Click Chemistry Tools
Bioconjugate Technology Company

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Lys-to-Lys Protein-Protein Conjugation Kit (Small Scale 100-500 µg)

Description

Lys-to-Lys Protein-Protein Conjugation Kit (Cat. # 1008) provides sufficient reagents to perform two protein-protein conjugation reactions. Start to finish, any two lysine containing proteins (100-500 µg) in a volume of 100 µL (1-5 mg/mL) can be efficiently conjugated in less than 3 hours.

Kit Contents:

Component	Amount
Tetrazine-PEG_5-NHS ester(Tz)	2 x 0.5 mg
Trans-Cyclooctene-PEG_4-NHS ester (TCO)	2 x 0.5 mg
DMSO	5 mL
BupH™Saline Buffer Pack	1 pack
Zeba™Spin Columns (0.5 mL)	8

Introduction

Lys-to-Lys Protein-Protein Conjugation kit provides all the necessary reagents to perform two protein-protein conjugation reactions. Conjugates are formed by an inverse-electron demand Diels-Alder cycloaddition reaction between *trans*-cyclooctene (TCO) and tetrazine (Tz) functional groups, a bioorthogonal click reaction characterized by exceptional kinetics ($k > 800\ M^{-1}s^{-1}$) and selectivity (Figure 1). The Tz/TCO reaction pair represents the most powerful tool available for catalyst-free protein–protein bioconjugation. Fast kinetics combined with exquisite specificity enables rapid conjugation (30-60 min) of lysine containing proteins to each other at low concentrations (e.g. 5-10 µM) with > 99% conversion of the limiting protein to conjugate in mild buffered media (e.g. PBS pH 7.5). Other features include long-term reactive stability of TCO and Tz functional groups on modified proteins stored in aqueous buffered media (e.g. maintaining > 90% reactivity after 1 monthat 4°C, pH 7.5). This stability avoids time-sensitive timing of reactions associated with classical chemistries based on thiol/maleimide chemistry, providing worry-free conjugation results.

ImportantInformation

- NHS esters are moisture-sensitive. Avoid moisture condensation by allowing product to come to room temperature before opening. Prepare working stock solutions immediately before use.
- For NHS ester reactions avoid buffers containing primary amines (e.g. Tris, glycine).

