KPL SureLINK™ Fluorescein (FITC) Labeling Kit

KPL SureLINK™ Fluorescein-X (FAM-X) Labeling Kit

Products	Catalog No.
KPL SureLINK™ Fluorescein (FITC) Labeling Kit, 5 Reactions	5610-0020 (82-00-01)
KPL SureLINK™ Fluorescein-X (FAM-X) Labeling Kit, 5 Reactions	5610-0021 (82-00-02)

TABLE OF CONTENTS

Section

PRODUCT DESCRIPTION

KPL SureLINK™ Fluorescein and Fluorescein-X Labeling Kits provide the reagents needed to label antibodies, proteins and other macromolecules with 5-FITC (Fluorescein-5-isothiocyanate) or 5-FAM-X (6-(Fluorescein-5-carboxamido) hexanoic acid, succinimidyl ester) through the modification of primary amines. These products contain individual vials of FITC or FAM-X which were packed under an inert environment to prevent deterioration during storage. The kits also contain organic solvent, modification buffer, and spin filters (for purifying the fluorophore conjugates).

BACKGROUND

5-FITC

5-FITC (Fluorescein-5-isothiocyanate) is a commonly used fluorescent dye (green) to label antibodies for immunodetection assays and is typically conjugated to the primary amine of proteins through the formation of thiourea bond. FITC has a molecular weight of 389.38 with the absorption and emission maximum at approximately 495 nm and 519 nm.

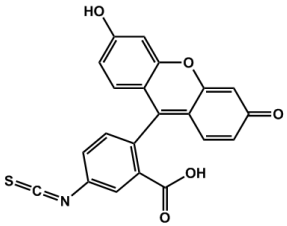

Figure 1. Chemical structure of 5-FITC (MW389.38)

5-FAM-X

5-FAM-X (6-(Fluorescein-5-carboxamido) hexanoic acid, succinimidyl ester) is a fluorescein derivative with a seven-atom aminohexanoyl spacer (known as “X”) between the fluorophore and the succinimidyl ester. The “X” spacer separates the fluorophore from the conjugated biomolecule. It can potentially reduce quenching, if the fluorescence quenching of the conjugated dyes upon conjugation is a problem. The succinimidyl ester of FAM-X conjugated to the primary amine of proteins through the formation of covalent carboxamide bonds. The stable carboxamide bonds are more resistant to hydrolysis which can

potentially reduce the release of free dyes from the conjugates during various treatments in immunoassays or during storage.

FAM-X has a molecular weight of 586.55 and shares the same absorption and emission maximum at approximately 495 nm and 519 nm as FITC.

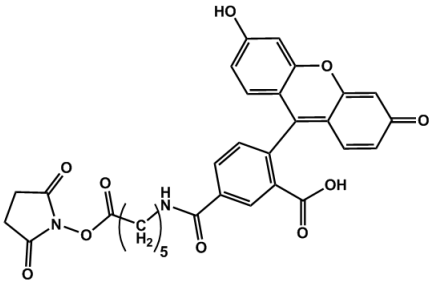

Figure 2. Chemical Structure of FAM-X (MW586.55)

PRODUCT COMPONENTS

5610-0020 (82-00-01), KPL SureLINK™ Fluorescein (FITC) Labeling Kit

Size: 5 Reactions

Kit Component	Part Number	Size	Quantity
KPL SureLINK™ FITC	5620-0020 (82-01-01)	5 x 0.3 mg	1
KPL Carbonate-bicarbonate Buffer Capsules	5640-0007 (82-01-02)	5 Capsules	1
KPL Anhydrous DMF	5640-0008 (82-01-03)	1.5 mL	1
KPL Spin-Pure Filters	5640-0001 (60-00-53)	5 Units	1
KPL Amber Reaction Tubes	5640-0009 (82-01-05)	10 Tubes	1

5610-0021 (82-00-02), KPL SureLINK™ Fluorescein-X (FAM-X) Labeling Kit

Size: 5 Reactions

Kit Component	Part Number	Size	Quantity
KPL SureLINK™ FAM-X	5620-0022 (82-02-01)	5 x 0.3 mg	1
KPL Borate Buffer (1X)	5640-0010 (82-02-02)	10 mL	1
KPL Anhydrous DMF	5640-0008 (82-01-03)	1.5 mL	1
KPL Spin-Pure Filters	5640-0001 (60-00-53)	5 Units	1
KPL Amber Reaction Tubes	5640-0009 (82-01-05)	10 Tubes	1

Kits utilize convenient single use dye vials to avoid the need to weigh out chemicals and to preserve chemical stability.

- Fluorescein kit 5610-0020 (82-00-01): 5 individual vials of FITC, each contains 0.3 mg FITC for labeling 100 µg – 2 mg proteins per reaction.
- Fluorescein-X kit 5610-0021 (82-00-02): 5 individual vials of FAM-X, each contains 0.3 mg FAM-X for labeling 100 µg – 3.5 mg proteins per reaction.

* Anhydrous DMF contains molecular sieves to maintain anhydrous conditions. FITC and FAM-X vials are packed in resealable foil bags with desiccants.

** The spin filters provided in the kits have the molecular weight cut off of 10 KDa.
*** Additional spin filters can be purchased 5640-0001 (60-00-53). The other components in the kits are not sold separately.

