Technical Bulletin

Tris(2-carboxyethyl)phosphine,

Immobilized on Agarose CL-4B

52486

Storage Temperature 4 °C
Do not freeze.
Synonyms: Trialkylphosphines, TCEP, TCEP Disulfide Reducing Gel

Product Description

Trialkylphosphines (TCEP) are highly effective agents for reducing disulfide bonds in proteins, peptides and other disulfide bond containing molecules[1,2] and are relatively non-reactive toward other functional groups[2]. The trialkylphosphine TCEP was first described by Levison *et al*.[3] as an odorless and efficient reductant of alkyl disulfides over a wide pH range. TCEP is stable in aqueous solutions and does not undergo the rapid oxidation that often occurs with other reducing agents such as dithiotreitol (DTT) and β-mercaptoethanol (BME, 2-ME).[4] TCEP does not interfere with commonly used sulfhydryl-reactive reagents (for example, maleimide crosslinkers).[5] Nevertheless, many protocols require recovery of the reduced sample separate from the reducing agent. Our Tris(2-carboxyethyl)phosphine, immobilized on Agarose CL-4B eliminates the need to use laborious and troublesome gel filtration methods to separate the reduced sample from the reducing agent. Immobilized TCEP Disulfide Reducing Gel may be adapted conveniently to a variety of scales and formats. Examples are given for batch, spin cup column and gravity-flow column procedures. For small-scale reductions, the most complete sample recovery is made using the spin-cup column procedure.

Important Product Information

- Disulfide reduction occurs over a wide range of pH (pH 4.0-9.0) and temperature (0-95 °C).
- Reduction can be performed in most buffers, as well as in ultrapure water.
- Most proteins will be reduced sufficiently without adding a denaturant such as guanidine. HCl; however, to ensure complete reduction, adding a denaturant will aid in exposing internal disulfides to the Immobilized TCEP.
- Protein type and concentration will ultimately determine the incubation time (Table 1).

Table 1. Suggested incubation times for reducing proteins at room temperature.

Sample Concentration (mg/mL)	Incubation Time
< 0.1	15 minutes
0.1-0.5	30 minutes
0.5-0.9	45 minutes
> 1	1 hour

- Optimize incubation time by monitoring time-point aliquots for sulfhydryls using Ellman's Reagent.
- Do not allow metals (spatulas, etc.) to contact the Immobilized TCEP as this will decrease its activity.
- Including 5-20 mM EDTA in the sample buffer during reduction will help prevent oxidation of generated sulfhydryl groups. Furthermore, adding EDTA (5-20 mM) to the sample buffer will help maintain activity of the Immobilized TCEP by chelating divalent metals such as Zn^{2+}, Cu^{2+} and Mg^{2+}, which otherwise will lower its activity.
- The tendency of –SH groups to reform disulfides after reduction is dependent on the concentration of free –SH groups generated and the elapsed time after reduction. Therefore, perform procedures using the reduced sample immediately after TCEP reduction.

Procedure for Reduction in Batch Format

(for 20-750µL samples)

Add a volume of TCEP reducing gel slurry equal to one to two times the volume of sample to a microcentrifuge tube. For example, use 25-50 µL of mixed slurry for a 25 µL protein/peptide sample.

1. Centrifuge the tube at ~ 1000 × g for 1 minute. Remove and discard the supernatant. If desired, the gel may be washed several times with sample buffer before adding sample to the tube. For example, add buffer, vortex briefly to resuspend gel, briefly centrifuge the tube and remove the supernatant.
2. Add protein/peptide solution to the washed gel. Vortex the tube and incubate the solution for the desired time and temperature (Table 1). For longer incubations, it may be helpful to place the tube on a rotating wheel or rocker platform to keep the gel in suspension.
3. Centrifuge the tube for 1 minute. Recover the supernatant containing the reduced protein/peptide.
   Note: Some loss of sample within the pelleted gel volume will occur. Although it will be diluted, additional sample can be recovered by washing the gel with buffer.
4. If desired, determine reduction efficiency (quantity of free –SH groups) by either Ellman's Reagent or SDS-PAGE using non-reducing conditions.

