EZ-Link™ TFP Ester-PEG4-DBCO and EZ-Link™ TFP Ester-PEG12-DBCO

Catalog Numbers C20040, C20039, C20043

Pub. No. MAN0026008 Rev. A.0

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™ TFP Ester-PEG4-DBCO and EZ-Link™ TFP Ester-PEG12-DBCO (referred hereafter as EZ-Link™ TFP-PEG(n)-DBCO) are heterobifunctional crosslinkers which contain an amine reactive tetrafluorophenyl (TFP) and a dibenzylcyclooctyne (DBCO) with a hydrophilic PEG spacer in between the two groups.

This guide describes a general protocol for labeling proteins using the amine reactive TFP-PEG(n)-DBCO reagents as well as guidelines for performing the second click reaction with an azide.

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 the first step of this procedure, the TFP group reacts with a primary amine in a protein, (bio)molecule, or surface to form a stable amide bond and a covalently bound DBCO moiety (see Figure 1). Once a protein, surface, or (bio)molecule is DBCO labeled it can undergo a copper free strain-promoted azide-alkyne cycloaddition (SPAAC) to form a stable triazole linkage with an azide labeled coupling partner (supplied separately) for the creation of diverse bioconjugates. Dibenzylcyclooctynes (DBCO) and azides are bioorthogonal coupling partners as they can react in a biological system without interfering with normal biochemical processes and as a result there is minimal off target labeling of macromolecules found in cells or lysates.

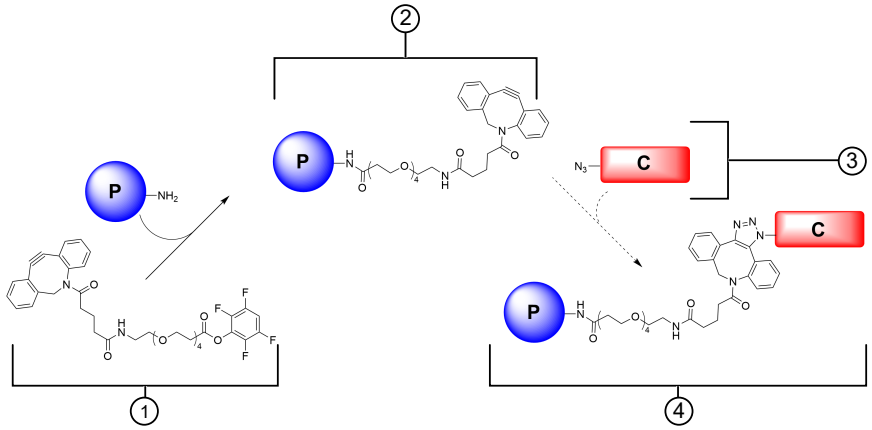
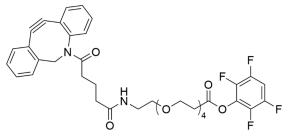
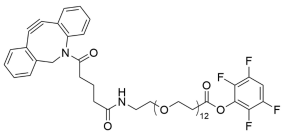


Figure 1 Two-step reaction scheme: Conjugation of a protein (P) to an azide-containing coupling partner (C) using EZ-Link™ TFP Ester-PEG4-DBCO or EZ-Link™ TFP Ester-PEG12-DBCO

① EZ-Link™ TFP Ester-PEG4-DBCO or EZ-Link™ TFP Ester-PEG12-DBCO
② DBCO-labeled protein
③ Azide coupling partner
④ Conjugated protein

Contents and storage

Contents	Structure	Cat. no.	Amount	Storage[1]
EZ-Link™ TFP Ester-PEG4-DBCO [2]		C20039	25 mg	≤–15°C Store desiccated.
EZ-Link™ TFP Ester-PEG4-DBCO, No-Weigh™ Format[3]		C20043	10 × 1 mg	
EZ-Link™ TFP Ester-PEG12-DBCO[4]		C20040	25 mg	

[1] The product is stable for 1 year when stored as directed.