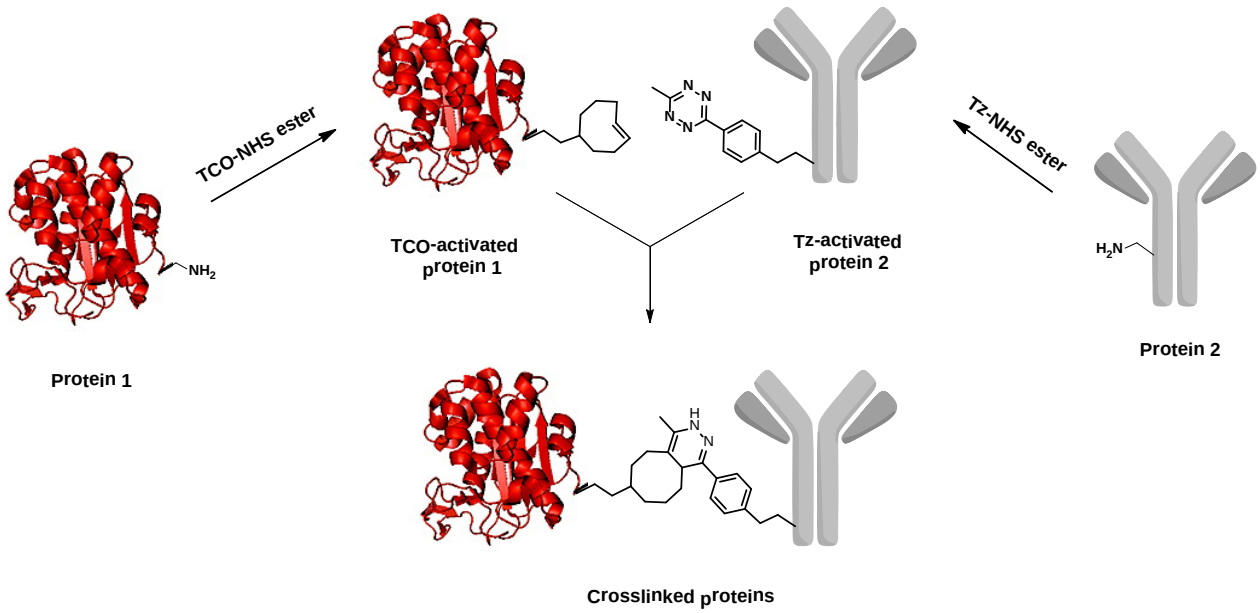

Figure 1. Schematic representation of protein-protein conjugation chemistry.

Protein Requirements

- Protocol requires 100-500 µg of each protein in a volume of 0.1 mL (1-5 mg/mL)
- Proteins must be highly purified and their molecular weight known
- Proteins must have available primary amines (e.g. lysine residues)

Additional Information

- When conjugating proteins at low concentrations (e.g. <5 µM), we recommend using a 4-5 fold molar excess over the limiting protein.
- Whenever possible, maintainthe protein used in excess at higher concentration than limiting protein. For example, if IgG is the limiting protein at 1 mg/mL keep the excess protein at 4-5 mg/mL for best results.
- Final protein-protein conjugates can be purified by a number of methods including size exclusion, ion exchange, affinity, or hydrophobic interaction chromatography. For certain applications, protein-protein conjugates can be used without further purification.

Materials Required but Not Provided

- UV-VIS spectrophotometer
- Microcentrifuge capable of handling 1.5 mL tubes
- Quartz semi-micro cuvette (50-100 µL)
- 1.5 mL microfuge tubes
- Pipettes and tips (P-10, P-100, P-1000)
- Ultrapure water (e.g. 18 MΩ-cm)
- Beaker, stir bar, and 6 N NaOH
- pH meter

Material Preparation

A. BupH™ Buffer Preparation

1. Dissolve BupH™ dry-blend buffer pack (provided)into 480 mL ultrapure water. Adjust the pH of the solution to 7.5 ± 0.05 with 6N NaOH. Adjust the final volume to 500mL with ultrapure water. For long-term storage sterile-filter the solution. Do not add sodium azide or Proclin 300 preservatives as these reagents interferewith protein determination (A280).

B. Protein Preparation

1. If protein #1 (100-500 µg) is lyophilized and free of exogenous amines (e.g. glycine or Tris), resuspend in 100µL BupH buffer (pH 7.5) to obtain a 1-5 mg/mL solution. Proceed to Tetrazine Labeling of Protein #1 as described in Section E.
2. If protein #2 (100-500 µg) is lyophilized and free of exogenous amines (e.g. glycine or Tris), resuspend in 100 µL BupH buffer (pH 7.5) to obtain a 1-5 mg/mL solution. Proceed to TCO Labeling of Protein #2 as described in Section F.
3. If either or both purified proteins (100-500 µg) are already in 100 µL buffer (e.g. PBS), for optimal results buffer exchange into BupH (pH 7.5) using the spin columns provided prior to Tz or TCO labeling. See Step C and Step D for buffer exchange of proteins.