Procedure for Reduction in Spin-cup Columns (for 50-750 µL samples)

1. Add an appropriate amount of mixed TCEP reducing gel slurry to a spin-cup column placed in a microcentrifuge tube. Use a volume of TCEP slurry equal to one to two times the volume of sample. For example, add 50-100 µL of mixed slurry to the spin cup for a 50 µL protein/peptide sample.
   Note: For 300-750 µL sample volumes, perform the reduction (incubation of sample with the gel) in a microcentrifuge tube as in the batch method described above. After incubation, transfer the gel/sample slurry in 400 µL portions to a spin cup column to recover the sample as in step 4 below.
2. Centrifuge at 1000 rpm in a microcentrifuge (~ 50 × g) for 30 seconds. Remove and discard the supernatant. If desired, the gel may be washed several times with sample buffer before adding sample to the tube: add buffer, vortex briefly to resuspend gel, centrifuge and discard flow-through.
3. Apply the sample to the top of the gel in the spin cup. Gently vortex or mix the sample and gel and incubate sample for the appropriate time (see Table 1). For longer incubations, it may be helpful to place the tube on a rotating wheel or rocker platform to keep the gel in suspension.
4. Place the spin cup in a new tube and centrifuge at ~ 50 × g for 1 minute. The collected flow-through is the reduced sample.
5. If desired, determine reduction efficiency (quantity of free –SH groups) by either Ellman's Reagent or SDS-PAGE using non-reducing conditions.

Procedure for Reduction in Gravity-flow Columns (for > 250 µL samples)

1. Choose a column size appropriate for a volume of TCEP reducing gel equal to at least two times the volume of sample.
2. With the lower column disc and bottom cap in place, gently pour the appropriate volume of gel slurry into the column. For example, pour 3 mL of mixed slurry to obtain a settled gel of 1.5 mL. Avoid creating air bubbles during filling. Allow gel to settle, add a top column disc if desired, then remove the bottom cap to drain the water.
   Note: Do not allow buffer to drain below the top of the gel bed to avoid introducing air bubbles, which will decrease flow rate and reduce capacity.
3. If desired, wash gel with two column volumes of ultrapure water or buffer.
4. Apply peptide or protein sample to the column. Cap the bottom of the column when the entire sample has entered the gel bed. Incubate column for desired time and temperature (Table 1). For peptide reduction, only 15 minutes is required.
5. Recover the sample from the column with buffer. Collect fraction volumes appropriate to the prepared column size. For example, collect 0.5-1 mL fractions from a 2 mL column to which 1 mL of sample was applied. Determine which fractions contain protein by measuring the absorbance at 280 nm relative to a buffer blank.

6. If desired, determine reduction efficiency (quantity of free –SH groups) by either Ellman's Reagent or SDS-PAGE using non-reducing conditions.

Procedure for Testing the Reducing Activity of the TCEP Disulfide Reducing Gel

This simple test can be performed to quantitate the amount of active TCEP on the gel. Additional reagents needed to perform this test are Ellman's Reagent and free TCEP.

1. Prepare 10 mL of a solution containing 40 mg (10 mM) Ellman's Reagent in 100mM Tris Buffer, pH 7.5. Allow 1 hour for the Ellman's Reagent to completely dissolve in the Tris Buffer at room temperature.
2. Prepare five free TCEP standards as follows:
    - Standard 1 (20 mM TCEP): Dissolve 57.5 mg free TCEP in 10 mL of ultrapure water
    - Standard 2 (4 mM TCEP): Dilute standard 1 with ultrapure water (2 mL standard 1 + 8 mL ultrapure water)
    - Standard 3 (2 mM TCEP): Dilute standard 2 with ultrapure water (5 mL standard 2 + 5 mL ultrapure water)
    - Standard 4 (1 mM TCEP): Dilute standard 3 with ultrapure water (5 mL standard 3 + 5 mL ultrapure water)
    - Standard 5 (0.5 mM TCEP): Dilute standard 4 with ultrapure water (5 mL standard 4 + 5 mL ultrapure water)
3. Combine 990 µL of the Ellman's Reagent solution with 10 µL of each TCEP standard. Measure the absorbance at 412 nm for each.
4. Combine 990 µL of the Ellman's Reagent solution with 10 µL of Immobilized TCEP Disulfide Reducing Gel slurry and measure the absorbance at 412 nm.
   Note: Wait for 1 minute after mixing before reading the absorbance to allow gel to settle to the bottom of the cuvette, where it will not interfere with absorbance measurement.
5. Prepare a standard curve for the TCEP standards. Determine the concentration of active TCEP immobilized on the gel by reference to the standard curve.

Note: Because the immobilized TCEP is 50% slurry, the actual concentration of active TCEP is approximately twice the values estimated by reference to the standard curve.

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

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