[2] EZ-Link™ TFP Ester-PEG4-DBCO has a molecular weight of 714.70 and a spacer arm length of 17.9 Å.

[3] 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.

[4] EZ-Link™ TFP Ester-PEG12-DBCO has a molecular weight of 1067.12 and a spacer arm length of 46.3 Å.

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 1 Materials required for TFP-PEG(n)-DBCO labeling and purification

Item	Source
Anhydrous dimethyl sulfoxide (DMSO)	D12345
Phosphate-buffered saline	28372
Zeba™ Dye and Biotin Removal Columns	A44296
1.5- or 2-mL microcentrifuge tubes	MLS
Variable-speed benchtop microcentrifuge	MLS

Table 2 *(Optional)* Additional materials required for coupling with an azide or determining the degree of DBCO labeling

Item	Source
Coupling with an azide	
Zeba™ Spin Desalting Columns	www.thermofisher.com
Slide-A-Lyzer™ Dialysis Cassettes	www.thermofisher.com
Degree of DBCO labeling	
NanoDrop™ Spectrophotometer	www.thermofisher.com

Procedural guidelines

- Do not use buffers that contain primary amines (e.g., Tris or glycine) or sulfhydryls because they will compete with the intended reaction. If necessary, dialyze or desalt samples into an appropriate buffer, such as PBS, before use.
- Do not use buffers that contain azides, which can react with DBCO.
- Dissolve the TFP-PEG(n)-DBCO in a dry water-miscible organic solvent such as DMSO or DMF before diluting into the final reaction buffer. The TFP-PEG(n)-DBCO does not easily dissolve directly in water or aqueous buffer.
- TFP-PEG(n)-DBCO is moisture sensitive. Equilibrate vial to room temperature before opening to prevent moisture condensation inside the vial.
- Use TFP-PEG(n)-DBCO immediately after reconstitution. Discard any unused portion.

- Conjugation with primary amines of proteins/peptides (i.e.,acylation) is favored at near neutral pH (6–9) and with concentrated protein solutions. For conjugation, use non-amine containing buffers at pH 7–9 such as PBS (20mM sodium phosphate, 150mM sodium chloride, pH 7.4; Product No. 28372); 20mM HEPES; 100mM carbonate/biocarbonate; or 50mM borate buffer.

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

Calculate EZ-Link™ TFP-PEG(n)-DBCO amount

See example calculations below to determine how much EZ-Link™ TFP-PEG(n)-DBCO to add.

1. Calculate millimoles of EZ-Link™ TFP-PEG(n)-DBCO reagent to add to the reaction for a 20-fold molar excess.

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{\text{20 mmol DBCO}}{\text{1 mmol protein}} = \textbf{mmol TFP-PEG(n)-DBCO}$$

2. Calculate microliters of 3.5 mM EZ-Link™ TFP-PEG(n)-DBCO reagent solution to add to the reaction.

   Note: For EZ-Link™ Maleimide-PEG4-DBCO No-Weigh Format, this can be achieved by adding 400 µL of anhydrous DMSO directly to the vial.

$$\text{mmol TFP-PEG\{n\}-DBCO} \times \frac{1 \times 10^6\ \mu\text{L}}{\text{L}} \times \frac{\text{L}}{\text{3.5 mmol TFP-PEG(n)-DBCO}} = \textbf{µL TFP-PEG(n)-DBCO}$$

   Example: For 1 mL of 1 mg/mL IgG (MW = 150,000 g/mol), add 38.1 µL of 3.5-mM TFP-PEG(n)-DBCO to the prepared sample.

