Click Chemistry Tools
Bioconjugate Technology Company

http://www.clickchemistrytools.com
tel: 480 584 3340
fax: 866 717 2037

Protein Modification with Bis-alkylating Reagents

Protein Requirements

- The protein to be reacted with a bis-alkylating reagent must be highly purified and its molecular weight known (e.g. 20-200 kDa).
- The protein must have available disulfide bonds.
- Proteins must be free of reducing agents (e.g. β-ME, DTT or TCEP) prior to labeling with a bis-alkylating reagent, if present these compounds must be removed (See Material Preparation).

Material Preparation

A. Preparation of PBS + 10 mM EDTA Buffer (pH 7.5)

1. Prepare PBS (100 mM Sodium Phosphate, 150 mM Sodium Chloride) + 10mM EDTA, pH 7.5. For long-term storage sterile-filter the solution. Do not add sodium azide or Proclin preservatives as these reagents interfere with protein A280 measurements.
2. Degassing and/or purging the buffer with nitrogen or argon is recommended for consistent labeling.

B. Protein Preparation

1. If the lyophilized protein (100-1000 µg) to be labeled is pure and free of exogenous thiols (e.g. DTT or β-ME), resuspend in 500 µL PBS + 10 mM EDTA (pH 7.5) to obtain a 0.5-5 mg/mL solution, proceed to Protein Reduction.
2. If the lyophilized protein is known to contain exogenous thiols (e.g. DTT, β-ME) resuspend in PBS + 10mM EDTA (pH 7.5) then proceed with buffer exchange, followed by Protein Reduction.
3. If the purified protein to be modified (100-1000 µg) is already in a suitable thiol-free buffer (e.g. MOPS, Tris-HCL, or PBS) at a concentration range from 0.5-5 mg/mL, proceed to Protein Reduction.

C. Equilibration of Desalting Column into PBS +10 mM EDTA (pH 7.5)

1. Choose a suitable desalting column. We recommend Pierce Zeba™ Spin Desalting Columns (7 k MWCO, 2mL).
2. Equilibrate the desalting column into PBS +10 mM EDTA (pH7.5) according to the manufacturer's instructions.

D. Buffer Exchange of Protein

1. Buffer exchange protein into PBS +10 mM EDTA (pH 7.5) equilibrated column according to the manufacturer's instructions.
2. Retain the eluate in the collection tube. Discard the used desalting column.
3. Protein is now buffer exchanged.

Protein Reduction

A. TCEP

1. Prior to labeling with a bis-alkylating reagent, proteins containing disulfide bonds must be reduced with TCEP to insure proper labeling.
2. Prepare 5-500 mM TCEP stock solution in ultrapure water, vortex to dissolve crystals completely.
3. Add an appropriate volume of TCEP to the protein solution to reach a final TCEP concentration of 2.5-5 times molar excess for each disulfide bond to be reduced. For IgG1, this corresponds to a 10-20 molar excess of TCEP versus antibody.
4. Allow the reduction reaction to incubate for 60 minutes at room temperature.
5. Prepare 2 desalting columns.
6. Buffer exchange TCEP reduced protein TWICE, using 2 consecutive columns

B. Immobilized TCEP Disulfide Reducing Gel

1. Using a pipette with the tip cut off, aliquot a volume of resin equal to 4 times the volume of protein solution to be reduced.
2. Centrifuge and wash the resin with PBS+10 mM EDTA (pH 7.5) according to the manufacturer's instructions.
3. Add the protein solution to the washed, packed resin. The volume ratio should be 2:1 compact resin: protein.
4. Pipette up and down to ensure proper mixing of the slurry. Do not vortex.
5. Incubate the slurry for 2-4 hours at 37°C
6. After reduction, centrifuge the resin according to the manufacturer's instructions. Remove the supernatant and proceed to the next step. (**Note**: for more efficient protein recovery, use a micro-spin column with a solid support to separate the reduced protein from the resin).

Labeling Reaction

The optimal amount of a bis-alkylating reagent reagent to use for each reaction depends on a number of factors. Usually 10-fold molar excess (2.5-fold molar excess per solvent accessible disulfide bond) of a bis-alkylating reagent over IgG results in a DOL around 4, however labeling at

high protein concentrations (>5 mg/mL) might require only 1.5-2 fold excess of a bis-alkylating reagent per solvent accessible disulfide bond. For Fab fragments labeling, the optimal molar excess of a bis-alkylating reagent is 2-2.5.

1. Determine molar excess of a bis-alkylating reagent to be used during the labeling reaction.
2. Determine the concentration of protein solution.
3. Add required amount of water-miscible solvent (e.g. DMSO) or ultrapure water to 5 mM stock solution, vortex vigorously for 2 minutes until the reagent is fully dissolved.
   Note: Bis-sulfone reagents are not water-soluble, water-miscible solvent should be used to make a stock solution.
   ThioLinker™ reagents are water-soluble, water or water-miscible solvent can be used to make a stock solution
4. Add the required volume of a bis-alkylating reagent to the protein solution to achieve the desired molar excess versus protein concentration. Pipette the mixture up and down several times to mix. Allow labeling reaction to proceed overnight at room temperature. **Note:** For the labeling of Fab fragments, the labeling reaction is usually complete after 4 hours.
5. Remove excess of a bis-alkylating reagent by desalting into PBS (or buffer of choice). **Note**: At this point in the procedure, EDTA is no longer required.

Troubleshooting

Problem	Possible Cause	Solution
Poor or lower than expected thiol bridging	Incorrect protein concentration and/or possible contaminants in protein sample.	Buffer exchange protein into reaction buffer (PBS pH 7.5 +10mM EDTA) using spin columns provided and confirm concentration of protein prior to labeling.
	Insufficient reduction	Ensure that proteins are fully reduced.
		Check TCEP expiration and ensure that stocks are being made immediately before use
	Oxygen in reaction buffer	De-gas buffer prior to use and/or purge with inert gas
	Reducing agent is present during reaction	Make certain that desalting is effectively removing TCEP after reduction
		Desalt reduced protein solution twice
Protein oligomerization or aggregation	Molar excess of a bis-alkylating reagent is too high for the protein of interest	Reduce the molar excess of a bis-alkylating reagent to 1.5x per disulfide contained in your protein.