INSTRUCTIONS

Thermo SCIENTIFIC

Pierce® NHS-Activated Agarose Slurry

2193.0

Number	Description
26200	**Pierce NHS-Activated Agarose Slurry,** 25 ml settled resin supplied in acetone Support: 6% crosslinked beaded agarose **Storage:** Upon receipt store the agarose slurry at 4°C. Product is shipped at ambient temperature.

Introduction

The Thermo Scientific Pierce NHS-Activated Agarose Resin allows simple and efficient covalent immobilization of proteins to a beaded-agarose support, providing a valuable tool for affinity purification of antibodies, antigens or other biomolecules. The activated agarose contains *N*-hydroxysuccinimide (NHS) functional groups that react with primary amines on proteins or other molecules to form stable amide linkages. The coupling reaction is performed in an amine-free buffer at pH 7-9. Coupling efficiency is typically greater than 80%, regardless of the ligand's molecular weight or pI. Once the ligand is immobilized, the prepared resin can be used for multiple affinity purification procedures. The crosslinked beaded agarose has fast linear flow potential, making it useful for gravity-flow and low- to medium-pressure applications.

Procedure for Protein Immobilization

The following coupling procedure is for 1 ml of settled resin in a 2 ml spin column; scale the procedure as needed.

A. Additional Materials Required

- Empty columns (e.g., 2 ml Pierce Centrifuge Column, Product No. 89896) and compatible centrifuge/collection tubes
- Coupling/Wash Buffer: 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 (PBS, Product No. 28372) or other amine-free buffer at pH 7-9
- Protein or peptide: Dissolve 1-20 mg protein or 1-2 mg peptide in 2-3 ml of Coupling/Wash Buffer. For proteins already in solution, dilute four-fold in Coupling/Wash Buffer or desalt/dialyze into Coupling/Wash Buffer. Completely remove primary amine-containing buffer (e.g., Tris or glycine).
- Quenching Buffer: 1 M ethanolamine, pH 7.4

B. Coupling Procedure

1. Invert bottle of agarose slurry several times. Using a wide-orifice or cut pipette tip, add 2 ml of the NHS-Activated Agarose Resin Slurry to a 2 ml spin column.

   Note: The slurry is supplied at 50% resin in acetone. During storage, acetone evaporation might result in a change of the resin percentage. If needed, add more acetone to adjust for evaporation.
2. Place the column containing the NHS-Activated Resin upright. Equilibrate column to room temperature and allow the resin to settle.
3. Remove top and bottom caps and place column in a collection tube. To remove storage solution, centrifuge column at 1,000 × *g* for 1 minute and discard the flow-through.

   Note: Do not allow resin bed to become dry at any time during the procedure.
4. Add 2 ml of ultrapure water to the column, centrifuge at 1,000 × *g* for 1 minute and discard the flow-through.
5. Add 2 ml of Coupling/Wash Buffer to the column, centrifuge at 1,000 × *g* for 1 minute and discard the flow-through.
6. Replace the bottom cap and add 2 ml of the protein solution to the column. Save 0.1 ml of the prepared sample for subsequent determination of coupling efficiency.

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7. Replace the top cap on the column and mix the reaction end-over-end for 1 hour at room temperature.

   Note: Approximately 80% of the reaction occurs in the first 30 minutes. The reaction may be extended to 2 hours at room temperature or overnight at 4ºC.

8. Remove top and bottom caps. Centrifuge column at 1,000 × *g* for 1 minute and save the flow-through.

9. Wash the column with 1 ml of Coupling/Wash Buffer. Centrifuge column at 1,000 × *g* for 1 minute and save the flow-through. Repeat this step once.

   Note: The saved flow-through and washes are used for determining the coupling efficiency by comparing the protein concentrations of these non-bound fractions to the starting sample (saved in Step 6). The NHS leaving group interferes with the BCA protein assay and absorbance measurements at 280 nm; for optimal results use the Thermo Scientific Pierce 660 nm Protein Assay (Product No. 22660) or dialyze the flow-through and wash fractions against PBS using a Thermo Scientific Slide-A-Lyzer Dialysis Cassette before measuring the absorbance.

C. Block Remaining Active Sites

1. Add 2 ml of Quenching Buffer to the resin and replace the bottom cap.
2. Replace the top cap and mix end-over-end for 15-20 minutes at room temperature.
3. Remove the top cap first and then the bottom cap. Place the column in a new collection tube and centrifuge at 1,000 × *g* for 1 minute. Discard the flow-through.
4. Wash column with at least 6 ml of Coupling/Wash Buffer. Monitor the final washes for the presence of protein by measuring the absorbance at 280 nm or by the Pierce 660 nm Protein Assay. Either proceed directly with affinity purification or prepare column for storage (Step 5).
5. For storage, wash column with 6 ml of PBS containing 0.05% sodium azide or other preservative. Replace bottom cap when 0.5-1 ml of buffer remains above the resin bed. Replace top cap and store column upright at 4°C.

General Protocol for Affinity Purification

Note: This protocol uses a column with a resin-bed volume of 1 ml. For columns with other bed volumes, adjust all solution (e.g., sample, wash, and elution) volumes accordingly. The amount of protein sample needed and incubation time are dependent upon the affinity system involved (e.g., antibody-antigen interaction) and must be optimized.

