

## Protein–Protein Crosslinking Kit

**Table 1.** Contents and storage information.

Material	Amount	Storage *	Stability
Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Component A, MW 312)	3 vials, 500 µg each	<ul style="list-style-type: none"> <li>• 2–6°C or ≤–20°C</li> <li>• Protect from moisture</li> </ul>	When stored properly, all kit components should be stable for at least 6 months.
Succinimidyl <i>trans</i> -4-(maleimidylmethyl)cyclohexane-1-carboxylate (SMCC) (Component B, MW 334)	3 vials, 500 µg each		
Tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP) (Component C, MW 287)	20 mg	<ul style="list-style-type: none"> <li>• 2–6°C</li> <li>• Protect from moisture</li> </ul>	
Dimethylsulfoxide (DMSO) (Component H)	1 mL		
Reaction tubes with stir bars (Component E)	6 tubes, 2 mL each	<ul style="list-style-type: none"> <li>• 2–6°C or ≤–20°C</li> </ul>	
Collection tubes (Component G)	6 tubes, 2 mL each		
<i>N</i> -ethylmaleimide (NEM) (Component D, MW 125)	20 mg	<ul style="list-style-type: none"> <li>• 2–6°C</li> <li>• DO NOT FREEZE</li> </ul>	
Spin columns (Component F)	6 columns, each containing 30,000 MW size exclusion resin in PBS		

\* For convenience, the entire kit may be stored at 2–6°C.

**Number of Labelings:** Sufficient reagents and purification media are provided to perform 3 protein–protein conjugations.

## Introduction

Invitrogen’s Protein–Protein Crosslinking Kit provides a convenient method for crosslinking small amounts (0.2–3 mg) of two different proteins—for instance an antibody and phycobili-protein such as R-phycoerythrin or an antibody and enzyme, such as horseradish peroxidase. This crosslinking is accomplished through the formation of a stable thioether bond.

With the kit, two heterobifunctional reagents—succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and succinimidyl *trans*-4-(maleimidylmethyl)cyclohexane-1-carboxylate (SMCC)—are used, respectively, to modify the lysine residues of one protein to thiols and to add thiol-reactive maleimide groups to the other protein. After deprotection of the thiolated protein by the reducing agent tris-(2-carboxyethyl)phosphine (TCEP), the two modified proteins are reacted to each other forming a stable thioether bond between them. *N*-ethylmaleimide (NEM) is provided for “capping” remaining free thiol groups on the thiolated protein to avoid possible nonspecific interactions. The kit contains ready-to-use spin columns that provide an extremely convenient and efficient method for purifying the thiol- or maleimide-modified proteins from excess modifying reagent. Sufficient reagents and purification media are provided to perform three protein–protein conjugations. The kit can also be readily modified for generating protein–peptide or protein–nucleic acid conjugates or for conjugating biomolecules to affinity matrices.

## Before You Begin

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**Caution** TCEP (Component C) is corrosive and NEM (Component D) is toxic and corrosive. Please handle these materials using appropriate safety equipment and practices.

### Materials Required but Not Provided

- **0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5.** Dissolve 11.5 g of  $\text{Na}_2\text{HPO}_4$ , 2.6 g of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  and 5.85 g of NaCl in 900 mL of deionized water ( $\text{dH}_2\text{O}$ ). Adjust pH to 7.5 with 5 M NaOH or 5 M HCl, if necessary. Add sufficient  $\text{dH}_2\text{O}$  to bring the volume to 1 L.
- **1 M sodium bicarbonate.** Dissolve 0.84 g of  $\text{NaHCO}_3$  in 10 mL  $\text{dH}_2\text{O}$ ; pH should be 8.3–8.5.

### Preparing the Protein

This kit is designed for conjugating small amounts (0.2–3 mg) of two different proteins (referred to as “protein X” and “protein Y” in this protocol) in a volume of up to 0.2 mL. The protocol is appropriate for all proteins containing free amino groups. Purified proteins should be at 1–15 mg/mL in an appropriate buffer (e.g. 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5) free of any amine-containing substances such as Tris, glycine, or ammonium ions. The protein solution should be at pH 7.5–8.5. Protein solutions containing relatively dilute buffers (e.g. 10–20 mM phosphate, borate, triethanolamine (TEA), or MOPS), can be pH adjusted to 7.5–8.5 by the addition of one tenth volume of 1 M sodium bicarbonate.

