EZ-Link™ DBCO Protein Labeling Kit

Catalog Number C20046

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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 Thermo Scientific™ EZ-Link™ DBCO Protein Labeling Kit (Cat. No. C20046) provides a convenient method to label proteins with dibenzylcyclooctyne (DBCO) for use in strain-promoted azide-alkyne cycloaddition (SPAAC) reactions. The kit uses EZ-Link™ TFP Ester-PEG4-DBCO, a heterobifunctional crosslinker that readily reacts with primary amines, forming covalently attached DBCO groups that can be further reacted with an azide-labeled coupling partner to produce diverse bioconjugates. The kit includes reagents for DBCO-labeling and purification of five 100-µL samples with a concentration of 0.5–5 mg/mL. Labeling and purification can be performed in as little as 90 minutes.

The kit includes the following reagents.

- **EZ-Link™ TFP Ester-PEG4-DBCO, No-Weigh™ Format—**Crosslinker that contains an amine-reactive tetrafluorophenyl (TFP) and azide-reactive DBCO group linked by a hydrophilic PEG spacer arm.
- **Zeba™ Dye and Biotin Removal Spin Columns—**Contain a ready-to-use resin that is designed for rapid removal of unconjugated crosslinker with exceptional protein recovery (typically >85%).
- **Dimethyl Sulfoxide (DMSO), Anhydrous—**Solubilizing agent for EZ-Link™ TFP Ester-PEG4-DBCO, No-Weigh™ Format.
- **BupH™ Phosphate Buffered Saline Pack—**Contains a dry-blend powder that is sufficient to prepare 500 mL of phosphate-buffered saline (PBS; 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2).

The kit is optimized for labeling proteins with molecular weights between 20 kDa and 150 kDa.

Procedure overview

DBCO and azides are bioorthogonal coupling partners, as they can react in a biological system without interfering with normal biochemical processes. As a result, there is minimal off-target labeling of macromolecules found in cells or complex cell lysates. In a typical conjugation procedure, labeling occurs in two steps. First, each coupling partner is independently labeled with a DBCO or azide group. Second, the two partners are introduced into the same system where labeling occurs without any additional reagents, such as a copper catalyst. The reaction between a DBCO and azide can be used for a variety of applications including protein-protein, protein-biomolecule, and protein-small molecule conjugations.

In this procedure, a 10- to 40-fold molar excess of EZ-Link™ TFP Ester-PEG4-DBCO is added to the protein sample. The reaction mixture is incubated at room temperature for 1 hour, during which the TFP group reacts with a primary amine to form a stable amide bond and a covalently attached DBCO group. After excess EZ-Link™ TFP Ester-PEG4-DBCO and other byproducts are removed, purified DBCO-labeled proteins can be used in SPAAC reactions to form stable triazole linkages with azide-labeled coupling partners (supplied separately). See Figure 1.

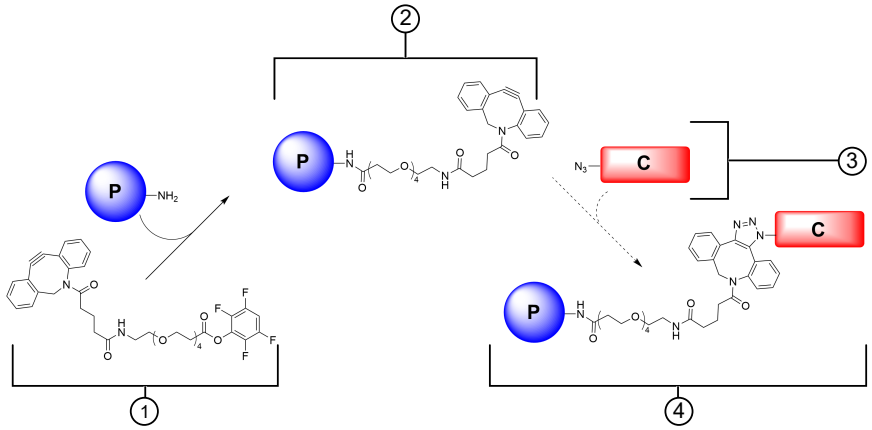

Figure 1 Two-step reaction scheme: Conjugation of a protein (P) to an azide-containing coupling partner (C) using EZ-Link™ TFP Ester-PEG4-DBCO