STORAGE AND STABILITY

- KPL SureLINK™ Fluorescein and Fluorescein-X Labeling kits are shipped at ambient temperature.
- Upon receipt, store all components at 2-8 ˚C and store KPL SureLINK™ FITC and FAM-X under desiccation. (Note: FITC and FAM-X vials are packed in resealable foil bags with desiccants.)
- Kits are stable for at least one year when stored at recommended conditions.

BEFORE YOU BEGIN

SAFETY AND HANDLING

- Read MSDS and all instructions thoroughly before using kits.
- Wear appropriate personal protective equipment when handling reagents.

OTHER REQUIRED SUPPLIES AND EQUIPMENT

- Antibody or protein, free of salts or contaminants. See Troubleshooting section for removing salts or other contaminants. Additional 1X Modification Buffer may be required for buffer exchange.
- Molecular biology grade water
- Shaker capable of gentle agitation
- Vortex
- Microcentrifuge

QUICK REFERENCE PROTOCOL

Rehydrate or transfer antibody/protein in an appropriate buffer

↓

Dissolve SureLINK™ FITC or FAM-X in DMF

↓

Add FITC or FAM-X to antibody/protein solution

↓

Incubate the reaction mixture at room temperature for 1 hours with gentle agitation

↓

Remove unconjugated FITC or FAM-X from antibody/protein using spin filter or by dialysis

↓

Measure degree of FITC or FAM-X incorporation

$$\text{IgG conjugate: F/P} = \frac{2.77 \times A_{495nm}}{A_{280nm} - (A_{495\text{nm}} \times 0.35)}$$

$$\text{Protein conjugate: F/P} = \frac{A_{495nm}/\varepsilon_{dye495\text{nm}}}{(A_{280nm} - (A_{495\text{nm}} \times 0.35))/\varepsilon_{protein280\text{nm}}}$$

Elapsed time: approximately 2 hr
Hands-on time: approximately 30 min

FLUORESCEIN (FITC) CONJUGATION PROTOCOL

This protocol describes the conjugation reaction for 0.5 mL of antibody (IgG) at a concentration of 0.5 mg/mL (0.25 mg, corresponding to 1.67 nmol IgG). This protocol uses a 60X molar excess of FITC to generate IgG-FITC conjugates with estimated F/P ratios of 4-7. Other volumes and concentrations can be used (see Calculations section). Other proteins can also be labeled using this protocol.

1. **Reconstitue conjugation buffer (1X carbonate-bicarbonate buffer, 0.1 M, pH 9.6).**
   Reconstitute the conjugation buffer using one carbonate-bicarbonate buffer capsule and 50 mL of molecular biology grade water. Gently pull apart the capsule to dislodge the powders into the water. Mix the solution until the powder has completely dissolved.

2. **Rehydrate or transfer the antibody into 0.5 mL of carbonate-bicarbonate buffer to obtain a 0.5 mg/mL solution using the provided reaction tube.**
   Buffers containing Tris, imidazole, glycine or other primary amines should not be used due to competition with the conjugation reaction. If the sample is stored in one of these buffers, exchange the buffer to the conjugation buffer by dialysis, using a spin filter or a desalting column. The unlabeled antibody should not contain any protein-based stabilizer, such as BSA. See more information in frequently asked questions and trouble shooting guide.

3. **Immediately prior to use, prepare a 4 mg/mL (10.27 nmol/µL) stock solution of KPL SureLINK™ FITC in anhydrous DMF by adding 75 µL of DMF to one vial of KPL SureLINK™ FITC (0.3 mg).**
   Tap down and equilibrate KPL SureLINK™ FITC vial to room temperature prior to opening to avoid the condensation of moisture. The fluorescein-5-isothiocyanate can potentially be hydrolyzed when exposed to water.

   (Note: If a high FITC molar excess is chosen, a higher concentration FITC stock solution should be prepared to reduce the percentage of DMF in the conjugation reaction. The percentage of DMF added should be less than 5% of the total reaction volume to minimize protein precipitation by DMF (Melnikova et al.).)

4. **Add 9.7 µl of 4 mg/mL KPL SureLINK™ FITC (99.6 nmol) to the antibody (0.5 mL at 0.5 mg/mL) or protein solution.**

Examples for conjugation reactions under other conditions:
Conjugation reactions at various conditions are listed in Table 1. When IgG solution at 0.5 mg/mL was used, the optimum FITC molar excess was determined to be 50-100 folds. When a higher concentration of IgG

solution is used, a higher incorporation rate of FITC is observed and thus a lower molar ratio is required. The reactions may be scaled up or scaled down as necessary, using the same volumetric ratio of protein to fluorophore. FITC-antibody conjugates with optimum sensitivity in ELISA are obtained at F/P ratios of 4-7. The incorporation rate of FITC and impact of F/P ratio will vary depending on the specific characteristic of the biomolecule to be labeled.

Table 1.

