Reductive Alkylation Kit

HAMPTON RESEARCH
Solutions for Crystal Growth

User Guide

Application

Reductive alkylation of lysine residues to change protein properties (pI, solubility and hydropathy) which may promote crystallization via improved crystal packing.

Features

- 6 Reductive alkylation reactions
- A surface-engineered protein ready for crystallization is produced within 24 hours
- Optimized protocol for selective alkylation of lysine residues
- Methanol free formaldehyde
- Flexible protocol allows for methylation, ethylation or isopropylation of lysine

Kit Contents

- Dimethylamine Borane Complex (Qty 6 x 6 mg)
- 1 M Formaldehyde (methanol free) (Qty 1 x 1 ml) for methylation
- 1 M Acetaldehyde (Qty 1 x 1 ml) for ethylation
- 1 M Acetone (Qty 1 x 1 ml) for isopropylation
- 1 M Glycine (Qty 1 x 1 ml)
- 50 mM DL-Dithiothreitol (DTT) (Qty 1 x 1 ml)

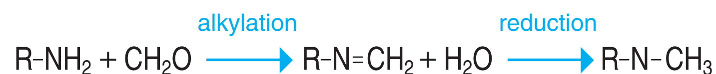
Discussion

Reductive alkylation of proteins has been successfully applied to obtain a significant number of high-quality crystals from proteins previously unable to be crystallized.[1,2] Alkylating the ε amino group of lysines alters the hydropathy, solubility and pI of the protein which may promote crystallization by altering sample-sample, sample-solvent and crystal packing interactions.[4]

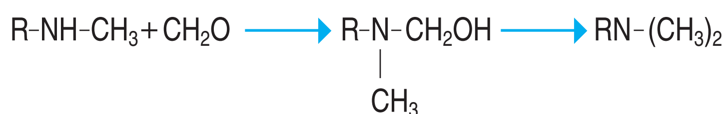
Reductive alkylation does not change the intrinsic charge on a protein but may change the isoelectric point (pI) slightly. The N-terminal amino group on the backbone will also be reductively alkylated. In general, alkylated proteins retain their original biochemical function.[5,6] This protocol is designed with the goal of generating a high degree of modification with few side reactions, resulting in a homogeneous population of protein.

With reductive methylation of lysine residues using Dimethylamine Borane Complex and formaldehyde, a Schiff based is formed between the ε amino group of lysine and formaldehyde that is then reduced to a secondary or tertiary amine.

Figure 1: Lysine methylation



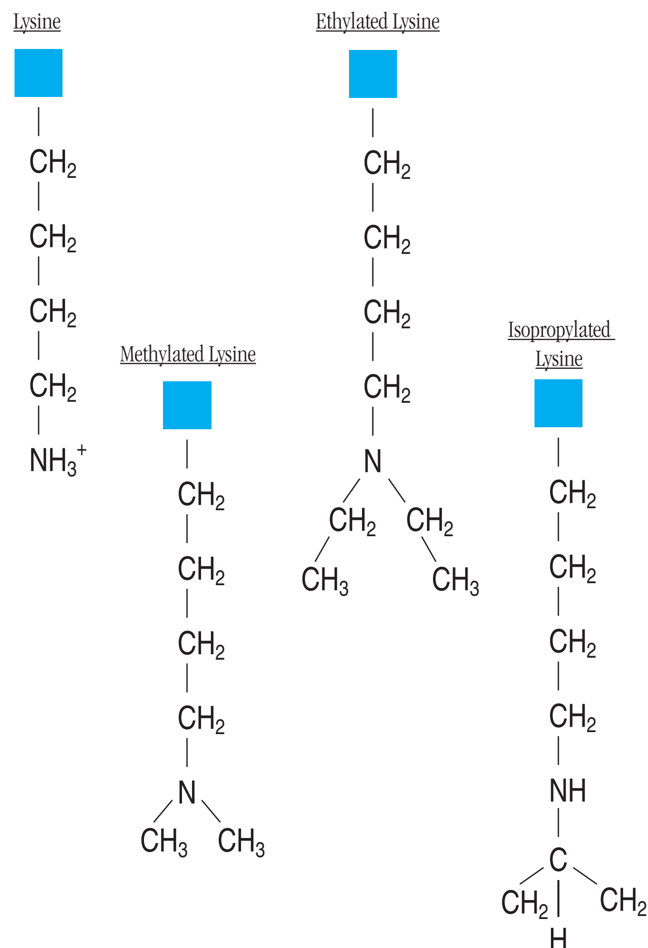
The partially methylated lysine reacts with formaldehyde to produce the final dimethylated lysine because the pKa of the monomethyllysine is lower than that of the lysine.