① EZ-Link™ TFP Ester-PEG4-DBCO
② DBCO-labeled protein
③ Azide coupling partner
④ Conjugated protein

Contents and storage

Table 1 EZ-Link™ DBCO Protein Labeling Kit (Cat. No. C20046)

Contents	Amount	Storage[1]
EZ-Link™ TFP Ester-PEG4-DBCO, No-Weigh™ Format (crosslinker)[2]	5 × 1 mg	≤–15°C Store desiccated.
Zeba™ Dye and Biotin Removal Spin Columns[3]	5 columns	2–8°C
Dimethyl Sulfoxide (DMSO), Anhydrous	1 × 3 mL	20–25°C
BupH™ Phosphate Buffered Saline Pack	1 pack	

[1] The kit is stable for 1 year when stored as directed.
[2] EZ-Link™ TFP Ester-PEG4-DBCO, No-Weigh™ Format has a molecular weight of 714.70 and a spacer arm length of 17.9 Å. The compound is clear to light amber and may not be visible at the bottom of the tube.
[3] The resin is supplied in a 0.1M NaCl/0.05% sodium azide solution.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Table 2 Materials required for DBCO-labeling and purification

Item	Source
Variable-speed benchtop microcentrifuge	MLS
1.5- or 2-mL microcentrifuge tubes	MLS
Vortex mixer	MLS
Deionized water	MLS
(Optional) NanoDrop™ Spectrophotometer, or equivalent UV-Vis spectrophotometer[1]	www.thermofisher.com

[1] Recommended to determine the degree of DBCO labeling.

Table 3 *(Optional)* Additional materials required for conjugation to an azide-labeled coupling partner

Item	Source
Zeba™ Spin Desalting Columns	www.thermofisher.com
Slide-A-Lyzer™ Dialysis Cassettes	www.thermofisher.com

Procedural guidelines

- Do not use buffers that contain primary amines (such as Tris or glycine), free ammonium ions, or azides because they will compete with the intended reaction. Dialyze or desalt samples into an appropriate buffer, such as PBS, before use. Impure proteins, such as antibodies in crude serum, will not label well.
- Reconstitute the EZ-Link™ TFP Ester-PEG4-DBCO crosslinker with DMSO (provided in the kit).

  Note: The crosslinker contains a hydrophilic PEG spacer arm to enhance solubility of DBCO-labeled proteins, however, the compound does not easily dissolve in water or other aqueous buffers.
- The crosslinker is moisture-sensitive. Equilibrate the vial to room temperature before opening to prevent moisture condensation inside the vial. Store unused vials in the foil pouch provided.
- Use the crosslinker immediately after reconstitution. Discard any unused portion.
- Do not reuse Zeba™ Dye and Biotin Removal Spin Columns.
- To achieve the desired results of the conjugate in your application, it may be necessary to optimize the labeling efficiency. See "Determine the degree of labeling (optional)" on page 5.

Guidelines for determining the molar excess of crosslinker to protein

Optimal crosslinker-to-protein molar ratios for reactions must be determined empirically. For optimal results, observe the following guidelines:

- Consider the concentration of the protein sample and the number of primary amino groups on the surface of the protein. Proteins at higher concentrations or with numerous amino groups require less crosslinker.
- For optimal results, we recommend the following molar excesses of crosslinker to protein.

  Note: The amount of crosslinker used may require optimization, depending on the desired degree of labeling.

Protein concentration	Recommended molar excess
0.5 to ≤1 mg/mL	20–40X
>1 to 5 mg/mL	10–20X

Before you begin

Prepare the reagents and protein sample

1. Prepare PBS solution—Add the contents of one BupH™ Phosphate Buffered Saline Pack to 500 mL of deionized water.
2. Prepare the protein sample at a concentration of 0.5–5 mg/mL in PBS solution. The final volume should be ≤120 µL.