C. Spin Column Equilibration into BupH (pH 7.5) prior to Buffer Exchange

1. Twist off the column's bottom closure and loosen the cap. Place each 0.5 mL spin column into a Clean 1.5 mL microfuge tube.
2. Centrifuge column at 1,500 x g for 2 minutes to remove storage solution. Place a pen mark on the side of the column where the compacted resin is slanted upward. Place column in centrifuge with the mark facing away from the center of the rotor in all subsequent centrifugation steps. Note-resin willappear white in color and compacted after centrifugation.
3. Add 0.3 mL BupH buffer (pH 7.5) to the top of each spin column, replace the cap and loosen.
4. Centrifuge at 1,500 x g for 2 minutes to remove buffer.
5. Repeat steps 3 and 4 two additional times, discarding buffer from collection tube after each spin.
6. Transfer equilibrated spin column (resin appears white and dry) into a new1.5 ml microfuge tube and immediately proceed to buffer exchange of protein.

D. Buffer Exchange of Protein

1. Buffer exchange protein into BupH (pH 7.5) equilibrated spin column by slowly applying 100 µL protein solution to the center of equilibrated resin bed.
2. Centrifuge at 1,500 x g for 2 minutes. Retain the eluate at bottom of 1.5 mL collection tube.
3. Protein is now buffer exchanged.

Tetrazine Labeling of Protein #1 (Limiting Protein)

1. Determine volume DMSO required to dissolve Tetrazine-PEG_5-NHS reagent provided (0.5 mg) by referring to the calculations in Appendix A.

2. Add required volume DMSO to tetrazine reagent, vortex for 2 minutes to completely dissolve.
3. Add 5 µL of tetrazine (20-fold molar excess) to 100 µL buffer exchanged protein #1 (limiting protein).
4. Allow reaction to proceed for 60 minutes at room temperature.
5. Remove excess reagent from labeled protein using a new buffer exchange spin column equilibrated in BupH (pH 7.5) by following Steps C and D. Retain final Tz-labeled protein #1 at bottom of collection tube.
6. Determine concentration of Tz-modified protein #1 (mg/mL) by measuring A280. Refer to calculations in Appendix A. Note-remove a 10 µL aliquot of Tz-modified protein and dilute into 90 µL BupH H (e.g. 1:10), then measure A280 using a semi-micro quartz cuvette (50-100 µL). Alternately, a Bradford or BCA protein assay can be performed using a 10 µL aliquot of the labeled protein.
7. Tetrazine-labeled protein is now ready for conjugation.

TCO Labeling of Protein #2 (Excess Protein)

1. Determine volume DMSO required to dissolve TCO-PEG_4-NHS reagent provided (0.5 mg). Refer to Appendix B for calculations.
2. Add required volume DMSO to TCO reagent, vortex for 2 minutes to completely dissolve.
3. Add 5 µL of TCO (20-fold molar excess) to 100 µL buffer exchanged protein #2.
4. Allow reaction to proceed for 60 minutes at room temperature.
5. Remove excess reagent from labeled protein using a new buffer exchange spin column equilibrated in BupH (pH 7.5) by following Steps C and D. Retain final TCO-labeled protein #2 at bottom of collection tube.
6. Determine concentration of TCO-labeled protein #2 (mg/mL) by measuring A280. Refer to calculations in Appendix B. Note- remove a 5µL aliquot of TCO-modified protein and dilute into 95 µL BupH H (e.g. 1:20), then measure A280 using a semi-micro quartz cuvette (50-100 µL). Refer to calculations in Appendix B. Alternately, a Bradford or BCA protein assay can be used to determine protein concentration if the protein's E1% or molar extinction coefficient are unknown.
7. TCO-labeled protein is now ready for conjugation.

Protein-Protein Conjugation

1. Select the desired protein-protein stoichiometry for your conjugation reaction (e.g. 1:3) Note-typical conditions usea 1.2-5 fold molar excess of the excess protein over the limiting protein.
2. Using the selected stoichiometry, calculate volume of TCO-modified protein (excess protein) to add to Tz-modified protein (limiting protein). Refer to calculations in Appendix C.
3. Mix required volume of TCO-modified protein with Tz-modified protein.
4. Allow the conjugation reaction to proceed for 60 minutes at room temperature.
5. Store protein-protein conjugates at 4°C until ready for use or purification.