- $R\text{-}NH_2$ is a lysine or N-terminal amino acid residue of the backbone

Alternatively, when acetaldehyde is substituted for formaldehyde, two ethyl groups are added to one lysine residue (ethylation) instead of two methyl groups. Or, when acetone is substituted for formaldehyde, one isopropyl group is added to the lysine (isopropylation) in place of the two methyl groups.

Figure 2: Native and alkylated lysines



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Instructions for Reductive Methylation

- The procedure is to be performed at 4°C.
- Maintain reagents at 4°C or on ice.
- The protein concentration should be between 1-10 mg/ml.
- The sample should be in a pH 7.5 buffer that does not contain any free amino groups.
- Avoid Tris and TRIS hydrochloride buffers.

Avoid Tris and TRIS hydrochloride buffers. HEPES is an appropriate buffer. As necessary, additional salts (Sodium chloride), polyols (Glycerol) and other additives may be included to maintain protein stability, homogeneity and solubility. These additives should be free of amino groups since such groups will interfere with the methylation of the sample.

1. Add 100 µl of deionized water to Dimethylamine Borane Complex (a 6 mg aliquot Dimethylamine Borane Complex), mix gently at 4°C to dissolve. The resulting solution is 1.0 M Dimethylamine Borane Complex.
2. Add 20 µl of Dimethylamine Borane Complex solution to 1.0 ml of protein.
3. Add 40 µl of 1.0 M Formaldehyde. Mix gently. Incubate the mixture at 4°C for 2 hours.
4. After the 2 hour incubation, add 20 µl of the Dimethylamine Borane Complex solution (prepared in step 1).
5. Add 40 µl of 1.0 M Formaldehyde. Mix gently. Incubate the mixture at 4°C for 2 hours.
6. After the second 2 hour incubation, add 10 µl of Dimethylamine Borane Complex solution (prepared in step 1). Incubate the mixture at 4°C overnight.
7. After the overnight incubation, stop the reagent by adding 125 µl of 1.0 M Glycine. Add 125 µl of 50 mM DTT (optional). Incubate the mixture at 4°C for 2 hours.

The protein should now be separated from the reaction products and protein aggregates by using extensive dialysis, size exclusion chromatography, microfiltration or protein precipitation. Following purification, concentrate the protein and perform crystallization screening.

Instructions for Reductive Ethylation

- The procedure is to be performed at 4°C.
- Maintain reagents at 4°C or on ice.
- The protein concentration should be between 1-10 mg/ml.
- The sample should be in a pH 7.5 buffer that does not contain any free amino groups.
- Avoid Tris and TRIS hydrochloride buffers.

HEPES is an appropriate buffer. As necessary, additional salts (Sodium chloride), polyols (Glycerol) and other additives may be included to maintain protein stability, homogeneity and solubility. These additives should be free of amino groups since such groups will interfere with the methylation of the sample.

1. Add 100 µl of deionized water to Dimethylamine Borane Complex (a 6 mg aliquot Dimethylamine Borane Complex), mix gently at 4°C to dissolve. The resulting solution is 1.0 M Dimethylamine Borane Complex.
2. Add 20 µl of Dimethylamine Borane Complex solution to 1.0 ml of protein.
3. Add 40 µl of 1.0 M Acetaldehyde. Mix gently. Incubate the mixture at 4°C for 2 hours.
4. After the 2 hour incubation, add 20 µl of the Dimethylamine Borane Complex solution (prepared in step 1).
5. Add 40 µl of 1.0 M Acetaldehyde. Mix gently. Incubate the mixture at 4°C for 2 hours.
6. After the second 2 hour incubation, add 10 µl of Dimethylamine Borane Complex solution (prepared in step 1). Incubate the mixture at 4°C overnight.
7. After the overnight incubation, stop the reagent by adding 125 µl of 1.0 M Glycine. Add 125 µl of 50 mM DTT (optional). Incubate the mixture at 4°C for 2 hours.

The protein should now be separated from the reaction products and protein aggregates by using extensive dialysis, size exclusion chromatography, microfiltration or protein precipitation. Following purification, concentrate the protein and perform crystallization screening.

Instructions for Reductive Isopropylation

- The procedure is to be performed at 4°C.
- Maintain reagents at 4°C or on ice.
- The protein concentration should be between 1-10 mg/ml.
- The sample should be in a pH 7.5 buffer that does not contain any free amino groups.
- Avoid Tris and TRIS hydrochloride buffers.

HEPES is an appropriate buffer. As necessary, additional salts (Sodium chloride), polyols (Glycerol) and other additives may be included to maintain protein stability, homogeneity and solubility. These additives should be free of amino groups since such groups will interfere with the methylation of the sample.

