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Methyltetrazine-PEG4-Maleimide

Important Product Information

- Molecules to be reacted with maleimide compounds must have free (reduced) sulfhydryls. Reduce peptide disulfide bonds with disulfide reducing reagents such as Immobilized TCEP Disulfide Reducing Gel (Pierce Biotechnology). Reduce disulfide bonds in high molecular weight proteins using 5 mM TCEP (1:100 dilution) for 30 minutes at room temperature, followed by TCEP removal using a desalting column. Proteins (e.g., antibodies) can be inactivated by complete reduction of their disulfide bonds. Selective reduction of hinge-region disulfide bonds in IgG can be accomplished with 2-Mercaptoethylamine•HCl (2-MEA). Sulfhydryls can be added to molecules using N-succinimidyl S-acetylthioacetate (SATA) or 2-iminothiolane•HCl (Traut's Reagent), which modify primary amines.
- Do not use buffers that contain sulfhydryl-containing components (e.g., DTT).
- 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 ~1,000 times more reactive toward a free sulfhydryl than to an amine.

Procedure for Sample Labeling

Additional Materials Required

- Water-miscible organic solvent such as dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF)
- Reducing reagents such as Immobilized TCEP Disulfide Reducing Gel (Pierce Biotechnology)
- Reaction buffer: Phosphate-buffered saline (PBS) or other sulfhydryl-free buffer at pH 6.5-7.5. Include 5-10 mM EDTA to help prevent the reoxidation of disulfides by trace divalent metals.
- (Optional): Quenching buffer: concentrated (0.5-1 M) cycteine, DDT or other thiol containing reducing agents
- (Optional): Spin Desalting Columns

Protein Labeling

- Prepare sulfhydryl-containing protein, prepared as described the Important Product Information section.
- Immediately before use, weigh a small quantity of Methyltetrazine-PEG4-Maleimide and dissolve it in dimethylformamide (DMF) or dimethylsulfoxide (DMSO) at a 5-20 mM concentration.
- Dissolve protein(s) in Conjugation Buffer at 0.1 mM (e.g., 5 mg in 1 ml for a 50 kDa protein).
- Add Methyltetrazine -PEG4-Maleimide solution to the dissolved protein(s) at 0.4 mM final concentration (ca. four-fold molar excess for 0.1 mM protein solution).

- **Note:** The reaction solution may appear cloudy as a result of the moderate aqueous solubility of DBCO-PEG4-Maleimide; usually, such solutions become clearer as the reaction proceeds. Many proteins will

precipitate when the DMF or DMSO concentration exceeds 10-15% of the final reaction volume; if protein solubility is not an issue, there is no limit to the DMF or DMSO concentration that may be used.

- Incubate reaction mixture for 1 hour at room temperature or for 2 hours at 4°C.
- Quench reaction by adding Quenching Solution at 10-50 mM final and incubating for 15 minutes at room temperature. Alternatively (or in addition) remove the excess nonreacted reagent by desalting or dialysis.

Copper-free Click Reaction

1. Prepare the azide-containing sample in reaction buffer.
2. Add DBCO-protein conjugate to azide-containing sample.

   Recommendation: Add 1 mole equivalent of limiting reagent to 1.2-2 molar equivalents of highest abundance reagent.
3. Incubate the reaction at room temperature for 30 min-12 hour.
4. The reaction is now ready for purification by size exclusion chromatography if required.

Troubleshooting

Problem	Possible Cause	Solution
No conjugation of Methyltetrazine with TCO	One or more sample is not labeled	Confirm molecules were labeled or repeat activation process
	Methyltetrazine -PEG4-Maleimide decomposed	Allow product to equilibrate to room temperature before opening
		Prepare new solutions in the indicated dry solvents
		Avoid buffers that contain sulfhydryl
	Excess reagent not quenched or removed	Remove non-reacted reagent by dialysis or desalting
Low conjugation of Methyltetrazine with TCO	Suboptimal reaction conditions	Increase incubation time
		Optimize conjugation conditions by altering molar excess
		Perform conjugation reactions at 37°C