Calculate the amount of crosslinker to add to the sample

To determine the optimal molar excess of crosslinker (relative to the protein concentration) to use in the reaction, see "Guidelines for determining the molar excess of crosslinker to protein" on page 3.

Use the following formula to calculate the amount (in millimoles) of crosslinker to add to the sample for a 20-fold molar excess.

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{\text{20 mmol crosslinker}}{\text{1 mmol protein}} = \textbf{mmol crosslinker}$$

Use the following formula to calculate the volume (in µL) of 3.5-mM crosslinker to add to the sample.

$$\text{mmol crosslinker} \times \frac{1 \times 10^6\ \mu\text{L}}{1\ \text{L}} \times \frac{1\ \text{L}}{3.5\ \text{mmol crosslinker}} = \textbf{µL crosslinker}$$

Example: For 100 µL of 1 mg/mL IgG (MW = 150,000 g/mol), add 3.8 µL of 3.5-mM crosslinker to the prepared sample.

$$0.100\ \text{mL IgG} \times \frac{1\ \text{mg protein}}{1\ \text{mL protein}} \times \frac{1\ \text{mmol IgG}}{1.5 \times 10^5\ \text{mg IgG}} \times \frac{\text{20 mmol crosslinker}}{\text{1 mmol IgG}} = \textbf{1.33} \times \textbf{10}^{\textbf{-5}}\ \textbf{mmol crosslinker}$$

$$1.33 \times 10^{-5}\ \text{mmol crosslinker} \times \frac{1 \times 10^6\ \mu\text{L}}{1\ \text{L}} \times \frac{1\ \text{L}}{3.5\ \text{mmol crosslinker}} = \textbf{3.8 µL crosslinker}$$

Protein labeling procedure

1 Prepare the crosslinker, then combine with the sample

1.1. Equilibrate one vial of crosslinker to room temperature.

1.2. Just before use, reconstitute the crosslinker.

a. Add 400 µL of Dimethyl Sulfoxide (DMSO), Anhydrous to one vial of EZ-Link™ TFP Ester-PEG4-DBCO.

b. Vortex or pipet up and down until the solution is homogenous.

IMPORTANT! Use the reconstituted crosslinker immediately. Hydrolysis begins upon reconstitution.

1.3. Add the appropriate molar excess of crosslinker to the prepared sample (see "Calculate the amount of crosslinker to add to the sample" on page 3).

1.4. Incubate the reaction mixture for 1 hour at room temperature or 2 hours on ice.

Discard the unused portion of the reconstituted crosslinker.

2 Prepare the Zeba™ Dye and Biotin Removal Spin Column

2.1. Twist to remove the bottom plug of the column, then loosen the cap. Do not remove the cap.

2.2. Place the column in a 1.5- or 2-mL microcentrifuge tube, then centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes. Discard the flow-through.

If you are using a fixed angle rotor, place a mark on the side of the column facing away from the rotor. For all subsequent centrifugation steps, ensure that the column is oriented in the same position.

IMPORTANT! Improper orientation of the column during centrifugation can result in inefficient removal of the crosslinker.

2.3. Remove the cap of the column, then add 400 µL of PBS solution to equilibrate the column and remove sodium azide from the resin.

IMPORTANT! Removal of sodium azide is critical for preserving the functionality of the labeled protein.

2.4. Centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes. Discard the flow-through.

3 Purify the DBCO-labeled protein

3.1. Place the prepared column in a new microcentrifuge tube, then remove the cap.

3.2. Slowly apply the reaction mixture (≤120 µL) to the center of the settled resin.

3.3. Centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes. Discard the column.

The purified DBCO-labeled protein is in the microcentrifuge tube.

3.4. *(Optional)* Proceed to determine the degree of labeling (see "Determine the degree of labeling (optional)" on page 5).

Use the DBCO-labeled protein immediately for conjugation, or store at 2–8°C, protected from light.