Volume of IgG solution (µL)	Protein conc. (mg/mL)	Total IgG amount (mg)	FITC molar excess (fold)	Volume of 4 mg/mL FITC stock (µL)	DMF % in reaction mixture
500	2	1.0	20	13	3.1%
500	1	0.5	50	16	3.1%
500	0.5	0.25	50	8	1.6%
500	0.5	0.25	100	16	3.1%
500	0.25	0.125	200	16	3.1%
500	0.1	0.05	500	16	3.1%

5. **Incubate the protein and the FITC together at room temperature for one hour with gentle agitation.**
6. **Remove the unconjugated FITC using a spin filter.**
   KPL recommends the following desalting procedure using the spin filters (MWCO 10 KDa) included in the kits:
   a. Insert the sample reservoir (unit with filter) on the top of filtrate receiver.
   b. Add the protein conjugate solution to the top section of the spin filter (sample reservoir). Each spin filter can hold 50 - 500 µL of sample. Cap the spin filter and place it into a centrifuge. Concentrate the sample by centrifugation at 12,000 – 14,000 x g for 10 minutes. The concentrated solution will remain in the top section of the spin filter. Dispose of the filtrate (bottom section) of the spin filter.
   c. Repeat as necessary until a buffer exchange of at least 200-fold is achieved. Note: The appropriate spin time depends on the protein concentration and the viscosity of the sample solution. Longer spin times will be required with increased protein concentration in the sample solution.

Desalting columns or dialysis cassettes can also be used to remove unconjugated fluorophore. Choose an appropriate molecular weight cutoff for your protein.

7. **The FITC-labeled protein is now ready to use. Store FITC conjugate at 2-8˚C. To determine the molar substitution ratio (MSR) of FITC to protein see the DETERMINING THE DEGREE OF FLUORESCEIN DYE INCORPORATION section.**
   Determine the degree of FITC incorporation prior to adding any stabilizers to the FITC-labeled protein, such as BSA.

FLUORESCEIN-X (FAM-X) CONJUGATION PROTOCOL

This protocol describes the conjugation reaction for 0.5 mL of antibody (IgG) at a concentration of 0.5 mg/mL (0.25 mg, corresponding to 1.67 nmol IgG). This protocol uses a 20X molar excess of FAM-X to generate IgG-FAM-X conjugates with estimated F/P ratios in the range of 6-15. Other volumes and concentrations can be used (see Calculations section). Other proteins can also be labeled using this protocol.

1. **Rehydrate or transfer the antibody into 0.5 mL of 1X borate (conjugation) buffer to obtain a 0.5 mg/mL solution using provided reaction tube.**
   Buffers containing Tris, imidazole, glycine or other primary amines should not be used due to competition with the conjugation reaction. If the sample is stored in one of these buffers, exchange the buffer to the conjugation buffer by dialysis, using a spin filter, or by using a desalting column. The conjugation buffer is 246 mM sodium borate, pH 8.4. The unlabeled antibody should not contain any protein-based stabilizer, such as BSA. See more information in frequently asked questions and trouble shooting guide.

2. **Immediately prior to use, prepare a 4 mg/mL (6.82 nmol/µL) stock solution of KPL SureLINK™ FAM-X in anhydrous DMF by adding 75 µL of DMF to one vial of SureLINK™ FAM-X (0.3 mg)**
   Tap down and equilibrate SureLINK™ FAM-X vial to room temperature prior to opening to avoid the condensation of moisture. The fluorophore can potentially be hydrolyzed when exposed to water.

   (Note: If a high FAM-X molar excess is chosen, a higher concentration FAM-X stock solution should be prepared to reduce the percentage of DMF in the conjugation reaction. The percentage of DMF added should be less than 5% of the total reaction volume to minimize protein precipitation by DMF (Melnikova et al.).)

3. **Add 5 µl of 4 mg/mL KPL SureLINK™ FAM-X (34 nmol) to the antibody (0.5 mL at 0.5 mg/mL) or protein solution.**

Examples for conjugation reactions under other conditions: Conjugation reactions at various conditions are listed in Table 2, for reference. When IgG solution at 0.5 mg/mL or above was used, the optimum FAM-X molar excess is 20 fold. When a low concentration of antibody is used, a higher molar excess of FAM-X should be used (for example, 100-fold at 0.1 mg/mL antibody). The reactions may be scaled up or scaled down as necessary, using the same volumetric ratio of protein to fluorophore.

Antibody conjugates with optimum sensitivity in ELISA are obtained at F/P ratios of 6-15. The incorporation rate of FAM-X and impact of F/P ratio will vary depending on the specific characteristic of the biomolecule to be labeled.

Table 2.

Volume of IgG solution (µL)	Protein conc. (mg/mL)	Total IgG amount (mg)	FAM-X molar excess (fold)	Volume of 4 mg/mL FAM-X stock (µL)	DMF % in reaction mixture
500	2	1.0	20	10	2.0%
500	1	0.5	20	5	1.0%
500	0.5	0.25	20	2.5	0.5%
500	0.1	0.05	100	2.5	0.5%

4. **Incubate the protein and the FAM-X together at room temperature for one hour with gentle agitation.**
5. **Remove the unconjugated FAM-X using a spin filter.**

   KPL recommends the following desalting procedure using the spin filters (MWCO 10 KDa) included in the kits:

   a. Insert the sample reservoir (unit with filter) on the top of filtrate receiver.

   b. Add the protein conjugate solution to the top section of the spin filter (sample reservoir). Each spin filter can hold 50 - 500 µL of sample. Cap the spin filter and place it into a centrifuge. Concentrate the sample by centrifugation at 12,000 – 14,000 x g for 10 minutes. The concentrated solution will remain in the top section of the spin filter. Dispose of the filtrate (bottom section) of the spin filter.

   c. Repeat as necessary until a buffer exchange of at least 200-fold is achieved. Note: The appropriate spin time depends on the protein

concentration and the viscosity of the sample solution. Longer spin times will be required with increased protein concentration in the sample solution.

