Controlled Protein-Protein Crosslinking Kit

Catalog Number 23456

Doc. Part No. 2161298 Pub. No. MAN0011431 Rev. B

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

Product description

The Controlled Protein-Protein Crosslinking Kit provides a quick, convenient way to couple any two proteins through an amine ($-NH_2$) functional group on one protein and a sulfhydryl (-SH) group on the other using Sulfo-SMCC as the crosslinking agent. Sulfo-SMCC is a heterobifunctional crosslinker that contains an *N*-hydroxysuccinimide (NHS) ester and a maleimide group. NHS esters react with primary amines at pH 7–9 to form covalent amide bonds. Maleimides react with sulfhydryl groups at pH 6.5–7.5 to form stable thioether bonds. This kit contains all the necessary reagents for modifying proteins to contain sulfhydryl groups and for determining reaction efficiency. Additionally, instructions are designed to provide a successful crosslinking experience regardless of level of expertise. The crosslinking strategies incorporated in these instructions are outlined in Figure 1.

The steps necessary to couple two proteins depend on the structure of Protein 2. If Protein 2 does not have a free sulfhydryl group available, either reduce an existing disulfide, as in reaction scheme A or add SATA to a primary amine, as in reaction scheme B. If Protein 2 has a free sulfhydryl, then it can be directly conjugated, as in reaction scheme C.

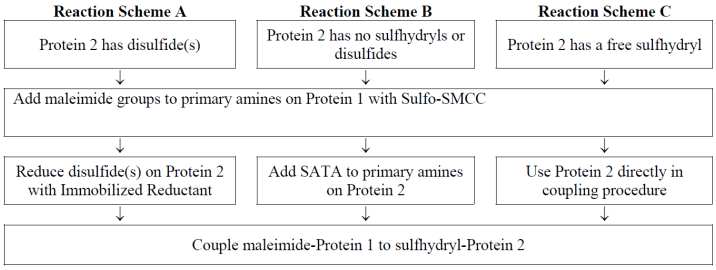

Figure 1 Coupling strategies for the Controlled Protein-Protein Crosslinking Kit

Note: Review this document in its entirety before beginning any of the included procedures.

Contents and storage

The contents of each kit are sufficient 10 antibody conjugate purifications.

Table 1 Controlled Protein-Protein Crosslinking Kit (Cat. No. 44920)

Item	Amount	Storage
Sulfo-SMCC Crosslinking Agent (Molecular Weight: 436.37 and Spacer Arm Length: 11.6 Å)	2 mg	Store kit at 4°C.
Activation/Conjugation Buffer (10X), contains sodium phosphate and EDTA	20 mL	
BupH™ Phosphate Buffered Saline Pack[1]	2 packs	
2-Mercaptoethylamine-HCl	6 mg	
Immobilized Reductant	0.2 mL	
SATA (Molecular Weight: 231.22 and Spacer Arm Length: 2.8Å)	2 mg	
Dimethylformamide	1 mL	
Hydroxylamine-HCl	5 mg	
Dextran Desalting Column, 5K MWCO, for sample volumes <1.2 mL	2 x 10 mL	
Cysteine-HCl	20 mg	
Ellman's Reagent (5,5'-dithio-bis-(2-nitrobenzoic acid)	2 mg	
Column Extender	2 each	
Bottom Plugs	2 each	
Porous Discs	2 each	
Porous Disc Insertion Tool	—	

[1] Each pack yields 500 mL of 0.1M phosphate, 0.15M sodium chloride; pH 7.2

Protein requirements

- The two proteins to be crosslinked must be highly purified and the molecular weights must be known.
- **Protein 1** must have an available primary amine (that is, N-terminus or a lysine residue) to react with the Sulfo-SMCC (Figure 2).

- **Protein 2** must have one of the following criteria: an available sulfhydryl group (-SH) as determined by Ellman's Reagent (see Procedure IV); a disulfide bond that can be reduced to generate free -SH groups (Figure 3); or an available primary amine so that the required -SH group can be added chemically (Figure 4).

Prepare Reagents

Prepare Phosphate Buffered Saline (PBS)

Dissolve the dry-blend buffer with 500 mL of ultrapure water. For long-term storage of excess buffer, sterile-filter the solution or add sodium azide to a final concentration of 0.02% and store at 4°C.

Prepare proteins

1. Dissolve 0.5–5.0 mg of Protein 1 and Protein 2 with up to 1 mL of PBS. For proteins already in solution, make a 1:1 dilution of the protein with PBS or dialyze against PBS.

