# EZ-Link™ Maleimide-PEG4-DBCO

Catalog Numbers C20041, C20044

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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™ Maleimide-PEG4-DBCO is a heterobifunctional crosslinker which contain a thiol/sulfhydryl maleimide and a dibenzylcyclooctyne (DBCO) with a hydrophilic PEG spacer in between the two groups.

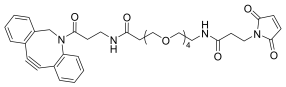
This guide describes a general protocol for labeling proteins using the thiol/sulfhydryl reactive Maleimide-PEG4-DBCO reagent as well as guidelines for performing the second click reaction with an azide.

### 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 Maleimide group reacts with a thiol/sulfhydryl group in a protein, (bio)molecule, or surface to form a stable thioether bond and a covalently bound DBCO moiety. 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.

## Contents and storage

| Contents[1] | Structure | Cat. no. | Amount | Storage[2] |
|---|---|---|---|---|
| EZ-Link™ Maleimide-PEG4-DBCO |  | C20041 | 25 mg | ≤–15°C<br>Store desiccated. |
| EZ-Link™ Maleimide-PEG4-DBCO No-Weigh Format[3] | | C20044 | 10 × 1 mg | |

[1] EZ-Link™ Maleimide-PEG4-DBCO and EZ-Link™ Maleimide-PEG4-DBCO No-Weigh Format have a molecular weight of 647.74 and a spacer arm length of 29.75 Å.
[2] The product is stable for 1 year when stored as directed.
[3] This compound is clear to light amber and may not be visible at the bottom of the tube.

## 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 Maleimide-PEG4-DBCO labeling and purification**

| Item | Source |
|---|---|
| Anhydrous dimethyl sulfoxide (DMSO) | D12345 |
| Phosphate-buffered saline or other sulfhydryl-free buffer, pH 6.5–7.5 | 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 extraneous sulfhydryl-containing components in the reaction buffers during conjugation (e.g., DTT). They react with the maleimide portion of the reagent, inhibiting and reducing conjugation efficiency of the intended target.
- Do not use buffers that contain azides, which can react with DBCO.
- Dissolve the EZ-Link™ Maleimide-PEG4-DBCO in a dry water-miscible organic solvent such as DMSO or DMF before diluting into the final reaction buffer. EZ-Link™ Maleimide-PEG4-DBCO does not easily dissolve directly in water or aqueous buffer.
- EZ-Link™ Maleimide-PEG4-DBCO is moisture-sensitive. Store product in the original container frozen with desiccant. Equilibrate vial to room temperature before opening to avoid moisture condensation onto the product.
- Prepare reagent solution immediately before use. The maleimide moiety will hydrolyze and become non-reactive in water; therefore, stock solutions cannot be prepared for storage. Discard any unused reconstituted reagent.
- A molecule must have a free (reduced) sulfhydryl to react with the maleimide moiety of EZ-Link™ Maleimide-PEG4-DBCO.
  - **To add sulfhydryls to molecules:** Use Pierce™ SATA (N-succinimidyl S-acetylthioacetate) (Cat. No. 26102) or Pierce™ Traut's Reagent (2-iminothiolane) (Cat. No. 26101), which modify primary amines.
  - **To reduce disulfide bonds in peptides:** Use Pierce™ Immobilized TCEP Disulfide Reducing Gel (Cat. No. 77712).
  - **To reduce disulfide bonds in proteins:** Use 5 mM (1:100 dilution) of Bond-Breaker™ TCEP Solution, Neutral pH (Cat. No. 77720), incubate for 30 minutes at room temperature, then pass two times through a desalting column (e.g., Zeba™ Spin Desalting Columns).