Troubleshooting

Problem	Possible Cause	Solution
No conjugation of tetrazine and TCO modified proteins	One or more proteins are not properly labeled with TCO or Tetrazine	Confirm purity and concentration of proteins prior to labeling process. Buffer exchange proteins into BupH (pH 7.5) if necessary.
	NHS-ester hydrolyzed	Allow product to equilibrate to room temperature before opening.
		Avoid buffers that contain primary amines such as Tris and glycine. Buffer exchange proteins before labeling if necessary.
	Excess labeling reagent improperly desalted	Remove excess un-reacted tetrazine and TCO reagents by desalting.
Low conjugation of TCO and Tetrazine labeled proteins	Suboptimal reaction conditions	Optimize conjugation conditions by altering molar excess
		Confirm proper concentration of protein #1-Tz and protein #2-TCO prior to conjugation (i.e. 1-5 mg/mL)

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Appendix A

Determine volume DMSO (µL) required to dissolve tetrazine reagent

1. Calculate millimoles tetrazine reagent required to label a protein with 20-fold molar excess of reagent, at a protein concentration (C_p), protein volume (V_p),and protein molecular weight MW_p:

$$mmol\ tetrazine\ reagent\ required = 20 \times C_p \times V_p \times \frac{1}{MW_p}$$

C_p	-	protein concentration (mg/mL)
V_p	-	volume of protein to be labeled (mL)
MW_p	-	protein molecular weight (daltons)

2. Calculate microliters anhydrous DMSO required to dissolve tetrazine reagent

$$\mu L\ DMSO\ required = \frac{mmol\ reagent\ in\ vial \times 5}{mmol\ reagent\ required} = \frac{0.00404}{mmol\ tetrazine\ required}$$

Example 1: Determine volume DMSO (µL) required to dissolve tetrazine reagent

To label 0.1 mL of a 1 mg/mL IgG solution (M.W. 150 kDa) with a 20-fold molar excess tetrazine reagent (M.W.618.64), dissolve reagent (0.5 mg) into 303.1 µL anhydrous DMSO, then add 5 µL of this stock to the antibody solution (0.1 mL) to achieve a 20-fold molar excess.

1. Calculate millimoles tetrazine required to label IgG with 20-fold molar excess

$$mmol\ tetrazine\ required = 20 \times C_p \times V_p \times \frac{1}{MW_p}$$

$$mmol\ tetrazine\ required = 20 \times 1.0\ mg/mL \times 0.1\ mL \times \frac{1}{150{,}000} = 0.000013333$$

2. Calculate volume anhydrous DMSO required to dissolve tetrazine reagent (0.5 mg)

$$\mu L\ DMSO\ required = \frac{0.00404}{mmol\ tetrazine\ required}$$

$$\mu L\ DMSO\ required = \frac{0.00404}{0.00001333} = 303.1\ \mu L$$

Example 2: Determine Concentration of Tz-labeled Goat IgG sample by A280 (mg/mL)

A Goat IgG antibody 0.1 mL at 2.0 mg/mL was labeled using a 20-fold molar excess Tetrazine reagent. After buffer exchange to remove excess reagent, a 1:20 dilution of the protein (5 uL in 95 uL BupH) measured A280 = 0.1224 Goat IgG E1% = 13.6 (A280 of a 10 mg/mL solution).

$$mg/mL = \frac{A280 \times dilution\ factor}{E1\% \times 0.1} = \frac{0.1224 \times 20}{1.36} = 1.8\ mg/mL$$

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Appendix B

Determine volume DMSO (µL) required to dissolve TCO reagent

1. Calculate millimoles TCO reagent required to label a protein with 20-fold molar excess of reagent, at a protein concentration (C_p), protein volume (V_p), and protein molecular weight MW_p:

$$mmol\ TCO\ reagent\ required = 20 \times C_p \times V_p \times \frac{1}{MW_p}$$

