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A Geno Technology, Inc. (USA) brand name

Sulfhydryl Immobilization Kit for Proteins

For Generation of Protein Affinity Columns
Through Free Sulfhydryls

(Cat. # 786-804)



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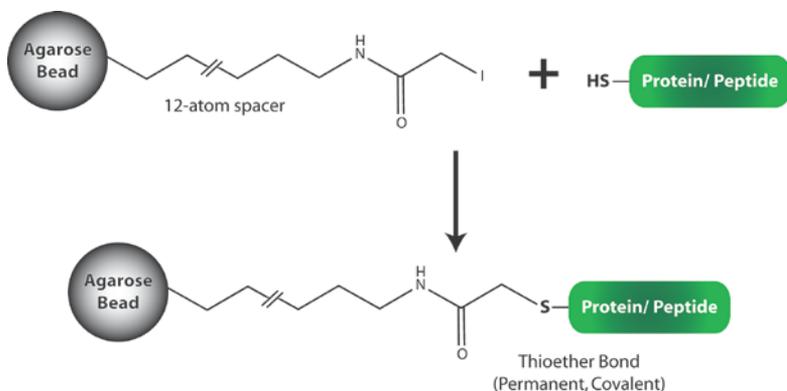
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INTRODUCTION

The Sulfhydryl Immobilization Kit for Proteins is designed for the simple and efficient coupling of proteins to a solid agarose support. The Sulfhydryl Coupling Resin Columns utilizes iodoacetyl groups that specifically react with free sulfhydryls to form covalent, permanent thioether bonds (see figure). The long spacer arm reduces steric hindrance and ensures greater binding of proteins and antibodies during affinity purification.

Proteins, including antibodies, must have free sulfhydryls for immobilization to the resin. A mild reducing agent, 2-Mercaptoethylamine, is supplied to reduce the hinge region disulfide bonds of antibodies, while preserving the functionally crucial disulfide bonds between the heavy and light chains.

The resulting columns can be used to study protein-protein interactions or for purification, via affinity chromatography. The columns, depending on the stability of the immobilized molecule, can be used several times without significant loss of activity.



ITEMS SUPPLIED (Cat. #: 786-804)

Part #	Description	Size
480S-A	Sulfhydryl Coupling Columns	5 x 2ml
2580	Optimizer Buffer™ II [5X]	25ml
102W-A	Wash Solution	60ml
105M	2-Mercaptoethylamine•HCl (MW=113.61)	50mg
295C	L-Cysteine. HCl	0.5g
320S-B	SpinOUT™ GT-600, 5ml	5

STORAGE CONDITION

The kit is shipped at ambient temperature. Upon arrival store at 4°C in the dark. Stable for 1 year.

IMPORTANT INFORMATION

- Perform immobilization steps at room temperature. Equilibrate all reagents to room temperature before use.
- Maleimides react with free sulfhydryls to form stable thioether bonds at pH 6.5-7.5. pH >7.5 significantly increases the reaction of amines with the maleimide groups.
- Some sulfhydryl-containing peptides and proteins may oxidize in solution and form disulfide bonds, which cannot react with maleimides. Disulfide bonds can be reduced to produce free sulfhydryls. The G-Biosciences Immobilized Reductant (Cat. # 786-148) enable peptide or protein reduction while recovering the sample in the absence of reducing agents.
- Ellman's Reagent (Cat. # BC87) can be used to determine the amount of free sulfhydryls. (See Appendix)
- For peptides or proteins lacking sulfhydryls, SATA (N-Succinimidyl-S-acetylthioacetate) (Cat. # BC96) or Traut's Reagent (2-Iminothiolane hydrochloride) (Cat. # BC95) can be used to add sulfhydryls via amine modification. (See Appendix)
- Ensure the protein solutions are free of particulates as these will inhibit column flow rates. We recommend filtering through a 0.45µm filter or a high speed centrifugation at 10,000g.
- Hydrophobic proteins may require additional washes or use of non-ionic detergent to minimize non-specific binding.

ADDITIONAL ITEMS REQUIRED

- 15ml centrifuge tubes
- 1X PBS with 0.05% sodium azide for column storage

PREPARATION BEFORE USE

1. Prepare 1X Optimizer Buffer™ II (1ml 5X Optimizer Buffer™ in 4ml de-ionized water). Prepare 25ml 1X Optimizer Buffer™ II for each column preparation
2. Prepare 1ml 1-10mg/ml protein solution in an aqueous buffer pH 6.0 supplemented with 5mM EDTA.

