Proteins other than whole IgG may have very different extinction coefficients, and thus, the formula will have to be adjusted for accurate determination of DOL.

Note: the antibody solution prepared in Step 3f is typically too concentrated for accurate absorbance measurement and should be diluted to approximately ~0.1 mg/mL. For example, if the labeled antibody is in ~0.5 mL PBS, which would roughly give an antibody concentration of ~2 mg/mL, you will need to perform a 1:20 dilution (i.e., dilution factor = 20) for spectral measurement.

- b) Calculate the DOL according to the formula:

$$\text{DOL} = (A_{\text{max}} \times \text{Mwt} \times \text{dilution factor}) / (\epsilon \times [\text{conjugate}])$$

In the above formula:

- A_{max} , "dilution factor" and [conjugate] are as defined in Step 4a;
- Mwt is the molecular weight of IgG: ~150,000;
- and ϵ is the molar extinction coefficient of XenoLight CF750: ~250,000.

5. Storage of Labeled Antibody

- a) Store the labeled antibody at 4 °C and protect from light.

Note: Long term storage of antibodies less than 0.1 mg/mL may result in binding to plastic vials and degradation of product.

Normalized Absorbance and Emission Spectra of XenoLight CF750 Conjugate

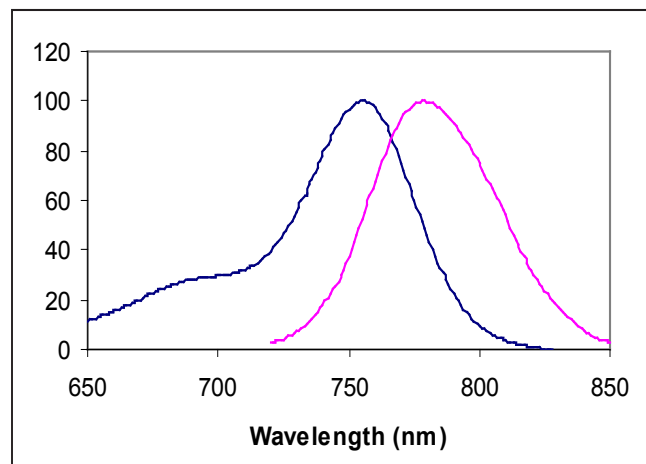


Figure 1. Absorbance and emission spectra of XenoLight CF750 conjugated goat anti-mouse IgG in pH 7.4 PBS buffer.

Caliper Life Sciences Offers the Following XenoLight Reagents

Catalog Number	Product Name	Unit size
122796	XenoLight D-Luciferin - K ⁺ Salt	1 g
125673	XenoLight CF680 Fluorescent Rapid Antibody Labeling Kit	3 labelings
125674	XenoLight CF750 Fluorescent Rapid Antibody Labeling Kit	3 labelings
125675	XenoLight CF770 Fluorescent Rapid Antibody Labeling Kit	3 labelings
125676	XenoLight CF680 succinimidyl ester, IR Fluorescent Dye	1 μ mole
125677	XenoLight CF750 succinimidyl ester, IR Fluorescent Dye	1 μ mole
125678	XenoLight CF770 succinimidyl ester, IR Fluorescent Dye	1 μ mole
125964	XenoLight CF DiR	25 mg

Label License

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