1. Add 100 µl of deionized water to Dimethylamine Borane Complex (a 6 mg aliquot Dimethylamine Borane Complex), mix gently at 4°C to dissolve. The resulting solution is 1.0 M Dimethylamine Borane Complex.
2. Add 20 µl of Dimethylamine Borane Complex solution to 1.0 ml of protein.

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3. Add 40 µl of 1.0 M Acetone. Mix gently. Incubate the mixture at 4°C for 2 hours.
4. After the 2 hour incubation, add 20 µl of the Dimethylamine Borane Complex solution (prepared in step 1).
5. Add 40 µl of 1.0 M Acetone. Mix gently. Incubate the mixture at 4°C for 2 hours.
6. After the second 2 hour incubation, add 10 µl of Dimethylamine Borane Complex solution (prepared in step 1). Incubate the mixture at 4°C overnight.
7. After the overnight incubation, stop the reagent by adding 125 µl of 1.0 M Glycine. Add 125 µl of 50 mM DTT (optional). Incubate the mixture at 4°C for 2 hours.

The protein should now be separated from the reaction products and protein aggregates by using extensive dialysis, size exclusion chromatography, microfiltration or protein precipitation. Following purification, concentrate the protein and perform crystallization screening.

Kit Storage

- Store between -20 to 4°C
- Best if used within 12 months of receipt
- For research use only

Comments

In some cases the reductive alkylation reaction leads to a significant (occasionally over 50%) amount of precipitated protein. In such instances the precipitated protein can be removed by centrifugation before purification of the soluble alkylated protein (supernatant). Alternatively, the reaction can be repeated with a lower protein concentration.

A 20-fold molar excess of formaldehyde over free amino group concentration should yield an essentially complete modification of all exposed lysine residue and the free N termini. The concentration of the protein can be adjusted where necessary according to the number of lysines present if the concentration of formaldehyde and reducing agent described here are used. Rayment reported the successful, complete modification of lysozyme with 6 lysines at 10 mg/ml and myosin subfragment with 100 lysines at 5 mg/ml.[4]

In some instances not all potential lysine sites may be alkylated. This may imply the unmodified residues were inaccessible to the reagents, were perhaps buried inside the protein or were masked by bound ligand.[2]

Prepare fresh Dimethylamine Borane Complex reagent just before beginning the reductive alkylation procedure and maintain the reagent at 4°C during the course of the procedure.

To assist in experimental planning, the total approximate reaction time from start to finish is 24 hours.
Reductive methylation will increase the mass of the protein 28 Da for each lysine residue or the N-terminal amine.

Reductive ethylation will increase the mass of the protein 55 Da for each lysine residue or the N-terminal amine.

Reductive isopropylation will increase the mass of the protein 41 Da for each lysine residue or the N-terminal amine.

In this protocol the reaction is quenched using Glycine (included). Any primary amine such as Tris or Ammonium sulfate can be used to quench the reaction. The use of glycine has the advantage of being thoroughly and successfully validated.[1] The use of Glycine does not introduce a Tris buffer variable, and does not risk precipitating the protein like Ammonium sulfate.

Mass spectrometry may be used as a quality control step. For methylation, assume 28 Da per lysine plus one more for the N termini. An incomplete methylation reaction may be identified due to the 28 Da per lysine residue difference between completely methylated, partially methylated and fully methylated sample

Alkylated protein may display reduced solubility[1,8], sometimes between two thirds and one third of the original sample. Use of the Hampton Research PCT kit can identify the appropriate sample concentration for protein crystallization.

Reductive alkylation may alter the original sample solubility such that crystallization conditions for the native sample will no longer yield crystals. Perform a thorough crystallization screen to identify new crystallization conditions.

Reductive alkylation can be used to produce a different crystal form of a previously crystallized sample.[1]

The addition of DTT reverses any modification of cysteine or methionine residues. If your sample will not tolerate a reducing agent (DTT), you may choose to skip this step.

You may wonder why alkylating a protein would improve its chances for crystallization. Through entropic effects, a high concentration of methylated or ethylated or isopropylated surface lysines may lead to a stabilized crystal lattice through side chain interactions. The hydrophobic nature of methylated lysines may favor protein-protein interactions and can alter sample-solvent interactions. Such interactions can drive the formation of certain protein complexes[9] and could promote crystallization of the sample.

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Technical Support

Please e-mail (tech@hrmail.com), fax (1-949-425-1611), or telephone (1-949-425-1321) your request to Hampton Research. Fax and e-mail Technical Support are available 24 hours a day. Telephone technical support is available 8:00 a.m. to 4:30 p.m. USA Pacific Standard Time.

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