

Protocol (05.05.2026)

Deprotection of Thiol-modified Oligonucleotides

Deprotection of Thiol-Modified Oligonucleotides Using DTT

Purpose

Prior to use Thiol-modified Oligonucleotides, it is necessary to cleave the disulfide bond with 100 mM DTT (pH 8,3 – 8,5) at room temperature for about 30 minutes. This protocol describes the procedure for reducing the disulfide bond in thiol-modified oligonucleotides to generate free thiol groups, which are required for downstream applications such as conjugation to maleimide-functionalized molecules or immobilization on surfaces.

Materials and Reagents

- Thiol-modified oligonucleotide (lyophilized)
- Dithiothreitol (DTT), 100 mM stock solution
- Buffer (e.g., 100 mM phosphate buffer, pH 8.3–8.5, or Tris-HCl buffer, pH 8.3–8.5)
- Nitrogen or argon gas (optional, for inert atmosphere storage)
- Microcentrifuge tubes (if performing small-scale deprotection)
- Centrifuge (if needed for sample concentration or buffer exchange)

Procedure

Step 1: Preparation of the Reducing Solution

- Prepare a 100 mM DTT solution in a suitable buffer (e.g., 100 mM phosphate buffer, pH 8.3–8.5).
- Ensure the solution is freshly prepared or stored at -20°C in aliquots to avoid oxidation.

Step 2: Cleavage of the Disulfide Bond

Before use, reduce the disulfide bond in thiol-modified oligonucleotides using the following conditions:

- Reagent: 100 mM DTT (Dithiothreitol) pH: 8.3 – 8.5
- Temperature: Room temperature ($\sim 20\text{--}25^{\circ}\text{C}$)
- Incubation Time: 30 minutes

Dissolve the thiol-modified oligonucleotide in a buffer of choice (if lyophilized).

Recommended concentration: 100–500 μM (depending on the downstream application).

Add an equal volume of 100 mM DTT solution to the oligonucleotide solution to achieve a final DTT concentration of 50 mM.

Incubate at room temperature for 30 minutes to ensure complete reduction.

After incubation, the reaction can be used directly or proceed with purification.

Step 3: Removal of Excess DTT (If Necessary)

For applications where residual DTT may interfere, remove excess DTT using one of the following methods:

Ethanol precipitation:

- Add 3 volumes of cold 100% ethanol and 0.1 volume of sodium acetate (3 M, pH 5.2).
- Incubate at -20°C for at least 30 minutes.
- Centrifuge at $\geq 12,000$ rpm for 10 minutes at 4°C .
- Remove the supernatant and wash the pellet with 70% ethanol.
- Air dry and resuspend in the desired buffer.

Spin column purification:

- Use a desalting column (e.g., NAP-5, Zeba, or Sephadex G-25) equilibrated with a suitable buffer.
- Load the sample and collect the purified oligonucleotide.

Dialysis (for large-scale preparations):

- Use a dialysis membrane (1–3 kDa cutoff) in a buffer of choice.
- Exchange buffer 3–4 times over several hours.

Step 4: Storage and Handling

To prevent disulfide reformation, store the deprotected oligonucleotides under one of the following conditions:

- Under an inert atmosphere (e.g., nitrogen or argon)
- In a solution containing 10 mM DTT

Store at -20°C for long-term stability.

Avoid repeated freeze-thaw cycles to minimize oxidation.

Notes and Considerations

pH is critical: Ensure the buffer remains at pH 8.3–8.5 for effective reduction.

If DTT removal is not required, keep the oligonucleotide in the presence of 10 mM DTT to maintain the reduced state.

For conjugation applications, use freshly reduced oligonucleotides immediately after purification.

Deprotection of Thiol-Modified Oligonucleotides Using TCEP

Purpose

Prior to use, it is necessary to cleave the disulfide bond in thiol-modified oligonucleotides, generating free thiol groups necessary for applications like conjugation to maleimide-functionalized molecules or immobilization on surfaces. Incorporating Tris(2-carboxyethyl)phosphine (TCEP) as an alternative reducing agent offers certain advantages over Dithiothreitol (DTT), such as greater stability and the absence of thiol groups, which can simplify downstream applications. Below is a protocol for the deprotection of thiol-modified oligonucleotides using TCEP.

Materials and Reagents

- Thiol-modified oligonucleotide (lyophilized)
- Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 0.5 M stock solution
- Desalted water
- 3 M Sodium acetate, pH 5.2
- Absolute ethanol (100%)
- Microcentrifuge tubes
- Centrifuge

Procedure

Step 1: Preparation of TCEP Reducing Solution

Prepare a 0.1 M TCEP solution by diluting the 0.5 M TCEP stock solution:

- Mix 80 μ L of 0.5 M TCEP with 320 μ L of desalted water to obtain 400 μ L of 0.1 M TCEP solution.
- It's recommended to prepare this solution fresh to ensure optimal reducing activity.