4 Conjugate the DBCO-labeled protein to an azide (optional)

The following procedure is an example application for this product in a SPAAC reaction. Specific applications will require optimization.

4 Conjugate the DBCO-labeled protein to an azide (optional) *(continued)*

4.1. Prepare the azide-labeled coupling partner (supplied separately) in an azide-free buffer, such as PBS.

For an antibody-small molecule conjugation reaction, we recommend a starting antibody concentration of 1 mg/mL.

4.2. Add the prepared azide-labeled coupling partner to the DCBO-labeled protein at a 1.5- to 10-fold molar excess.

For an antibody-small molecule conjugation reaction, we recommend starting with a 7.5-fold molar excess of azide-labeled coupling partner to DBCO-labeled protein.

4.3. Incubate the reaction mixture for 4–12 hours at room temperature.

Note: Incubation at 4°C can be extended to overnight (>12 hours) if needed.

4.4. Depending on your application, proceed in one of the following ways.

- Use the conjugated protein immediately for the intended application.
- Proceed to purify the conjugated protein. See "Purify the DBCO-labeled protein" on page 4.

5 Determine the degree of labeling (optional)

The efficiency of the conjugation reaction can be determined by measuring the absorbance of the protein at 280 nm and the absorbance of the DBCO group at its excitation maximum (309 nm). We recommend using a NanoDrop™ Spectrophotometer for convenience. No dilution or cuvettes are needed; 1–2 µL of the protein sample can be added directly onto the pedestal.

5.1. For samples with high concentrations, dilute a small amount of the purified conjugate with PBS.

5.2. Measure the absorbance of the protein at 280 nm (A_{280}) and the DBCO group at 309 nm (A_{309}).

5.3. Calculate the concentration of the protein in the sample using the following formula.

$$\text{Protein concentration (M)} = \frac{[A_{280} - 0.90(A_{309})] \times \text{Dilution factor}}{203{,}000}$$

Note: 203,000 is the molar extinction coefficient (ε) in $cm^{-1}M^{-1}$ of a typical IgG at 280 nm and is also suitable for IgA, IgD, and IgE. In this equation, 0.90 is a correction factor for the DBCO contribution to A_{280}.

5.4. Calculate the degree of labeling (DOL) using the following formula.

$$\text{DOL} = \frac{\text{Moles of DBCO}}{\text{Moles of protein}} = \frac{A_{309} \times \text{Dilution factor}}{12{,}000 \times \text{Protein concentration}}$$

Note: 12,000 is the approximate molar extinction coefficient in $cm^{-1}M^{-1}$ of the DBCO group.

Troubleshooting

Observation	Possible cause	Recommended action
No conjugation of DBCO with azide	One or more coupling partners were not labeled.	Confirm molecules were labeled or repeat the activation process.
	The EZ-Link™ TFP Ester-PEG4-DBCO crosslinker was hydrolyzed.	Allow the product to equilibrate to room temperature before opening.
	Labeling was insufficient.	Increase the molar excess of labeling reagent.
	Excess reagent was not quenched or removed.	Remove non-reacted reagent by dialysis or desalting.
Low conjugation of DBCO and azide	Reaction conditions were suboptimal.	Optimize the conjugation conditions by altering the molar excess or increasing the concentration.
		Perform conjugation reactions at 37°C.
		Increase the incubation time.

Related products

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Product	Cat. No.
EZ-Link™ Maleimide-PEG4-DBCO No-Weigh Format	C20044
EZ-Link™ Biotin-PEG12-DBCO	C20042
SiteClick™ Antibody Azido Modification Kit	S20026
NHS-Azide	88902
NHS-PEG4-Azide	26130
Click-iT™ ManNAz Metabolic Glycoprotein Labeling Reagent	C33366
Zeba™ Dye and Biotin Removal Spin Columns	A44296

Limited product warranty

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Revision	Date	Description
A.0	6 December 2021	New manual for EZ-Link™ DBCO Protein Labeling Kit.

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