If one of the proteins contains native thiols (as does  $\beta$ -galactosidase, for example), steps 1.1–1.6 and steps 4.1–4.5 should be skipped, since the native thiols can be reacted directly with the maleimide groups added to the second protein (protein Y). Please note that protein Y, which will be used to form the maleimide derivative, should NOT have any free thiols. Invitrogen’s Thiol and Sulfide Quantitation Kit (T6060) can be used to assay for free thiols on proteins.

Conjugations with phycobiliproteins may optionally employ commercially obtained pyridyl-disulfide derivatives of R-phycoerythrin or B-phycoerythrin. Invitrogen has a pyridyldisulfide derivative of R-phycoerythrin (P806) that can be used in this manner. If these derivatives are used, the thiolation of protein X (steps 1.1–1.6), including the protein-conjugate purification for protein X (steps 3.1–3.7), can be skipped.

## Experimental Protocol

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### Thiolating of Protein X

- 1.1 Transfer 200  $\mu\text{L}$  of a 1–15 mg/mL solution of protein X in pH 7.5–8.5 buffer to one 2 mL reaction tube (containing a stir bar).
- 1.2 Calculate the amount of SPDP solution required for the reaction. For proteins of MW  $\geq 100,000$ , about 1.5–3 thiols/molecule of protein produce the best yield of conjugate with minimal formation of aggregates. We recommend using a molar ratio (MR) of about 5:1 (SPDP:protein) for protein concentrations of 5–15 mg/mL, or 10:1 for protein concentrations

of 1–4 mg/mL, since the reactivity of proteins with SPDP decreases upon dilution of the protein solution. Fewer thiols/molecule of protein may be necessary for optimal conjugation with proteins of MW significantly less than 100,000, and the MR (SPDP:protein) may need to be reduced accordingly.

Using the equation below, the amount ( $\mu\text{L}$ ) of 5 mg/mL SPDP stock solution needed to achieve a given MR can be calculated:

$$\mu\text{L of 5 mg/mL SPDP} = \frac{\text{mg of protein X}}{5 \text{ mg/mL SPDP}} \times \frac{312}{\text{MW of protein X}} \times \text{MR} \times 1000$$

where 312 is the MW of SPDP and 1,000 is a unit conversion factor.

- 1.3 Allow the vial of DMSO to thaw and warm to room temperature. Immediately before starting the reaction, prepare a 5 mg/mL SPDP stock solution by adding 100  $\mu\text{L}$  of DMSO to one vial of SPDP (Component A; 500  $\mu\text{g}$ ). Pipet up and down to completely dissolve the contents of the vial. Because SPDP readily hydrolyzes, solutions should be made immediately prior to use.
- 1.4 While stirring, add the appropriate amount of SPDP stock solution to the reaction tube containing the solution of protein X. Mix thoroughly. Discard the leftover SPDP stock solution.
- 1.5 Stir the reaction at room temperature for 1–1.5 hours.
- 1.6 Proceed to steps 3.1–3.7 for purifying the thiolated protein X free from excess SPDP.

### Creating a Maleimide Derivative of Protein Y

The maleimide derivative of protein Y should be prepared immediately before it is reacted with the thiolated, deprotected protein X (see *Crosslinking the Protein X–SH and Protein Y–Maleimide*). The maleimide derivative is unstable and should be used within 3 hours after it is made.

