INSTRUCTIONS

Immobilized TCEP Disulfide Reducing Gel

77712

1325.3

Number	Description
77712	**Immobilized TCEP Disulfide Reducing Gel**, 5mL of gel (10mL of 50% slurry in ultrapure water). TCEP (Tris[2-carboxyethyl] phosphine hydrochloride) immobilized onto 4% crosslinked beaded agarose. **Loading:** Gel has an effective (functional) TCEP concentration > 8mM (i.e., 8µmol/mL of gel). **Storage:** Upon receipt store at 4°C. Product is shipped at ambient temperature. Do not freeze.

Introduction

Trialkylphosphines 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 (e.g., maleimide crosslinkers).[5] Nevertheless, many protocols require recovery of the reduced sample separate from the reducing agent. The Thermo Scientific Immobilized TCEP Disulfide Reducing Gel 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 (see Related Thermo Scientific Products Section).

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

- Optimize incubation time by monitoring time-point aliquots for sulfhydryls using Ellman's Reagent (Product No. 22582).
- Do not allow metals (spatulas, etc.) to contact the Immobilized TCEP as this will decrease its activity.
- Including 5-20mM EDTA in the sample buffer during reduction will help prevent oxidation of generated sulfhydryl groups. Furthermore, adding EDTA (5-20mM) 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)

1. 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 25-50µL of mixed slurry for a 25µL protein/peptide sample.
2. 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.
3. 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.
4. 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.
5. If desired, determine reduction efficiency (quantity of free –SH groups) by either Ellman's Reagent (Product No. 22582) or SDS-PAGE using non-reducing conditions.

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

1. Add an appropriate amount of mixed TCEP reducing gel slurry to a spin-cup column (Product No. 69700) placed in a microcentrifuge tube (Product No. 69715). Use a volume of TCEP slurry equal to one to two times the volume of sample. For example, add 50-100µL of mixed slurry to the spin cup for a 50µL protein/peptide sample.

   Note: For 300-750µL 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 400µL 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 (Product No. 22582) or SDS-PAGE using non-reducing conditions.

Procedure for Reduction in Gravity-flow Columns (for > 250µL 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 3mL of mixed slurry to obtain a settled gel of 1.5mL. 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 0.5-1mL fractions from a 2mL column to which 1mL of sample was applied. Determine which fractions contain protein by measuring the absorbance at 280nm relative to a buffer blank.
6. If desired, determine reduction efficiency (quantity of free –SH groups) by either Ellman's Reagent (Product No. 22582) 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 (Product No. 22582) and free TCEP (Product No. 20490).

1. Prepare 10mL of a solution containing 40mg (10mM) 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 (Product No. 20490) standards as follows:

   Standard 1 (20mM TCEP): Dissolve 57.5mg free TCEP in 10mL of ultrapure water
   Standard 2 (4mM TCEP): Dilute standard 1 with ultrapure water (2mL standard 1 + 8mL ultrapure water)
   Standard 3 (2mM TCEP): Dilute standard 2 with ultrapure water (5mL standard 2 + 5mL ultrapure water)
   Standard 4 (1mM TCEP): Dilute standard 3 with ultrapure water (5mL standard 3 + 5mL ultrapure water)
   Standard 5 (0.5mM TCEP): Dilute standard 4 with ultrapure water (5mL standard 4 + 5mL ultrapure water)
3. Combine 990µL of the Ellman's Reagent solution with 10µL of each TCEP standard. Measure the absorbance at 412nm for each.
4. Combine 990µL of the Ellman's Reagent solution with 10µL of Immobilized TCEP Disulfide Reducing Gel slurry and measure the absorbance at 412nm.

   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.

Troubleshooting

Problem	Possible Cause	Solution
Incomplete reduction of sample	Insufficient amount of gel used	Use the recommended amount of gel
	Insufficient incubation time	For proteins, incubate for the recommended time. For peptides, attempt a brief incubation
	Disulfides were not accessible in protein	Include a denaturant such as guanidine•HCl (Product No. 24115) in the buffer **Note:** Do not use urea because it can form cyanates that will readily react with free sulfhydryl groups.
	Excessive incubation time	Do not exceed a 2 hour incubation
Reducing capacity of gel is dimished, as determined by testing with Ellman's Reagent (see below)	Product was more than 1 year old	Purchase new product
	Product was stored in buffer (e.g., PBS) rather than as provided in ultrapure water	
	Product was exposed to metals (in a storage buffer or by contact with metal utensils)	
Loss of overall protein/peptide	Batch method was used	Use spin cup column procedure
	Collected incorrect fractions from column	Check other fractions for protein
	Protein nonspecifically bound to the agarose resin support	Modify the buffer used (e.g., increase salt content or alter the pH of buffer, or use 10-30% DMSO)

Related Thermo Scientific™ Products

69700	**Pierce™ Spin Cups – Paper Filter**, 50 columns
69705	**Pierce Spin Columns – Screw Cap with Luer-Lok Adapters**, 25 columns
29922	**Disposable Polypropylene Columns**, for 1-5mL bed volumes, 100 columns
89868	**Pierce Centrifuge Columns, 0.8mL capacity,** 50 columns
89896	**Pierce Centrifuge Columns, 2mL capacity,** 25 columns
89897	**Pierce Centrifuge Columns, 5mL capacity,** 25 columns
89898	**Pierce Centrifuge Columns, 10mL capacity,** 25 columns
22582	**Ellman's Reagent**, 5g
20490	**TCEP•HCl**, 1g
24115	**Guanidine•HCl Solution (8M)**, 200mL

Cited References

1. Ruegg, U.T. and Rudinger, J. (1977). Reductive cleavage of cystine disulfides with tributylphosphine. *Methods Enzymol* **47**:111-26.
2. Kirley, T.L. (1989). Reduction and fluorescent labeling of cyst(e)ine-containing proteins for subsequent structural analysis. *Anal Biochem* **180**:231.
3. Levison, M.E., *et al.* (1969). Reduction of biological substances by water-soluble phosphines: Gamma-globulin. *Experentia* **25**:126-7.
4. Han, J.C., *et al.* (1994). A procedure for quantitative determination of Tris(2-carboxyethyl)phosphine, an odorless reducing agent more stable and effective than dithiothreitol. *Anal Biochem* **220**:5-10.
5. Haugland, R.P. and Blalgat, M.K. (1998). Preparation of Avidin Conjugates. *In* J.D. Pound (ed.) Immunological Protocols, Humana Press Inc., Totowa, NJ, **80**:189-90.