C_p	-	protein concentration (mg/mL)
V_p	-	volume of protein to be labeled (mL)
MW_p	-	protein molecular weight (daltons)

2. Calculate microliters anhydrous DMSO required to dissolve TCO reagent

$$\mu L\ DMSO\ required = \frac{mmol\ reagent\ in\ vial \times 5}{mmol\ reagent\ required} = \frac{0.0048585}{mmol\ TCO\ required}$$

Example 1: Determine volume DMSO (µL) required to dissolve TCO reagent

In order to label 0.1 mL of a 5 mg/mL HRP solution (M.W. 44 kDa) with a 20-fold molar excess TCO reagent (M.W. 514.56), dissolve reagent (0.5 mg) into 21.37 µL anhydrous DMSO, then add 5 µL of this stock to the HRP solution to achieve a 20-fold molar excess.

1. Calculate millimoles TCO required to label HRP with 20-fold molar excess

$$mmol\ TCO\ required = 20 \times C_p \times V_p \times \frac{1}{MW_p}$$

$$mmol\ TCO\ required = 20 \times 5.0\ mg/mL \times 0.1\ mL \times \frac{1}{44{,}000} = 0.00022727$$

2. Calculate volume anhydrous DMSO required to dissolve TCO reagent (0.5 mg)

$$\mu L\ DMSO\ required = \frac{0.0048585}{mmol\ TCO\ required}$$

$$\mu L\ DMSO\ required = \frac{0.0048585}{0.00022727} = 21.38\ \mu L$$

Example 2: Determine Concentration of Buffer Exchanged TCO-labeled Protein by A280

HRP at 5.0 mg/mL was labeled using a 20-fold molar excess TCO reagent. After buffer exchange to remove excess reagent, a 1:20 dilution of the protein (5 uL in 95 uL BupH) measured A280 = 0.0950. HRP E1% 280 = 4.56 (A280 of a 10 mg/mL solution).

$$mg/mL = \frac{A280 \times dilution\ factor}{E1\% \times 0.1} = \frac{0.095 \times 20}{1.30.4566} = 4.17\ mg/mL$$

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Appendix C

Calculate volume of protein in excess (V_{excess}) to mix with volume of limiting protein ($V_{limiting}$)

$$V_{excess} = molar\ excess \times \frac{C_{limiting} \times V_{limiting}}{C_{excess}} \times \frac{MW_{excess}}{MW_{limiting}}$$

molar excess	desired molar excess of excess protein (e.g. 3-fold)
V_{excess}	volume of excess protein, mL
$V_{limiting}$	volume of limiting protein, mL
C_{excess}	concentration of excess protein, mg/mL
$C_{limiting}$	concentration of limiting protein, mg/mL
MW_{excess}	molecular weight of excess protein
$MW_{limiting}$	molecular weight of limiting protein

Example 1: Determine volume of protein in excess (V_{excess}) to add to limiting protein ($V_{limiting}$)

Calculate volume of TCO-labeled protein in excess (TCO-HRP @5.0 mg/ml), 44kDa) to mix with 0.1 mL tetrazine-labeled limiting protein (Tetrazine-IgG @ 1.8 mg/mL, 150 kDa) using a 3-fold molar excess TCO-HRP:

$$V_{excess} = 3 \times \frac{1.8\ mg/mL \times 0.1\ mL}{5.0\ mg/mL} \times \frac{44{,}000}{150{,}000} = 0.03168\ mL\ \ or\ 31.68\ \mu L$$

Therefore, 31.68 µL of TCO-labeled HRP (5.0 mg/mL) must be added to 0.1 mL tetrazine-labeled IgG- (1.8 mg/mL) to initiate the conjugation at a 1:3 stoichiometry.