A. Additional Materials Required

- Binding/Wash Buffer: Phosphate-buffered saline (PBS, Product No. 28372), Tris-buffered saline (TBS, Product No. 28379) or other buffer that is compatible with the intended affinity interaction
- Sample: Prepare antigen or other molecule in Binding/Wash Buffer or dilute sample 1:1 in Binding/Wash Buffer
- Elution Buffer: Thermo Scientific IgG Elution Buffer (Product No. 21004) or 0.1-0.2 M glycine•HCl, pH 2.5-3.0
- Neutralization Buffer (optional): 1 ml of high-ionic strength alkaline buffer such as 1 M phosphate or 1 M Tris; pH 9

B. Procedure

Note: Throughout the procedure, do not allow the resin bed to become dry; replace bottom cap as soon as buffer drains down to the top of resin bed.

1. Equilibrate the prepared affinity column to room temperature.
2. Remove top and bottom caps and place column in a collection tube. Centrifuge the column at 1,000 × *g* for 2 minutes to remove the storage solution.
3. Equilibrate column by adding 1 ml of Binding/Wash Buffer and centrifuge at 1,000 × *g* for 2 minutes. Discard buffer from collection tube. Repeat this step twice.
4. Add sample to column and allow it to enter the resin bed. Replace top and bottom caps on the column.
5. Incubate the column. Optimal reaction time depends on the specific affinity interaction. Typically, 2 hours at room temperature with end-over-end mixing is sufficient, or the incubation can be extended to overnight at 4ºC.

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6. Remove top cap and bottom caps from column and place column in new collection tube. Centrifuge the column at 1,000 × *g* for 2 minutes. Save the flow-through to analyze binding efficiency.
7. Wash the column with 3 ml of Binding/Wash Buffer. Centrifuge at 1,000 × *g* for 2 minutes.
8. Elute the bound protein by applying 8 ml of Elution Buffer. Collect 1 ml (or 0.5 ml) fractions. The pH of each fraction can be adjusted to neutral by adding 50 µl of Neutralization Buffer per 1 ml of collected eluate.
9. Monitor elution using the Pierce 660 nm Protein Assay. Alternatively, measure the absorbance at 280 nm of the eluted fractions. Pool fractions of interest and exchange into an appropriate storage buffer by desalting or dialysis.

C. Column Regeneration and Storage

Note: Regenerate the column immediately after elution to prevent damage to the immobilized molecule by the low-pH elution buffer.

1. Wash column with 8 ml of Binding/Wash Buffer to remove any residual protein and neutralize the elution buffer.
2. Equilibrate column with 4 ml of Binding/Wash Buffer containing 0.05% sodium azide.
3. Replace bottom cap and add 2 ml of Binding/Wash Buffer to the column and cap the top. Store column upright at 4°C.

Troubleshooting

Problem	Possible Cause	Solution
Low coupling efficiency	Primary amine-containing buffer not completely removed before coupling	Dialyze or desalt sample to completely remove Tris or glycine
Protein is not soluble in Coupling Buffer	Molecule is hydrophobic	Dissolve molecule in Coupling Buffer containing up to 4 M guanidine•HCl or 20% DMSO
Affinity column has reduced binding capacity with time	Immobilized sample was damaged by time, temperature or elution conditions	Prepare a new affinity column
	Nonspecifically bound material has reduced capacity	Wash column with high salt (~1 M NaCl) to remove nonspecifically bound material

Additional Information Available from our Web Site

- Tech Tip #12: Prepare molecules with poor solubility for immobilization on affinity supports
- Tech Tip #27: Optimize elution conditions for immunoaffinity purification
- Tech Tip #7: Remove air bubbles from columns to restore flow rate
- Tech Tip #29: Degas solutions for use in affinity and gel filtration columns

Related Thermo Scientific Products

89868 **Pierce Centrifuge Columns,** 0.8 ml, 50 centrifuge columns and 50 screw caps

89896 **Pierce Centrifuge Columns, 2 ml (resin bed capacity),** gravity or centrifuge compatible, 25 units

89897 **Pierce Centrifuge Columns, 5 ml (resin bed capacity),** gravity or centrifuge compatible, 25 units

89898 **Pierce Centrifuge Columns, 10 ml (resin bed capacity),** gravity or centrifuge compatible, 25 units

28372 **BupH™ Phosphate Buffered Saline,** each dry-blend pack makes 500 ml with water, 40 packs

21004 **IgG Elution Buffer,** low-pH elution buffer for general protein affinity purifications, 1 L

22660 **Pierce 660 nm Protein Assay**

References

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Hermanson, G.T., *et al.* (1992). *Immobilized Affinity Ligand Techniques*. Academic Press, Inc.: San Diego, CA.

Lasch, J. and Koelsch, R. (1978). Enzyme leakage and multipoint attachment of agarose-bound enzyme preparations. *Eur. J. Biochem*. **82:**181-6.

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Current versions of product instructions are available at www.thermo.com/pierce. For a faxed copy, call 800-874-3723 or contact your local distributor.

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