- 2.1 Transfer 200  $\mu\text{L}$  of a 1–15 mg/mL protein Y solution in pH 7.5–8.5 buffer to one 2 mL reaction tube containing a stir bar. Note that protein Y should not contain free thiols. Invitrogen's Thiol and Sulfide Quantitation Kit (T6060) can be used to assay for free thiols on proteins.
- 2.2 Calculate the amount of SMCC solution required for the reaction. Since maleimides are fairly unstable in aqueous solution, particularly above pH 8, we recommend using a higher MR of SMCC to protein Y than the MR used for SPDP and protein X. For proteins of MW  $\geq 100,000$ , a MR of 10:1 (SMCC:protein) is used for protein concentrations of 5–15 mg/mL, and a MR of 20:1 is used for concentrations of 1–4 mg/mL. The MR may need to be reduced for proteins of a MW significantly less than 100,000. The equation below can be used to calculate the amount of 5 mg/mL SMCC stock solution needed to achieve a given MR:

$$\mu\text{L of 5 mg/mL SMCC} = \frac{\text{mg of protein Y}}{5 \text{ mg/mL SMCC}} \times \frac{334}{\text{MW of protein Y}} \times \text{MR} \times 1000$$

where 334 is the MW of SMCC and 1,000 is a unit conversion factor.

- 2.3 Allow the vial of DMSO to thaw and warm to room temperature. Immediately before starting the reaction, add 100  $\mu\text{L}$  of DMSO to one vial of SMCC (Component B; 500  $\mu\text{g}$ ) to prepare a

5 mg/mL solution. Pipet up and down to completely dissolve the contents of the vial. Because SMCC readily hydrolyzes, solutions should be made immediately prior to use.

- 2.4 While stirring, add the appropriate amount of SMCC stock solution to the reaction tube containing protein Y solution. Mix thoroughly. Discard the leftover SMCC stock solution.
- 2.5 Stir the reaction at room temperature for 1–1.5 hours.
- 2.6 Proceed to steps 3.1–3.7 for purifying the protein Y–maleimide conjugate free from excess SMCC.

### **Purifying the Derivatized Proteins**

If the molecular weight of the crosslinked protein is less than 30,000, then the provided spin columns should not be used. Dependent on the size of the proteins, one could alternatively use HPLC, dialysis, or a resin with a lower cutoff molecular weight.

- 3.1 Use a new spin column for each sample to be purified. Remove the top cap of the column, then remove the bottom closure.

**Note:** the order of the removal of the caps is important.

- 3.2 Allow the column buffer (0.1 M phosphate, 0.1 M NaCl, 2 mM EDTA, 2 mM azide, pH 7.5) to drain from the column by gravity. Discard this flowthrough.

- 3.3 Place each column in a 2 mL collection tube (note this tube does not contain a stir bar) and centrifuge the column and tube for 3 minutes at  $1100 \times g$  using a swinging bucket rotor. Discard the buffer in the collection tube and save the empty collection tube for step 3.5. The spin column is now ready for purifying thiol- or maleimide-modified proteins from steps 1.1–1.6 or steps 2.1–2.6.

- 3.4 After the reaction of protein X and SPDP or protein Y and SMCC is complete, inspect the reaction mixture carefully. If any precipitate has formed, centrifuge the sample for 5 minutes in a microfuge. Apply the entire reaction mixture, or the supernatant if centrifugation was necessary, to the center of the column in a dropwise fashion. Allow the solution to absorb into the gel bed.

**Note:** It is important to not load more than 250  $\mu\text{L}$  of the reaction mixture onto each spin column, since these purification columns will not adequately remove SPDP or SMCC from sample volumes larger than 250  $\mu\text{L}$ , and the excess SPDP or SMCC will interfere with the subsequent crosslinking reaction.

- 3.5 Place the spin column in the empty collection tube and centrifuge it for 5 minutes at  $1100 \times g$ .
- 3.6 The collection tube now contains the purified thiolated protein X or the maleimide derivative of protein Y in 0.1 M phosphate, 0.1 M NaCl, 2 mM EDTA, 2 mM azide, pH 7.5. Typically about 80–90% of the protein added to the conjugation reaction is recovered as the modified protein. After purification, the SPDP–modified protein X can be stored up to two weeks at  $4^\circ\text{C}$  before being deprotected to generate free thiol (SH) groups (see steps 4.1–4.5). The puri-

fied protein Y maleimide should be used for crosslinking with deprotected thiolated protein within 3 hours; the crosslinking reaction is outlined in steps 5.1–5.8.