$$\text{1 mL IgG} \times \frac{\text{1 mg protein}}{\text{1 mL protein}} \times \frac{\text{1 mmol IgG}}{1.5 \times 10^5\ \text{mg IgG}} \times \frac{\text{20 mmol DBCO}}{\text{1 mmol IgG}} = \textbf{1.33} \times \textbf{10}^{\textbf{-4}}\ \textbf{mmol TFP-PEG(n)-DBCO}$$

$$1.33 \times 10^{-4}\ \text{mmol TFP-PEG(n)-DBCO} \times \frac{1 \times 10^6\ \mu\text{L}}{\text{L}} \times \frac{\text{L}}{\text{3.5 mmol TFP-PEG(n)-DBCO}} = \textbf{38.1 µL TFP-PEG(n)-DBCO}$$

Procedure for protein conjugation

Label protein

1. Prepare protein at a concentration of 1–5 mg/mL in PBS or other suitable buffer that is azide and amine free.

2. Immediately before use, prepare a 3.5 mM solution of TFP-PEG(n)-DBCO in anhydrous DMSO or DMF. Vortex and mixed well to fully dissolve the TFP-PEG(n)-DBCO.

   Note: If using EZ-Link™ TFP Ester-PEG4-DBCO, No-Weigh™ Format, add 400 µL of anhydrous DMSO directly to the vial to make a 3.5 mM solution . The vial can accommodate a total volume of 700 µL if a more dilute solution is desired.

3. Add the appropriate amount of TFP-PEG(n)-DBCO (see Calculations Section) to the protein solution.

4. Incubate reaction for 2 hours at room temperature or overnight on ice.

5. Remove unreacted TFP-PEG(n)-DBCO with suitable purification media. We recommend using Zeba™ Dye and Biotin Removal Columns.

   Note: Columns must first be equilibrated with an azide free buffer prior to protein purification

Store the labeled protein at 2–8°C, protected from light.

(*Optional*) Couple EZ-Link™ TFP-PEG(n)-DBCO labeled conjugate to an azide

The following protocol is an example application for this product for strain-promoted azide-alkyne cycloaddition (SPAAC). Specific applications will require optimization.

1. Prepare the azide containing moiety in an azide free buffer such as PBS.

   Note: For an antibody-small molecule conjugation reaction, a starting antibody concentration of 1 mg/mL is recommended. Lower concentrations may increase reaction times

2. Add the DBCO labeled conjugate to the azide labeled moiety

   Note: A molar excess of 1.5–10 equivalents of one of the coupling partners can be used to increase conjugation efficiency. For an antibody-small molecule conjugation reaction, 7.5 equivalents excess of one of the coupling partners is recommended.

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

   Note: The reaction can be incubated at 4°C; however, this must be done overnight for at least 12 hours.

The conjugate is now ready for purification or can be used directly depending on application. Sodium azide can be added following the DBCO/azide coupling to a final concentration of 0.02% (w/v) for long term storage, if preferred.

(*Optional*) Determine degree of DBCO labeling

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.

Note: Excessive dilution of some proteins with low intrinsic A_{280} may prevent you from deriving accurate A_{280} values for your samples. Use only a portion of your protein conjugate sample and dilute it only to the minimum volume necessary for your cuvettes and spectrophotometer to avoid readings below the optimal range for your instrument.

1. For samples with high concentrations, dilute a small amount of the purified conjugate into PBS or other suitable buffer.

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

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}.

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 activation process.
	TFP-ester was hydrolyzed.	Allow product to equilibrate to room temperature before opening.
	Labeling was insufficient.	Increase the molar excess of labeling reagent.

Observation	Possible cause	Recommended action
No conjugation of DBCO with azide *(continued)*	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 conjugation conditions by altering molar excess or increasing concentration.
		Perform conjugation reactions at 37°C.
		Increase 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

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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

Revision history: Pub. No. MAN0026008

Revision	Date	Description
A.0	9 December 2021	New manual for EZ-Link™ TFP Ester-PEG4-DBCO and EZ-Link™ TFP Ester-PEG12-DBCO.