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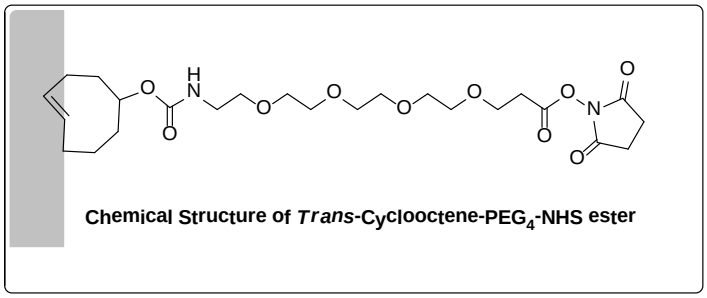
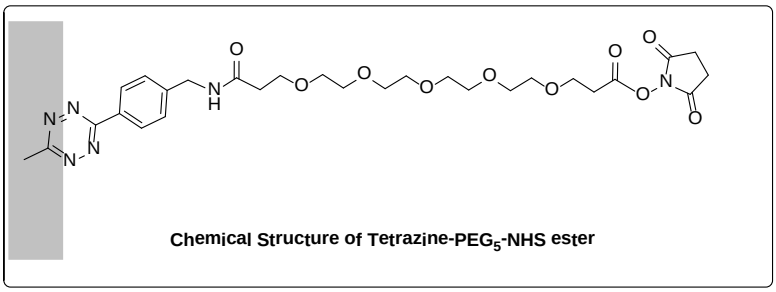
Appendix D



Chemical Structure of *Trans*-Cyclooctene-PEG_4-NHS ester



Chemical Structure of Tetrazine-PEG_5-NHS ester

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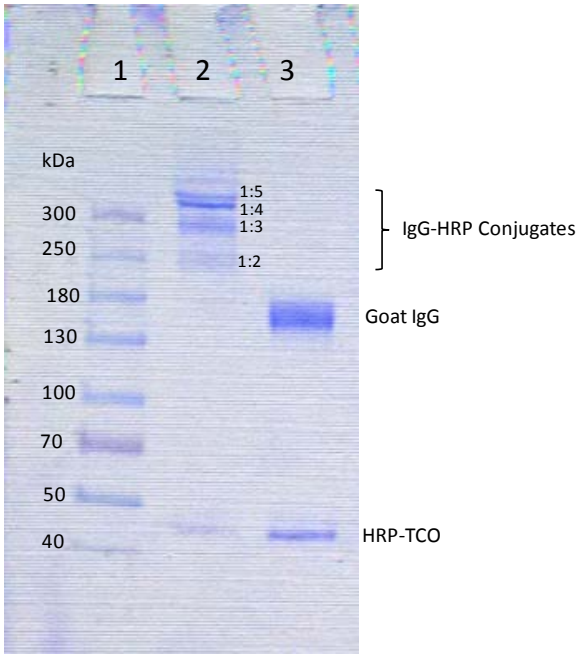
Appendix E

Example 1: 0.1 mL Goat IgG at 2mg/mL in BupH buffer (pH 7.5) was labeled using a 20-fold molar excess tetrazine reagent. Similarly, 0.1 mL HRP (500 µg) at 5.0 mg/mL in BupH buffer (pH 7.5) was labeled using a 20-fold molar excess TCO reagent. After removal of excess reagent and determining each protein concentrations, 43.6 µL HRP-TCO at 4.04 mg/mL (3-fold excess) was added to 0.1 mL IgG-Tetrazine at 2 mg/mL or 0.1 mL IgG (unmodified). After 60 minutes, an aliquot (1 µL) from each conjugation reaction was analyzed by SDS-PAGE.



Lane 1. High Range Molecular Weight Protein Marker
Lane 2. Positive Control IgG-Tz + HRP-TCO (1:3 ratio) Crude Conjugation Reaction-1 µL
Lane 3. Negative Control IgG (unmodified) + HRP-TCO (1:3 ratio) Crude Conjugation Reaction-1 µL