PROTOCOL

A. Reduce & Desalt Protein

1. Allow the 2-Mercaptoethylamine•HCl to warm to room temperature and then open the vial and weigh out ~6mg.
2. Add the 1ml protein solution to the 6mg 2-Mercaptoethylamine•HCl and incubate at 37°C for 1.5 hours.
3. Prepare the SpinOUT™ GT-600 column by briefly centrifuging at 1,000g for 1 minute in a 15ml centrifuge tube and then removing the top cap then bottom tab. Allow the storage buffer to drain out.
4. Add 2ml 1X Optimizer Buffer™ II to the SpinOUT™ column and centrifuge at 1,000g for 1 minute. Repeat this step once.
5. Allow the reduced protein sample to cool to room temperature and slowly add to the SpinOUT™ column. Once the sample has entered the column, add 100µl 1X Optimizer Buffer™ II.
6. Centrifuge column at 1,000g for 1 minute. The flow-through is the reduced protein in coupling buffer. If required a protein assay can be performed at this stage.
7. Adjust the volume to 2-3ml with 1X Optimizer Buffer™ II and reserve a 100µl aliquot to be used to assess coupling efficiency (Starting Material).

B. Immobilize Protein on Sulfhydryl Coupling Resin

1. Invert the Sulfhydryl Coupling Columns 5-6 times to suspend the resin. Remove the top cap and then bottom tab and centrifuge for 1 minute at 1,000g in a 15ml centrifuge tube to remove the storage buffer.
2. Add 2ml 1X Optimizer Buffer™ II and centrifuge at 1,000g for 1 minute. Repeat this step once, and then apply the bottom rubber stopper.
3. Slowly apply the reduced protein sample to the Sulfhydryl Coupling Column. Apply the top cap and incubate at room temperature for 15 minutes with end-over-end mixing or rocking.
4. Incubate the column upright for a further 30 minutes at room temperature without mixing.
5. After incubation, remove the top then bottom caps and collect the flow-through in a clean tube. Save the flow-through to determine the coupling efficiency.
NOTE: *The coupling efficiency is determined by measuring and comparing the concentration of the protein in Starting Material (Section A, Step 7) and in the flow-through (Section B, Step 5). The concentration may be determined either by UV absorbance at 280nm or by a suitable protein assay.*
6. Wash the column with at least 2ml Wash Solution and centrifuge at 1,000g for 1 minute. Repeat this wash step three more times.
7. Wash the column with 2ml 1X Optimizer Buffer™ II and centrifuge at 1,000g for 1 minute. Repeat this wash step once. Place the stopper on the bottom of the column.

C. Block Non-Specific Sites

1. Add 15.8mg L-Cysteine. HCl to 2ml 1X Optimizer Buffer™ II and slowly add to the column.
2. Apply the top cap and incubate at room temperature for 15 minutes with end-over-end mixing or rocking.
3. Incubate the column upright for a further 30 minutes at room temperature without mixing.
4. Remove the top then bottom caps and allow to drain. The column can now be stored or used for affinity purification.
5. For storage, wash the column with 2ml 1X PBS supplemented with 0.05% sodium azide. Repeat three times. Seal the bottom of the column and add 2ml 1X PBS supplemented with 0.05% sodium azide and apply the top cap. Store upright at 4°C.

TROUBLESHOOTING

Issue	Possible Cause	Suggested Resolution
Low level of immobilization	Low or no free sulfhydryls	Immediately desalt and start coupling procedure after reducing agent treatment to prevent disulfide bonds reforming
	Reducing agent inhibiting binding	
Loss of binding capacity after several uses	Immobilized ligand altered by temperature, affinity purification conditions or time	Generate a new column
	Binding sites and pores blocked by non-specific binding or particulates	Filter samples through a 0.45µm filter or centrifuge at 10,000g to remove particulates
		Use high salt concentrations, non-ionic detergents to reduce non-specific binding.

APPENDIX 1: GENERAL AFFINITY PURIFICATION PROTOCOL

The following protocol is a simple spin method that allows for rapid affinity purification. The columns can be used for gravity flow methods. The concentration of protein and binding conditions are dependent of the affinity interactions and will need to be optimized for each situation.

Additional Components

Binding/ Wash Buffer: PBS, TBS or other aqueous buffer that supports the desired affinity interaction. Degas the buffers to avoid introducing air bubbles.