2. Reconstitute proteins containing free sulfhydryls immediately before performing the crosslinking procedure to minimize disulfide formation.

   Note: If proteins are in buffers that contains primary amines (for example, Tris or glycine), these compounds must be thoroughly removed by dialysis or desalting, as they will quench the Sulfo-SMCC and SATA reactions.

Activate maleimide of Protein 1 (60 minutes)

Note: In the following reaction (Figure 2), five-fold molar excess of Sulfo-SMCC is added to the protein, which generally results in 1–3 moles of maleimide per mole of protein. See Table 2 in the "Appendix" on page 5 for the volume of Sulfo-SMCC in PBS to add per milliliter of Protein 1 to achieve five-fold excess.

The Sulfo NHS ester of Sulfo-SMCC reacts with primary amines on lysine residues resulting in the protein containing an available sulfhydryl-reactive maleimide group.

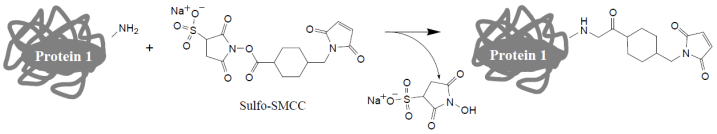

Figure 2 Reaction scheme for maleimide activation of Protein 1

1. Invert the desalting column several times to suspend the resin. Position the column upright in a test tube or clamp and allow the resin to settle for several minutes.

2. Remove the top cap from the column and carefully pipette the storage solution (contains 0.02% sodium azide) until 5–10 mm of solution remains above the resin bed.

3. *(Optional)* Using the open end of the supplied porous disc insertion tool, insert and slide a porous disc to within 1 mm of the resin bed. The top porous disc provides a stop-flow function that prevents disturbance and drying of the resin bed during use.

4. Twist off the column's bottom end tab.

5. Equilibrate column by adding 30 mL of PBS and allowing it to flow through.

6. Add 2 mL of PBS to the vial containing 2 mg Sulfo-SMCC and mix thoroughly to dissolve.

7. Immediately add approximately 5-fold molar excess (see Table 2) of the Sulfo-SMCC solution to Protein 1 and incubate for 30 minutes at room temperature.

8. To remove nonreacted Sulfo-SMCC, apply approximately 1 mL of the maleimide-Protein 1 reaction mixture to the equilibrated desalting column.

9. Add PBS to the desalting column. Collect 0.5 mL fractions and measure the absorbance at 280 nm of each fraction to locate the protein peak. Pool fractions that contain most of the protein.

10. Determine concentration of the maleimide-Protein 1 (that is, the pooled fractions) by comparing its absorbance at 280 nm with the absorbance of the original protein solution. Alternatively, determine protein concentration using the Thermo Scientific™ Coomassie Plus™ (Bradford)™ Assay Kit (Cat. No. 23236).

11. Use the maleimide-Protein 1 for the protein-protein crosslinking procedure (Procedure III, Figure 5). To determine the maleimide content of Protein 1, perform Procedure V.

STOPPING POINT The maleimide-activated protein may be stored frozen before or after desalting provided the protein is not adversely affected by freezing.

Modify sulfhydryl of Protein 2

Reduce disulfide bonds (90 minutes) (Protocol for reaction scheme A)

Immobilized reductant containing 2-MEA reduces disulfide bonds resulting in available sulfhydryl groups able to react with maleimides.

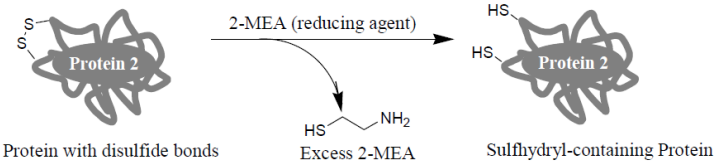

Figure 3 Reaction scheme for disulfide reduction of Protein 2

1. Prepare PBS-EDTA by mixing 90 mL PBS, pH 7.2, with 10 mL Conjugation Buffer (10X).

2. Add 100 µL Conjugation Buffer (10X) to the 6 mg of 2-Mercaptoethylamine-HCl (MEA). Mix thoroughly to dissolve.

3. To activate the Immobilized Reductant, add 25 µL of the MEA solution to the vial of Immobilized Reductant and mix thoroughly. Incubate the mixture for 15 minutes.