Desalting columns or dialysis cassettes can also be used to remove unconjugated fluorophore. Choose an appropriate molecular weight cutoff for your protein.

6. **The FAM-X-labeled protein is now ready to use. Store FAM-X conjugate at 2-8˚C. To determine the molar substitution ratio (MSR) of FAM-X to protein see the DETERMINING THE DEGREE OF FLUORESCEIN DYE INCORPORATION section.**

Determine the degree of FAM-X incorporation prior to adding any stabilizers to the FAM-X-labeled protein, such as BSA.

DETERMINING THE DEGREE OF FLUORESCEIN DYE INCORPORATION

The molar substitution ratio (MSR) of fluorescein (FITC or FAM-X) to protein is referred to as the F/P ratio. The F/P molar ratio refers to the ratio of moles of fluorescein (F) to moles of protein (P) in the conjugate. Two methods of determining an F/P molar ratio are described below.

Option 1. Determine F/P ratio by measuring absorbance at 280 and 495 nm, if the protein extinction coefficient is available

Transfer the labeled protein sample into a cuvette and measure the absorbance at both 280 nm (A_{280nm}) and 495 nm (A_{495nm}) using a spectrophotometer. Both absorption values should fall within the linear range of the spectrophotometer. If necessary, dilute the sample with a high-pH buffer. Record the A_{280nm}, A_{495nm}, and dilution factor (DF).

Notes:
(1). The unconjugated FITC or FAM-X must be removed from the labeled protein in order to determine the degree of fluorescein dye incorporation.
(2). The A_{280nm} is used to calculate the amount of protein in the sample. The A_{495nm} is used to calculate the amount of fluorescein dye in the sample.
(3). If dilution is necessary, the sample can be diluted into a high-pH buffer (greater than pH 7), such as the conjugation buffer, borate (pH 8.4) or carbonate/bicarbonate (pH 9.6) buffer. The absorbance of fluorescein is sensitive to pH. See frequently asked questions for more information.

(4). The labeled protein can be measured directly in the cuvette and then recovered.

Calculation 1A. Determine F/P ratio for antibody (IgG) conjugates based on extinction coefficient

A simplified formula is used to determine an F/P ratio for IgG conjugates by utilizing the extinction coefficients (ε) of IgG and fluorescein. The below formula is based on the assumption that same dilution factor (DF) was used to obtain A_{280nm} and A_{495nm}.

$$\mathrm{F/P} = \frac{2.77 \times A_{495nm}}{A_{280nm} - (A_{495\mathrm{nm}} \times 0.35)}$$

Note: ($\varepsilon_{\text{IgG (280 nm)}}$/ $\varepsilon_{\text{fluorescein (495 nm)}}$) = (210,000/75,800) = 2.77

Calculation 1B. Determine F/P ratio for protein conjugates based on extinction coefficient

F/P ratio can be determined using A_{280nm}, and A_{495nm} based on extinction coefficients (ε) of the protein and fluorescein. The below formula is based on the assumption that same dilution factor (DF) was used to obtain A_{280nm} and A_{495nm}.

$$\mathrm{F/P} = \frac{[fluorescein]}{[\mathrm{P}rotein]} = \frac{A_{495nm}/\varepsilon_{dye495\mathrm{nm}}}{(A_{280nm} - (A_{495\mathrm{nm}} \times 0.35))/\varepsilon_{protein280\mathrm{nm}}}$$

Notes:

(1). $\varepsilon_{dye495\mathrm{nm}}$ = 75,800 $M^{-1}cm^{-1}$ for FITC and FAM-X

$\varepsilon_{protein280\mathrm{nm}}$ = 210,000 $M^{-1}cm^{-1}$ for IgG

The absorbance of fluorescein is sensitive to pH. The variation of extinction coefficient depends on the pH of buffer used in absorbance measurement (Bioconjugate Techniques, Greg T. Hermanson, see more information in frequently asked questions).

(2). Determine extinction coefficient (ε)f a protein

The extinction coefficient (ε) of a protein can be estimated based on:

(a). the molecular weight of the protein,

(b). the absorbance of the protein solution at 280 nm, and

(c). the concentration (w/w, or mg/mL) of the protein solution.

$$\varepsilon = E_{280nm}^{1mg/mL} \times MW = E_{280nm}^{0.1\%} \times MW = 0.1 \times E_{280nm}^{1\%} \times MW$$

Example: $E_{280nm}^{1mg/mL}$= 1.4 (absorbance of 1mg/mL IgG solution at 280 nm) and MW = 150,000 g/mol

$$\varepsilon = E_{280nm}^{1mg/mL} \times MW = 1.4 \times 150{,}000 = 210{,}000\ M^{-1}cm^{-1}$$

(3). The A_{280nm} of the protein is adjusted due to the absorbance of fluorescent dye at 280 nm. There is a minor contribution of absorbance at 280 nm due to the fluorescent dye. This contribution is approximately 35% of the absorbance at 495 nm (The and Feltkamap, 1970). Thus, the corrected A_{280} can be determined according to the following equation:

A_{280} (corrected) = A_{280} – (A_{495} X 0.35)

(4). Determining molar concentration of fluorescein and protein:

$A_{\_\_nm} = \varepsilon cl$ (Beer's law), so $c = A_{\_\_nm}/\varepsilon l$

Example: (based on 1 cm cuvette)

$$\text{fluorescein concentration (M)} = \frac{A_{495nm} \times DF}{\varepsilon_{dye495nm} \times 1cm}$$

(Note: If the protein has natural fluorescence, the A_{495nm} may also need to be adjusted.)

$$\text{protein concentration (M)} = \frac{(A_{280nm} - (A_{495nm} \times 0.35)) \times DF}{\varepsilon_{protein280nm} \times 1cm}$$

Abbreviations:

- $A_{\_\_nm}$ Absorbance at the given wavelength (nm)
- ε Protein or dye molar extinction coefficient ($M^{-1}cm^{-1}$) at given wavelength
- c Concentration
- ℓ Path length in cm (usually 1 cm for a cuvette)
- DF Dilution factor
- $E_{280nm}^{1mg/mL}$ Absorbance of 1 mg/mL protein at 280 nm, $E_{280nm}^{0.1\%}$

Option 2. Determine F/P ratio based on protein concentration, if the extinction coefficient of the protein is unavailable

If you do not know the molar extinction coefficient of the protein at 280 nm, or the A_{280nm} is not measurable, measure the total protein concentration by an alternative method, such as the BCA (Pierce) or Bradford (Bio-Rad) assay. These assays will consume your sample and are not necessary for IgG conjugates. The absorbance at 495 nm (A_{495nm}) for fluorescein in conjugates can be obtained using a spectrophotometer (see details in above option 1).

1. Determine molar concentration of fluorescein (based on 1 cm cuvette)

$$\text{fluorescein concentration (M)} = \frac{A_{495nm} \times DF}{75{,}800 \times 1cm}$$

2. Determine molar concentration of protein

Given the molecular weight (MW, g/mol) and concentration of the labeled protein (mg/mL) from an alternative method, such as the BCA or Bradford Assay.

$$\text{Protein concentration (M)} = \frac{\text{protein concentration (mg/mL)}}{\text{MW (g/mol)}}$$

3. Determine F/P molar ratio of the conjugates

The F/P molar ratio of conjugates can be obtained through dividing fluorescein molar concentration by protein molar concentration.

$$\text{F/P} = \frac{[\,fluorescein]}{[\text{Pr}\,otein]}$$

CALCULATIONS

Part 1: Determining the desired fluorophore:antibody (or protein) molar ratio for the conjugation reaction.

Various fluorophore:antibody molar ratios can be used with the SureLINK™ fluorophore kits. In general, the degree of fluorophore incorporated depends on the following:

1. The concentration of protein to be labeled (Figure 3 and 4). Higher protein concentrations (≥0.5 mg/mL) allow for the incorporation of fluorophore at higher rates.
2. The molar excess of fluorophore added. As seen in Figure 3 and 4, higher molar excesses of fluorophore improve incorporation.
3. The availability of primary amines.
4. The incubation time and temperature of the conjugation reaction.

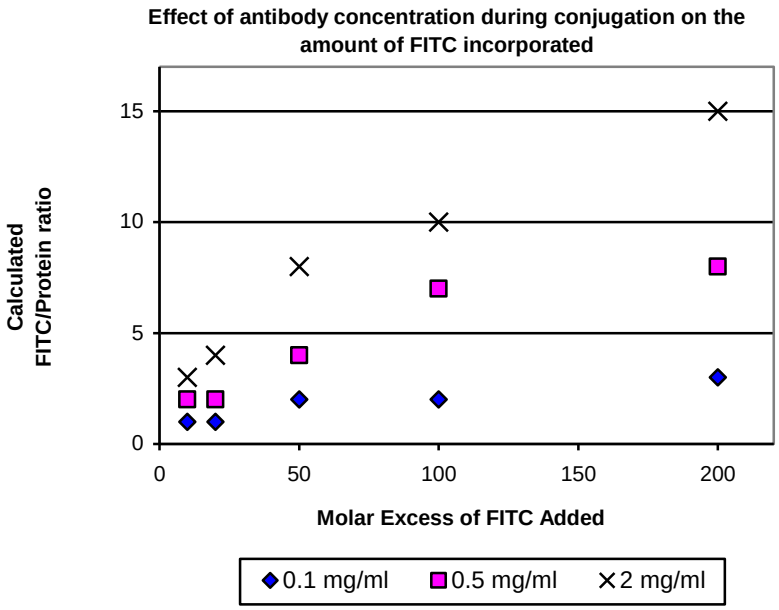

Fig. 3. FITC incorporation rate with IgG: The FITC incorporation ratio was determined empirically under different conditions. IgG (goat anti-mouse IgG) at concentrations of 0.1 mg/ml, 0.5 mg/ml and 2 mg/ml were conjugated to FITC at molar excesses of 10-fold to 200-fold.