3.7 Discard the used spin column.

### Deprotecting the Thiolated Protein X

4.1 Calculate the amount of 1 mg/mL TCEP stock solution required to achieve a MR of 5:1 (TCEP:protein X) using the formula below:

$$\mu\text{L of TCEP} = \frac{\text{mg of protein X}}{1 \text{ mg/mL TCEP}} \times \frac{286.7}{\text{MW of protein X}} \times 5 \times 1000$$

where 286.7 is the MW of TCEP, 5 is the desired MR and 1,000 is a unit conversion factor.

- 4.2 Weigh out 3–5 mg of TCEP powder (Component C) and dissolve it in 3–5 mL of 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5, to make a 1 mg/mL solution of TCEP.
- 4.3 Add the appropriate amount of TCEP stock solution to the thiolated protein X solution. Mix well.
- 4.4 Incubate the sample at room temperature for 10–15 minutes.
- 4.5 Proceed immediately to the next section. The deprotected thiolated protein X (protein X–SH) is now ready to be conjugated with the purified protein Y–maleimide. There is no need to remove any remaining TCEP, since this reagent generates free thiols on protein X without producing products that interfere with the subsequent conjugations.

### Crosslinking the Protein X–SH and Protein Y–Maleimide

- 5.1 Combine protein X–SH from step 4.5 (or native thiol-containing protein, see *Preparing the Protein*) and protein Y–maleimide at a MR of about 1:1. Mix thoroughly.
- 5.2 Incubate the mixture for 3 hours at room temperature. Alternatively, the mixture can be incubated for 1 hour at room temperature plus overnight at 4°C. During this time, a stable thioether bond is formed between protein X and protein Y.
- 5.3 Determine the amount of NEM (Component D) necessary to “cap” the remaining free thiols and stop the reaction. Generally, the appropriate volume of 1 mg/mL NEM solution to use is equal to the volume of 5 mg/mL SPDP solution calculated in step 1.2 for the thiolation reaction. If the native protein X contains free thiols, NEM should not be used.
- 5.4 Weigh 3–5 mg of NEM (Component D) and dissolve it in 3–5 mL of 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5, to make a 1 mg/mL solution. Note that it may take up to 5 minutes for the NEM to completely dissolve. We recommend using a clear glass tube for making this solution so that it will be easy to confirm that all the NEM crystals have dissolved. Stirring or shaking the tube speeds up this process.
- 5.5 Add the appropriate amount of NEM solution to the reaction mixture from step 5.2. Incubate the sample for 30 minutes at room temperature.
- 5.6 The crosslinking of protein X and protein Y is now complete. The conjugation mixture should contain mostly the protein X–protein Y conjugate, with small amounts of free protein

X and protein Y. This mixture can now be used directly in the desired biological application. Since only small amounts of proteins were crosslinked, further purification is not practical, nor is it usually necessary. The final concentration of conjugate (mg/mL) can be estimated by assuming a 50–70% recovery of the starting amounts of protein X and protein Y (the sum, in mg) and dividing by the final volume (in mL). Since some proteins may aggregate during the conjugation procedure and cause higher background signals in the biological application, it is recommended that the conjugate solution be centrifuged for 5 minutes in a microcentrifuge before use. Only the supernatant should then be used.

- 5.7 If desired, when more than 3 mg total protein is used for crosslinking, the protein–protein conjugate can be purified by size-exclusion column chromatography. Generally, BioRad Bio-Gel A-0.5 or A-1.5 chromatography matrices are useful in this application. Amicon Centricon filters with 50 kD or 100 kD cutoff may also be useful to purify the conjugate, or at least to eliminate the smaller unconjugated protein component.
- 5.8 If the concentration of the conjugate is  $\leq 1$  mg/mL, 1–10 mg/mL of bovine serum albumin (BSA), gelatin, or other protein should be added as a stabilizer. Store the conjugate solution at 4°C. We recommend adding a small amount of preservative, such as sodium azide or thimerosal, when possible. If both proteins of the conjugate are stable upon freezing and thawing, the conjugate may be frozen in small aliquots for long-term storage.

## Product List Current prices may be obtained from our website or from our Customer Service Department.

Cat #	Product Name	Unit Size
P6305	Protein–Protein Crosslinking Kit *3 conjugations* .....	1 kit

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### Toll-Free Ordering for USA:

Order Phone: (800) 438-2209  
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