4. Centrifuge the mixture to pellet the activated Immobilized Reductant. Remove and discard the supernatant.

5. Suspend and wash the activated Immobilized Reductant four times using 1 mL of PBS-EDTA for each wash. Remove the wash buffer by centrifugation.

6. Add 1 mL of Protein 2 and 50 µL of Conjugation Buffer (10X) to the activated Immobilized Reductant and mix thoroughly. Incubate the mixture for 60 minutes at 37°C.

   Note: Higher temperature (up to 65°C) may be required to unfold protein and completely reduce buried disulfide bonds. When incubating at 65°C, shorten incubation time to 10–20 minutes.

7. Briefly centrifuge mixture to pellet the Immobilized Reductant.

8. Remove and retain supernatant containing sulfhydryl-Protein 2 for the crosslinking procedure outlined in Procedure III. Sulfhydryl groups are unstable, therefore, once sulfhydryls are generated, initiate the crosslinking procedure within 1 hour. Refrigerate the reduced sulfhydryl-Protein 2 until ready to initiate crosslinking. To determine sulfhydryl content, perform Procedure IV.

Add sulfhydryl groups by reaction with SATA (180 minutes) (Protocol for reaction scheme B)

Note: In the following reaction (Figure 4), 10-fold molar excess of SATA is added to Protein 2, which generally results in 1–5 sulfhydryl groups per protein. See Table 3 in the Appendix for the volume of SATA in DMF to add to Protein 2 to achieve 10-fold excess.

The NHS ester of SATA reacts with primary amines on lysine residues. Hydroxylamine de-protects the latent sulfhydryl groups, which are able to react with maleimides.

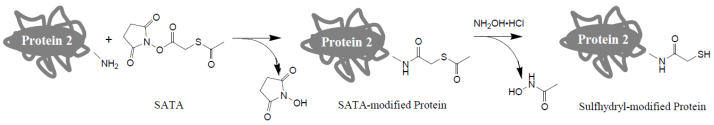

Figure 4 Reaction scheme for sulfhydryl modification of Protein 2

1. Prepare PBS-EDTA by mixing 90 mL PBS, pH 7.2, with 10 mL Conjugation Buffer (10X).

2. Invert the desalting column several times to suspend the resin, then position the column upright in a test tube or clamp and allow the resin to settle for several minutes.

3. Remove the top cap from the column and carefully pipette the storage solution (contains 0.02% sodium azide) until 5–10 mm of solution remains above the resin bed.

4. *(Optional)* Using the open end of the supplied porous disc insertion tool, insert and slide a porous disc to within 1 mm of the resin bed. A top porous disc provides a "stop-flow" function that prevents disturbance and drying of the resin bed during use.

5. Twist off column bottom end tab. Equilibrate column by adding 30 mL of PBS-EDTA and allowing it to flowthrough.

6. Add 500 µL of DMF to the vial containing 2 mg of SATA and mix thoroughly to dissolve.

7. Add a 10-fold molar excess of SATA/DMF solution to 1 mL of Protein 2 solution (see Table 3). Incubate reaction for 30 minutes at room temperature.

   Note: The SATA-modified protein may be stored at –20°C provided protein is not adversely affected by freezing.

8. Add 100 µL of Conjugation Buffer (10X) to the vial containing 5 mg of Hydroxylamine•HCl and mix thoroughly to dissolve.

9. To de-protect the latent sulfhydryl, add 100 µL of hydroxylamine solution to the SATA-modified Protein 2. Incubate mixture for 2 hours at room temperature.

10. To remove nonreacted reagents, apply approximately 1 mL of de-protected sulfhydryl-Protein 2 to the equilibrated desalting column.

11. Add PBS-EDTA to the Desalting Column. Collect 1 mL fractions and measure the absorbance at 280 nm of each fraction to locate the protein. Pool fractions containing most of the protein.

12. Determine concentration of sulfhydryl-Protein 2 (that is, the pooled protein sample) by comparing its absorbance at 280 nm with the absorbance of the original protein solution. Alternatively, determine protein concentration using the Coomassie Plus™ (Bradford)™ Assay Kit (Cat. No. 23236).

13. Use sulfhydryl-Protein 2 for crosslinking as outlined in Procedure III. Sulfhydryl groups are unstable, therefore, initiate the crosslinking procedure within 1 hour of sulfhydryl modification. Refrigerate the sulfhydryl-Protein 2 until ready to initiate the crosslinking reaction. To determine sulfhydryl content, perform Procedure IV.