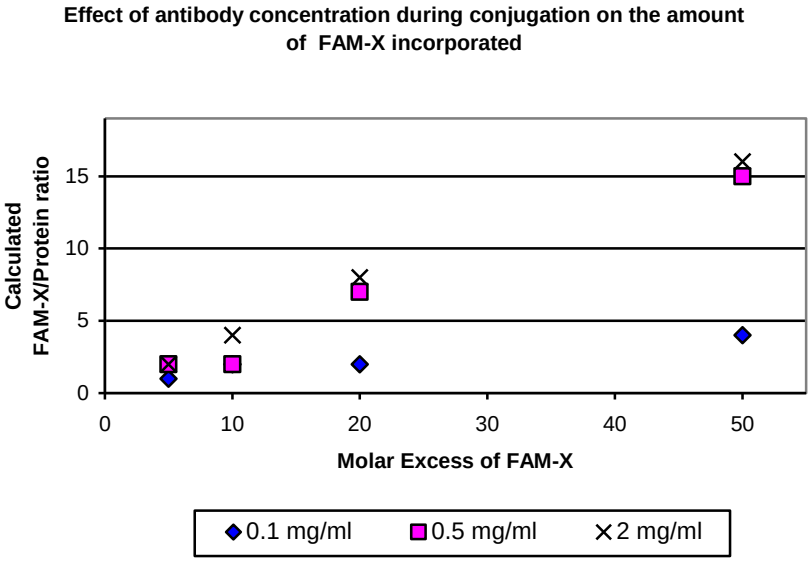

Fig. 4. FAM-X incorporation rate with IgG: The FAM-X incorporation ratio was determined empirically under different conditions. IgG (goat anti-mouse IgG) at concentrations of 0.1 mg/ml, 0.5 mg/ml and 2 mg/ml were conjugated to FAM-X at molar excesses of 5-fold to 50-fold.

Part 2: Determining the amount of KPL SureLINK™ FITC or FAM-X for the conjugation reaction.

The volume of FITC or FAM-X stock solutions required for the conjugation reaction can be determined once the desired FITC or FAM-X molar excess ratio (See Part 1.) has been determined.

$$\text{Volume (µL) of 4 mg/mL FITC for conjugation reaction} = \frac{1000 \times (\text{protein amount in mg}) \times (\text{FITC molar excess ratio})}{(\text{protein molecular weight in KDa}) \times (10.27\ \text{nmole/µL})}$$

$$\text{Volume (µL) of 4 mg/mL FAM-X for conjugation reaction} = \frac{1000 \times (\text{protein amount in mg}) \times (\text{FAM-X molar excess ratio})}{(\text{protein molecular weight in KDa}) \times (6.82\ \text{nmole/µL})}$$

Example: 0.25 mg of IgG (MW 150 KDa) is labeled using a 60-fold FITC molar excess.

Volume of 4 mg/mL FITC = (1000 x 0.25 x 60) / (150 x 10.27) = 9.7 µL

Part 3: Calculating the percentage of DMF in the conjugation reaction.

DMF exposure may cause irreversible conformational changes to antibodies and other proteins. These conformational changes may have impact on long term stability and antigen-binding activity. It is preferred to limit the percentage of DMF in conjugation reactions to less than 5% (Melnikova et al.).

If the DMF percentages exceed 5%, higher concentration FITC or FAM-X stock solutions should be prepared to reduce the final concentration of DMF in the conjugation reaction.

RECOMMENDED USE OF FITC CONJUGATES

KPL SureLINK™ FITC Labeling kits can be used to label antibodies or other proteins for a variety of purposes. Antibody-FITC conjugates are widely used in a variety of applications, including immunoassays, flow cytometry, and immunofluorescence microscopy. The conjugate concentration that will provide the best signal-to-background in your specific assay may vary and should be determined for each conjugate. Include positive and negative controls in each immunoassay for proper review of experimental results and successful troubleshooting.

Use of FITC conjugates in various assays

There is an optimum molar ratio of fluorophore to protein for the performance of FITC-labeled protein conjugates in immunoassays. The ideal F/P ratio for a given FITC-protein conjugate will be dependent on the number and location of modified lysine groups in a particular protein. Different antibodies may show different behavior based on the locations of lysine groups in the antibody.

Figure 5 shows an example for goat anti-mouse IgG antibody conjugated to various amounts of FITC dye and tested in an ELISA. In this experiment, the fluorescence intensity of the antibody-FITC conjugate reaches a maximum at an F/P (fluorophore-to-protein) ratio of 4-7. Once the molar excess of FITC goes beyond a certain point, the resulting fluorescent protein conjugates have a lower intensity, possibly due to steric hindrance or quenching between proximal fluorescein moieties.

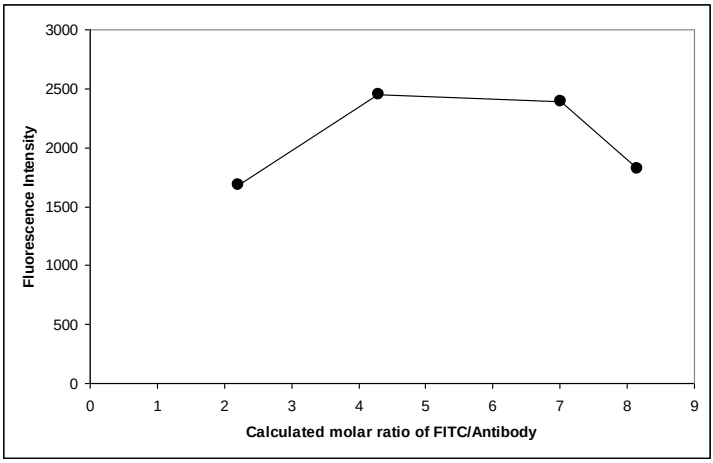

Figure 5. Relationship between F/P and fluorescence intensity for a FITC conjugate of goat anti-mouse IgG antibody. IgG was detected in ELISA with FITC-labeled antibody at various F/P ratios.