Sample: Dissolved or exchanged in Binding/Wash Buffer

Elution Buffer: 0.1-0.2M Glycine•HCl at pH2.5-3.0

Neutralization Buffer: 1M Tris•HCl pH 8.5

Procedure

1. Allow the column and buffers to warm to room temperature.
2. Remove top then bottom caps and centrifuge the column at 1,000g for 1minute in a 15ml centrifuge tube. Discard the storage buffer.
3. Equilibrate the resin with 3 column volumes (CV) of Binding/Wash Buffer.
4. Add ≤ 2 ml sample and allow the sample to enter the resin bed. Replace the bottom cap and then add 0.2ml Binding/Wash Buffer. Replace the top cap.
5. Incubate the column at room temperature with end-over-end mixing or rocking for 15-60 minutes.
6. Remove top then bottom caps and centrifuge the column at 1,000g for 1minute in a 15ml centrifuge tube. Add 1ml Binding/Wash Buffer and centrifuge again. Combine and save both flow-throughs to evaluate binding efficiency and capacity.
7. Wash the resin with 4CV of Binding/Wash Buffer.
8. Elute the protein with 1CV of Elution Buffer and collect in a centrifuge containing 100 μ l Neutralization Buffer. Repeat this step 2-3 times more. Samples can be used directly for SDS-PAGE or can be dialyzed or desalted for other downstream applications.
9. Equilibrate the column by adding 2CV Binding/Wash buffer and allow it to flow-through the resin.
10. Cap the bottom of the column and add 2CV Binding/Wash Buffer supplemented with 0.05% sodium azide for long term storage. Store upright at 4°C.

APPENDIX 2: ELLMAN'S REAGENT (DTNB) ASSAY

1. Make 10mM DTNB stock solution by dissolving 40mg DTNB in 10ml 0.1M Tris-HCl pH 7.5. The stock solution can be stored at 4°C for 3 months. Dilute the stock solution 100 fold with 0.1M Tris-HCl pH 7.5 to make 0.1mM DTNB working solution.
2. Aliquot 950µl of 0.1mM DTNB work solution to each 1.5ml centrifuge tube. Add 50µl test sample and mix by brief vortexing. Set a blank by adding 50µl of 0.1M Tris-HCl pH 7.5 to 950µl of 0.1mM DTNB work solution.

NOTE: *The test sample may need to be diluted before applied to the assay and the dilution factor should be recorded. The 50µl test sample applied to the assay reaction should have a sulfhydryl concentration less than 0.5mM. Concentrations exceeding 0.5mM free sulfhydryl will result in high absorbance values and less accurate estimation of the concentration based on the extinction coefficient.*

3. Incubate 2 minutes at room temperature.
4. Measure the absorbance of the test sample with a spectrophotometer against blank at 412nm.
5. Calculate the concentration of free sulfhydryls in the sample from the molar extinction coefficient of NTB ($14.15 \text{ mM}^{-1} \text{ cm}^{-1}$) as follow:
mM free sulfhydryls = Absorbance / (path length x 14.15) x 20 x dilution factor
Path length is the cuvette path length in centimeter (cm); 20 is the dilution factor of 50µl sample to 1ml assay volume

APPENDIX 3: USE OF SATA TO ADD SULFHYDRYLS

SATA (N-Succinimidyl S-Acetylthioacetate) (Cat. # BC96) introduce protected sulfhydryls into proteins, peptides and other molecules. It is a NHS esters of S-acetylthioacetic acid.

1. Immediately before reaction, dissolve ~7mg SATA in 0.5ml DMSO to give ~55mM solution.
2. Combine 1ml 2-10mg/ml protein solution in PBS with 10µl 55mM SATA.
3. Incubate at room temperature for 30 minutes
4. Desalt the solution with a desalting column equilibrated with PBS. We recommend G-Biosciences SpinOUT™ GT-600 (Cat. # 786-170).
5. Identify the fraction with the protein using absorbance at 280nm or a suitable assay.
6. Combine 1ml SATA-modified protein with 100µl 0.5M hydroxylamine, 25mM EDTA in PBS.
7. Incubate for 2 hours at room temperature.
8. Desalt as before using PBS supplemented with 10mM EDTA.

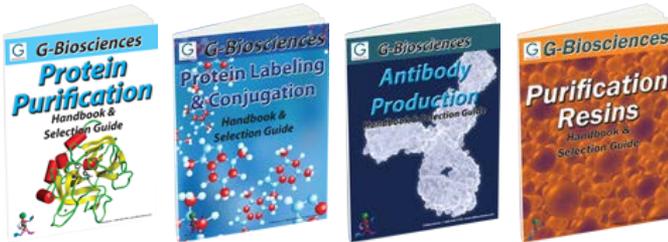
APPENDIX 4: USE OF TRAUT'S REAGENT TO ADD SULFHYDRYLS

Traut's Reagent (2-Iminothiolane) (Cat. # BC95) is a cyclic thioimide compound for thiolation of primary amines.

1. Dissolve the protein or peptide in a non-amine buffer at pH8.0. The addition of 2-5mM EDTA will prevent oxidation of generate sulfhydryls into disulfide bridges.
2. Add 2 to 20 fold molar excess of Traut's reagent to the protein solution.
NOTE: A 2mg/ml solution of Traut's reagent in water or buffer is a 14mM stock solution.
3. Incubate the solution for 1 hour at room temperature.
4. Desalt the solution with a desalting column equilibrated with PBS with 2-5mM EDTA. We recommend G-Biosciences SpinOUT™ GT-600 (Cat. # 786-170).

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