Step 2: Reduction of Disulfide Bonds

Dissolve the lyophilized thiol-modified oligonucleotide in 400 μ L of the freshly prepared 0.1 M TCEP solution.

Vortex the mixture gently to ensure complete dissolution.

Incubate the solution at room temperature ($\sim 20\text{--}25^\circ\text{C}$) for 1 hour, vortexing intermittently to facilitate the reduction process.

Step 3: Precipitation of Reduced Oligonucleotide

Add 50 μ L of 3 M sodium acetate (pH 5.2) to the reaction mixture and vortex briefly.

Add 1.5 mL of absolute ethanol to the mixture and vortex to mix thoroughly.

Incubate the solution at -20°C for at least 20 minutes to precipitate the oligonucleotide.

Step 4: Recovery of Reduced Oligonucleotide

Centrifuge the mixture at 12,000 rpm ($\sim 13,400 \times g$) for 10 minutes at 4°C.

Carefully decant the supernatant without disturbing the pellet.

Wash the pellet with 1 mL of 70% ethanol to remove residual salts and TCEP.

Centrifuge again at 12,000 rpm for 5 minutes at 4°C.

Remove the supernatant and air-dry the pellet briefly (avoid over-drying).

Resuspend the purified, reduced oligonucleotide in an appropriate volume of desalted water or your buffer of choice.

Step 5: Storage and Handling

To prevent reoxidation, store the reduced oligonucleotide under an inert atmosphere (e.g., nitrogen or argon) or in a solution containing 10 mM TCEP.

For long-term storage, aliquot the solution and store at -20°C .

Avoid repeated freeze-thaw cycles to maintain oligonucleotide integrity.

Notes and Considerations

The pH of the TCEP solution should be neutral to slightly acidic; avoid alkaline conditions as TCEP is more stable at lower pH.

If immediate use of the reduced oligonucleotide is planned, purification steps can be minimized; however, for sensitive applications, thorough purification is recommended.

DTT or TCEP?

DTT and TCEP are both used to reduce disulfide bonds in thiol-modified oligonucleotides, but TCEP is more stable in solution, works over a broader pH range, and does not require a buffer for activity. DTT, while effective, oxidizes quickly and requires removal after deprotection, whereas TCEP is more hydrophilic and easier to eliminate, making it preferable for bioconjugation applications (attachment of fluorophores, enzymes, proteins, peptides, or nanoparticles to oligos for targeted detection, drug delivery, and molecular diagnostics, including thiol-maleimide coupling, amine-NHS ester reactions, click chemistry and enzymatic labeling). For more details:

1. Reducing Mechanism

DTT: A thiol-based reducing agent that works through a disulfide exchange reaction, converting S-S bonds into free thiols. It requires mildly basic conditions (pH 7–8) to be effective.

TCEP: A phosphine-based reducing agent that directly reduces disulfides to free thiols without requiring a basic pH.

2. Stability and Storage

DTT: Oxidizes rapidly in solution, losing effectiveness over time. It must be freshly prepared for optimal performance.

TCEP: More stable in aqueous solutions, does not oxidize as quickly, and can be stored longer.

3. Reaction Conditions

DTT: Works efficiently but often requires a buffered solution (e.g., TE buffer at pH 7.5–8.5) to maintain reducing power.

TCEP: Functions well in a broader pH range (pH 4–9) and does not require a buffer for activity.

4. Removal & Purification

DTT: Needs to be removed after deprotection, usually via gel filtration, precipitation, or column purification, since residual DTT can interfere with downstream applications.

TCEP: More hydrophilic and easier to remove, often not requiring additional purification steps.

5. Compatibility with Downstream Applications

DTT: Leaves thiol groups reactive, which may lead to unwanted oxidation if not properly handled.

TCEP: Generates more stable thiol products with less tendency to reoxidize, making it preferred for applications like bioconjugation.

In conclusion:

- **DTT** is commonly used for temporary reduction before oligonucleotide conjugation: *if you need a fast reduction and plan to purify the oligonucleotide afterward, DTT is a good choice.*
- **TCEP** is preferred when long-term stability of the thiol state is required, particularly in aqueous environments: *if you require a more stable and cleaner reduction process, especially for bioconjugation, TCEP is the better option.*

References

Getz E. B. *et al. Analytical Biochemistry.* 1999; 273: 73-80