RECOMMENDED USE OF FAM-X CONJUGATES

KPL SureLINK™ FAM-X Labeling kits can be used to label antibodies or other proteins for a variety of purposes. FAM-X is a good substitute for FITC for most applications, including flow cytometry and immunofluorescence microscopy. As with FITC conjugates, the FAM-X conjugate concentration that will provide the best signal-to-background in your specific assay may vary and should be determined for each conjugate.

FAM-X conjugates of protein can be prepared with a higher F/P ratio than conjugates prepared with FITC. In contrast to FITC, the spacer arm of FAM-X allows for greater separation of the fluorophores from each other and from the protein, leading to less quenching between proximal fluor molecules and less steric hinderance. SeraCare has prepared FAM-X conjugates with optimum F/P ratios of 6-15 that are about 1.5-fold more active as compared to FITC conjugates in ELISA assays. FAM-X conjugates also have slightly better photostability as compared to FITC conjugates.

FREQUENTLY ASKED QUESTIONS

Q: What biomolecules can be conjugated with the KPL SureLINK™ FITC or FAM-X Labeling Kit?
A: Proteins, peptides, and oligonucleotides can be conjugated with FITC or FAM-X. Proteins and peptides can be modified using primary amino groups on lysine residues and the N-terminus. Modified oligonucleotides with primary amino groups are suitable for conjugation.

Q: What determine the degree of fluorescein incorporation?
A: The degree of fluorescein incorporation depends on the concentration of protein, the molar excess of fluorescein added, the availability of primary amines in protein, and the incubation temperature and time of conjugation reaction.

Q: Is free fluorescein a concern following the conjugation protocol?
A: In immunoassays such as ELISA and Western blotting, the effect of free fluorescein is minimal as most of the unconjugated fluorescein is removed in the washing steps. However, free fluorescein must be removed in order to quantitate the amount of fluorescein incorporation.

Q: How can I determine the number of primary amines on my protein?
A: Without knowledge of the protein sequence, the number of primary amines can be estimated by multiplying the MW of the protein by 0.0006. This formula estimates the number of lysine residues given an average weight of 110 Daltons for an amino acid, and an average lysine content of 6.6% in a protein (Dayhoff, M.O., 1978). Due to steric or functional constraints, some lysine residues may not be available for conjugation.

Q: Unlabeled protein samples are stored in buffer with Tris, sodium azide, or glycine. Can I still conjugate the proteins to KPL SureLINK™ FITC or FAM-X?
A: Tris, sodium azide and glycine will react with the SureLINK™ FITC or FAM-X in the conjugation reaction. Remove them by buffer exchange against the conjugation buffer, then begin conjugation protocol

Q: Do I have to dissolve KPL SureLINK™ FITC or FAM-X in DMF?
A: KPL SureLINK™ FITC or FAM-X is soluble in either DMSO or DMF. As with NHS esters and isothiocyanate, exposure to aqueous buffers should be limited to prevent inactivation of FITC and FAM-X by hydrolysis.

Q: How long can you store rehydrated KPL SureLINK™ FITC or FAM-X?
A: It is not recommended that you store SureLINK™ FITC or FAM-X solution for future use. The 0.3 mg sizes of SureLINK™ FITC or FAM-X is designed for single use, although multiple conjugates can be made.

Q: Why do I have to store KPL SureLINK™ FITC or FAM-X under desiccation?
A: The desiccation helps to remove moisture to prevent inactivation of the isothiocyanate and NHS ester by hydrolysis. The FITC and FAM-X are packed in air-tight zip-lock bag with desiccants.

Q: My protein normally has an absorbance at 495 nm. Can I still quantitate the amount of fluorescein?
A: If your unlabeled protein has an absorbance at 495 nm, you may subtract the portion of the A_{495nm} due to the unlabeled protein if you know the extinction coefficient at 495 nm.

Q: My unlabeled protein contains a protein-based stabilizer such as BSA. How do I prepare my sample for conjugation?

A: BSA may be removed by commercially available albumin removal kits, such as Bio-Rad Affi-gel blue. If the unlabeled protein and contaminating protein are sufficiently different in size, an appropriate gel filtration column can be used to purify the sample. The contaminating protein can also be removed by immunoprecipitation, if you have an antibody to recognize the contaminating protein.

Q: Adding more KPL SureLINK™ FITC or FAM-X brings the percentage of DMF in the conjugation reaction above 5%. Can I still use this molar ratio of FITC or FAM-X?
A: Yes. Instead of dissolving FITC or FAM-X in DMF at 4 mg/mL, dissolve it at higher concentration in order to bring the percentage of DMF in the final reaction to less than 5%.

Q: Do I have to purify my sample using the spin filters included in the KPL SureLINK™ FITC or FAM-X Labeling Kits?
A: No, you do not have to use the included 10,000 MWCO spin filters. The excess biotin can be removed with a variety of desalting columns or dialysis cassettes. For conjugation to antibodies, 50,000 MWCO Vivaspin 6 centrifugal concentrators also work well.