Crosslink Maleimide-Protein 1 to Sulfhydryl-Protein 2 (60 minutes)

The double bond of the maleimide reacts with sulfhydryl groups at pH 6.5–7.5 to form a stable thioether bond.

Figure 5 Reaction scheme for crosslinking maleimide-activated Protein 1 to sulfhydryl-containing Protein 2

1. Mix maleimide-Protein 1 and sulfhydryl-Protein 2 in approximately equal molar amounts.

   Note: If the concentrations of the proteins are approximately equal, reacting equal mass amounts of the two proteins will achieve sufficient crosslinking.

2. Incubate mixture for 60 minutes at room temperature.

3. Store conjugate at 4°C. For long-term storage (that is, > 1 month), mix conjugate with an equal volume of 50% ethylene glycol or 100% glycerol and store at –20°C.

Determine sulfhydryl content

1. Prepare PBS-EDTA by mixing 90 mL PBS, pH 7.2, with 10 mL Conjugation Buffer (10X).

2. Add 1 mL of PBS-EDTA solution to the vial containing 2 mg Ellman's Reagent and mix for 5-10 minutes to dissolve completely. This 2 mg/mL stock solution of Ellman's Reagent is stable for up to 1 week stored frozen.

3. Dilute a portion of the stock Ellman's Reagent 1:2 with PBS-EDTA (that is, add 1 part Ellman's Reagent to 2 parts PBS-EDTA) to make the 1.0 mg/mL working Ellman's Reagent.

4. To prepare the cysteine (-SH group) standards, add 2 mL of PBS-EDTA solution to the vial containing 20 mg of cysteine and mix thoroughly to dissolve. This 10 mg/mL stock cysteine solution is stable for up to 1 week stored frozen.

5. Dilute a portion of the stock cysteine 1:200 with PBS-EDTA to make a 0.05 mg/mL working cysteine solution (285 nmol/mL).

6. Prepare 10 serial dilutions of the working cysteine solution (that is, dilute the 0.05 mg/mL working cysteine 1:1 with PBS-EDTA solution, then dilute that 0.025 mg/mL solution 1:1, etc.). These 10 dilutions will be used as cysteine standards.

7. In separate wells of a microplate, pipette duplicate samples containing 100 µL of each of the following:
   - PBS-EDTA solution (reagent blank)
   - Each of the 10 cysteine standards
   - Sulfhydryl-Protein 2

   Note: If the concentration of sulfhydryl-Protein 2 is low (that is, 0.5–1.0 mg/mL), use 200 µL of samples rather than 100 µL. Also use 200 µL of PBS-EDTA solution and 200 µL of each standard.

8. To each 100 µL sample in the microplate, add 10 µL of Ellman's Reagent (0.5 mg/mL) and mix well.

   Note: If using 200 µL samples, add 20 µL of working Ellman's Reagent to each sample.

9. Use a plate reader to measure the absorbance of each well at 405 nm. Absorbance measurement at 412 nm or 420 nm is also acceptable.

10. Calculate sulfhydryl content by either of the following methods:
    - Plot the absorbance values of the cysteine standards. Determine slope of the curve and the Y-intercept and determine sulfhydryl content as follows:

      $$\text{sulfhydryl content (moles)} = \frac{A_{405} \text{ of the sample} - \text{Y-intercept}}{\text{slope}}$$

    - Alternatively, divide the absorbance value of each cysteine standard by its cysteine content (mole cysteine) then calculate the average absorbance units (average AU per mole of cysteine) for all the standards. Determine sulfhydryl content as follows:

      $$\text{sulfhydryl content (moles)} = \frac{A_{405} \text{ of the sample}}{\text{average AU per mole cysteine}}$$

11. Using the results from Step 10 on page 4 and the molecular weight of the protein, calculate the number of sulfhydryls per protein molecule as follows:

    $$\text{sulfhydryl/protein} = \frac{\text{moles of cysteine in sample}}{\text{moles of protein}}$$

Indirectly determine maleimide content

1. Prepare PBS-EDTA by mixing 90 mL PBS with 10 mL Conjugation Buffer (10X).

2. Prepare cysteine standards and reagent blank as in Steps 2 on page 4–6 on page 4 of Procedure IV. Use 200 µL of each standard and blank per well.

