

## Methyltetrazine-PEG4-NHS Ester

### Important Product Information

- NHS esters are moisture-sensitive. To avoid moisture condensation onto the product always let vial come to room temperature before opening; be careful to limit exposure to moisture and restore under an inert atmosphere. The NHS-ester moiety readily hydrolyzes and becomes non-reactive; therefore, prepare stock solutions immediately before use. Stock solutions in anhydrous solvents can be kept for several days (freeze when not in use).
- Hydrolysis of the NHS ester is a competing reaction. 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 (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4); 20 mM HEPES; 100 mM carbonate/bicarbonate; or 50 mM borate buffer.
- Do not use buffers that contain primary amines, (e.g., Tris, glycine).
- Dissolve Methyltetrazine-PEG4-NHS ester in a dry water-miscible organic solvent such as DMSO or DMF before diluting in final reaction buffer. Methyltetrazine-PEG4-NHS ester is soluble in aqueous buffers up to 1.8 mM.

### Procedure for Sample Labeling

#### Additional Materials Required

- Water-miscible organic solvent such as dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF)
- Reaction buffer: Phosphate-buffered saline (PBS) or other buffer at pH 5-9
- Quenching buffer: 1 M Tris-HCl, Ph 8.0
- Spin Desalting Columns

#### Protein Derivatization

- Prepare proteins in PBS.
- Immediately before use, prepare 10 mM of Methyltetrazine-PEG4-NHS reagent in DMSO or DMF.
- Add the NHS reagent to the protein sample at a final concentration of 0.5-2 mM. If the protein concentration is  $\geq 5$  mg/ml, use a 10-fold molar excess of the reagent. For samples  $< 5$  mg/ml, use a 20- to 50-fold molar excess.
- Incubate the reaction at room temperature for 30 minutes or on ice for 2 hours.
- Stop the reaction by adding Quenching Buffer to a final concentration of 50-100 mM Tris.
- Incubate the reaction at room temperature for 5 minutes or on ice for 15 minutes.
- Remove non-reactive reagent by dialysis or desalting.

## TCO-Tetrazine Ligation Reaction

1. Prepare the tetrazine-containing sample in reaction buffer.
2. Add TCO-protein conjugate to tetrazine -containing sample.

**Recommendation: Add 1 mole equivalent of limiting reagent to 1.1-2.0 mol equivalents of highest abundance reagent.**

3. Incubate the reaction at room temperature for 30-60 min. Incubation at 4°C requires 30-120 min.
4. The reaction is now ready for purification.

## Troubleshooting

Problem	Possible Cause	Solution
No conjugation of TCO with tetrazine	One or more sample is not labeled	Confirm molecules were labeled or repeat activation process
	NHS-ester hydrolyzed	Allow product to equilibrate to room temperature before opening
		Prepare new solutions in the indicated dry solvents
		Avoid buffers that contain primary amines such as Tris and glycine
Excess reagent not quenched or removed	Remove non-reacted reagent by dialysis or desalting	
Low conjugation of TCO and tetrazine	Suboptimal reaction conditions	Increase incubation time
		Optimize conjugation conditions by altering molar excess
		Perform conjugation reactions at 37°C