Q: What is the extinction coefficient of FITC?
A: The absorbance of fluorescein is sensitive to pH. The variation of extinction coefficient depends on the pH of buffer used in absorbance measurement (conjugation buffer: borate buffer, pH 8.4, carbonate/bicarbonate buffer, pH 9.6). The extinction coefficient of FITC at pH is 81,000-85,000. At pH 7.8 the absorbance of FITC conjugates decreases by 8% (Bioconjugate Techniques, Greg T. Hermanson).

TROUBLESHOOTING GUIDE

Problem 1: During the labeling reaction, the protein precipitates

Causes and/or Observations	Possible Solution
• The percentage of DMF in the conjugation reaction is too high.	Dissolve the KPL SureLINK™ FITC or FAM-X in anhydrous DMF at a higher concentration. It is preferably to maintain DMF concentrations to less than 5% in the conjugation reaction.

Problem 2: Low fluorescein incorporation

Causes and/or Observations	Possible Solutions
• The isothiocyanate of FITC or NHS-ester bond of the FAM-X has been hydrolyzed during storage, compromising the labeling reaction.	Upon receipt, store the KPL SureLINK™ FITC or FAM-X at 4˚C in a dessicator. Equilibrate FITC or FAM-X vial to room temperature prior to opening to avoid the condensation of moisture.
• Contaminants are present in the protein sample that carry a primary amino or a nucleophilic group (ex. Tris, glycine and azide), reducing the efficiency of the modification reaction.	Dialyze the protein sample thoroughly against conjugation buffer prior to the modification step.

Problem 3: Weak Level of Detection in ELISA or Western Blot

Causes and/or Observations	Possible Solutions
• Low level of fluorescence	Increase the concentration of the protein during the conjugation reaction. In general, this is more important than the amount of fluorescein added.
• Other proteins are present in the protein sample—compromising the preparation of the desired fluorescein conjugate.	Fractionate the protein sample over an acrylamide gel electrophoresis to visualize the protein contamination. Depending on the level of impurity, additional protein purification steps may be required.
• Poor recovery of protein after conjugation using spin filter	If the protein is <10 KDa, it may pass through the spin filter. Use a lower molecular weight cutoff spin filter.

Problem 4: High level of Background in ELISA or Western Blot

Causes and/or Observations	Possible Solution
• The concentration of the fluorescein conjugate is too high.	Titrate and optimize the amount of conjugate required for each immunoassay.

RELATED PRODUCTS

Product/Application Group	Product Name	Size	Catalog Number
Protein Labeling Kits & Reagents	KPL SureLINK™ HRP Conjugation Kit KPL SureLINK™ HRP Conjugation Kit	6 x 0.1mg rxn 6 x 1.0 mg rxn	5610-0022 (84-00-01) 5610-0023 (84-00-02)
	KPL SureLINK™ AP Conjugation Kit KPL SureLINK™ AP Conjugation Kit KPL SureLINK™ Chromophoric Biotin Labeling Kit KPLSureLINK™ Chromophoric Biotin	3 x 0.5 mg rxn 3 x 0.1 mg rxn 5 x 1 mg 10 mg	5610-0026 (85-00-02) 5610-0025 (85-00-01) 5610-0027 (86-00-01) 5620-0030 (86-00-03)
	Spin-Pure Filters	5 per pack	5640-0001 (60-00-53)

REFERENCES

Melnikova, Y. et al., (2000) Antigen-Binding Activity of Monoclonal Antibodies after Incubation with Organic Solvents. Biochemistry (Moscow) 65 (11), 1256-1265.

Bioconjugate Techniques, (1996) Greg T. Hermanson, Chapter 8, page 304.

The, T. H. & Feltkamp, T. E. W., (1970) Conjugation of Fluorescein Isothiocyanate to Antibodies. Immunology 18(6), 865-873.

Dayhoff, M.O. (1978) Atlas of Protein Sequence and Structure, Suppl. 2, National Biomedical Research Foundation, Washington.

Notes:

For Research Use Only

The products listed herein are for research use only and are not intended for use in human or clinical diagnosis. Nothing disclosed herein is to be construed as a recommendation to use these products in violation of any patents. The information presented above is believed to be accurate. However, said information and product are offered without warranty or guarantee since the ultimate conditions of use and the variability of the materials treated are beyond our control. We cannot be responsible for patent infringements or other violations that may occur with the use of these products. No claims beyond replacement of unacceptable material or refund of purchase price shall be allowed. All claims regarding product performance must be made within 30 days following date of delivery.

Trademarks

SureLINK is a trademark of SeraCare Life Sciences.

Disclaimer

The recommendations of this bulletin are provided solely for the benefit of users who need practical guidance on immunoassay procedures. Because experimental conditions for the use of the suggested products are beyond the control of SeraCare, it is impossible for SeraCare to implicitly guarantee the performance of the mentioned products for any and all assay procedures. Users who need additional information or technical support should call Technical Services at 800/676-1881 or 301/948-7755 for assistance.

SeraCare Life Sciences

508.244.6400 • 800.676.1881 Toll Free • 508.634.3334 Fax
www.seracare.com