3. In a separate well, mix 100 µL of working cysteine (0.05 mg/mL) reagent and 100 µL of maleimide-Protein 1. Incubate plate for 1 hour at room temperature.

4. In separate wells, prepare the following two controls:
   - 200 µL of maleimide-Protein 1
   - 100 µL of working Cysteine (0.05 mg/mL) + 100 µL PBS-EDTA

5. Incubate plate for 1 hour at room temperature. Add 20 µL of working Ellman's Reagent (0.5 mg/mL) to each well.

6. Measure the absorbance of each well at 405 nm. Divide the absorbance value of each cysteine standard by its cysteine content (mole cysteine) then calculate the average absorbance units (average AU per mole of cysteine) for all standards.

7. The maleimide content of the protein (moles of maleimide/mole of Protein 1) will equal the number of moles of cysteine bound to the protein. Use the average AU per mole of cysteine to determine sulfhydryl content as follows:
   - *Calc 1*: Original moles of cysteine added to maleimide-Protein 1

     $$\frac{A_{405} \text{ of the cysteine control}}{\text{average AU per mole of cysteine}}$$

   - *Calc 2*: Moles of cysteine remaining after incubation with maleimide-Protein 1

     $$\frac{(A_{405} \text{ of the protein:cysteine mixture}) - (A_{405} \text{ of the protein})}{\text{average AU per mole of cysteine}}$$

   - Moles of maleimide/mole of Protein 1 equal to moles of cysteine bound to the protein sample = *Calc 1 – Calc 2*.

Appendix

Adding the amount of Sulfo-SMCC indicated in Table 2 generally results in 1–3 maleimides per protein (mole/mole). Actual maleimide incorporation depends on the number of accessible amines on the protein.

Table 2 Volume of Sulfo-SMCC in PBS to add per milliliter of a protein of known concentration and molecular weight (MW)

For example, for a 50 kDa protein at 2 mg/mL, add 88 µL of the Sulfo-SMCC solution to 1 mL of protein.

Protein MW × 10^3	Protein concentration					
	0.5 mg/mL	1 mg/mL	2 mg/mL	3 mg/mL	4 mg/mL	5 mg/mL
5	220 µL	440 µL	880 µL	1,200 µL	–	–
10	110 µL	220 µL	440 µL	660 µL	880 µL	1,100 µL
25	44 µL	88 µL	176 µL	264 µL	352 µL	868 µL
50	22 µL	44 µL	88 µL	132 µL	176 µL	434 µL
100	11 µL	22 µL	44 µL	66 µL	88 µL	217 µL
200	6 µL	11 µL	22 µL	33 µL	44 µL	109 µL

Adding the amount of SATA (10-fold molar excess) indicated in Table 3 generally results in 1–5 sulfhydryl groups per protein (mole/mole). Actual sulfhydryl incorporation depends on the number of accessible amines on the protein.

Table 3 Volume of SATA in DMF to add per milliliter of protein of known concentration and molecular weight (MW)

For example, for a 50 kDa protein at a concentration of 2 mg/mL, add 22 µL of SATA/DMF to 1 mL of protein.

Protein MW × 10^3	Protein concentration (mg/mL)					
	0.5 mg/mL	1 mg/mL	2 mg/mL	3 mg/mL	4 mg/mL	5 mg/mL
5	56 µL	110 µL	220 µL	280 µL	440 µL	–
10	28 µL	55 µL	110 µL	170 µL	220 µL	280 µL
25	11 µL	22 µL	44 µL	68 µL	88 µL	112 µL
50	6 µL	11 µL	22 µL	34 µL	44 µL	56 µL
100	3 µL	6 µL	11 µL	17µL	22 µL	28 µL
200	1.4 µL	2.8 µL	6 µL	9 µL	11 µL	14 µL

Related products

Product name	Cat. No.
Sulfo-SMCC (sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate), 50 mg	22322
SATA (*N*-succinimidyl S-acetylthioacetate), 50 mg	26102
Ellman's Reagent (5,5'-dithio-bis-(2-nitrobenzoic acid), 5 g	22582
Glycerol, 1 L	17904
Ethylene Glycol (50% aqueous), 200 mL	29810

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: Pub. No. MAN0011431 B

Revision	Date	Description
B	10 May 2024	• Instructions for using the bottom column closure were added to the document. • The document was updated to the current template, with associated updates to the warranty, trademarks, and logos.
A00	17 October 2015	Baseline document for Controlled Protein-Protein Crosslinking Kit.