    **Note:** Complete reduction of disulfide bonds can inactivate proteins (e.g., antibodies). Selective reduction of hinge-region disulfide bonds in IgG can be accomplished with Pierce™ Mercaptoethylamine-HCl (2-MEA; Cat. No. 20408).
- Ensure excess EZ-Link™ Maleimide-PEG4-DBCO is removed after the maleimide-labeling procedure to ensure an efficient conjugation reaction.
- Do not reuse Zeba™ Dye and Biotin Removal Spin Columns.
- The maleimide group reacts predominantly with free sulfhydryls at pH 6.5-7.5, forming stable thioether bonds. At pH values > 7.5, reactivity toward primary amines and hydrolysis of the maleimide groups can occur. At pH 7, the maleimide group is ~1000 times more reactive toward a free sulfhydryl than to an amine

## Guidelines for determining optimal amount of EZ-Link Maleimide-PEG4-DBCO

The optimal amount of EZ-Link™ Maleimide-PEG4-DBCO to use for each reaction depends on a number of factors. For optimal results, observe the following guidelines:

- The extent of labeling can be controlled by regulating the reagent-to-target molar ratio in the reaction.
- Use 5- to 20-fold molar excess of reagent as a starting point for protein solutions >1 mg/mL.
- When labeling more dilute solutions, a greater relative molar fold excess of reagent may be necessary to achieve the same results.

## Before you begin

### Calculate EZ-Link™ Maleimide-PEG4-DBCO amount

Example calculations for IgG modification (MW = 150,000 g/mol) are provided below.

1. Calculate the quantity in millimoles of EZ-Link™ Maleimide-PEG4-DBCO to add to the reaction for a 20-fold molar excess.

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{\text{20 mmol DBCO}}{\text{1 mmol protein}} = \textbf{mmol Maleimide-PEG4-DBCO}$$

2. Calculate microliters of 3.9 mM EZ-Link™ Maleimide-PEG4-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 uL of DMSO to the vial.

$$\text{mmol Maleimide-PEG4-DBCO} \times \frac{1 \times 10^6\ \mu\text{L}}{1\ \text{L}} \times \frac{1\ \text{L}}{3.9\ \text{mmol Maleimide-PEG4-DBCO}} = \textbf{µL Maleimide-PEG4-DBCO}$$

   **Example:** For 1 mL of 2 mg/mL IgG (MW = 150,000 g/mol), add 68.2 µL of 3.9-mM Maleimide-PEG4-DBCO to the prepared sample.

$$1\ \text{mL IgG} \times \frac{1\ \text{mg protein}}{1\ \text{mL protein}} \times \frac{1\ \text{mmol IgG}}{1.5 \times 10^5\ \text{mg IgG}} \times \frac{20\ \text{mmol DBCO}}{1\ \text{mmol IgG}} = \textbf{2.66} \times \textbf{10}^{-4}\ \textbf{mmol Maleimide-PEG4-DBCO}$$

$$2.66 \times 10^{-4}\ \text{mmol Maleimide-PEG4-DBCO} \times \frac{1 \times 10^6\ \mu\text{L}}{\text{L}} \times \frac{\text{L}}{3.9\ \text{mmol Maleimide-PEG4-DBCO}} = \textbf{68.2 µL Maleimide-PEG4-DBCO}$$

## Procedure for protein conjugation

### Label protein

1. Dissolve protein to be modified in sulfhydryl and azide-free buffer at pH 6.5–7.5 at a concentration of >1 mg/mL.

   **Note:** Protein already in sulfhydryl-free buffer at pH 6.5–7.5 may be used without buffer exchange or dilution.

2. Immediately before use, make a 3.9 mM stock solution of EZ-Link™ Maleimide-PEG4-DBCO in anhydrous DMSO or DMF (400 µL per mg). Vortex and mix well to fully dissolve the Maleimide-PEG4-DBCO.

   **Note:** If using EZ-Link™ Maleimide-PEG4-DBCO No-Weigh Format, add 400 µL of anhydrous DMSO directly to the vial to make a 3.9 mM stock solution. The vial can accommodate a total volume of 700 µL if a more dilute solution is desired.

3. Add the appropriate volume of the EZ-Link™ Maleimide-PEG4-DBCO (see Calculations Section) to the protein solution.

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

   **Note:** Except for possible degradation or microbial growth, there is no harm in reacting longer than the specified time.

5. Remove unreacted EZ-Link™ Maleimide-PEG4-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™ Maleimide-PEG4-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. |
| | Maleimide was hydrolyzed. | Allow 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 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™ TFP Ester-PEG4-DBCO, No-Weigh™ Format | C20043 |
| 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 | 9 December 2021 | New manual for EZ-Link™ Maleimide-PEG4-DBCO. |

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