INSTRUCTIONS

Pierce™ Protein G IgG Plus Orientation Kit

44990

0760.3

Number	Description
44990	**Protein G IgG Plus Orientation Kit,** sufficient reagents for preparing 2 × 2mL affinity columns

Kit Contents:

Protein G Columns, 2 each, containing 2mL of Protein G agarose resin (4mL total slurry volume) in 0.02% sodium azide

Disuccinimidyl Suberate (DSS), 2 × 13mg

BupH™ Phosphate Buffered Saline Pack (Crosslinking Buffer), contains 0.15M NaCl, 0.1M Na_2PO_4, pH 7.2 when reconstituted with 500mL of ultrapure water

Blocking Buffer for Orientation Kit, 6mL, contains 0.1M ethanolamine, pH 8.2

IgG Elution Buffer, 15mL, pH 2.8 (contains primary amine)

Antibody Binding/Wash Buffer, 120mL, contains 50mM sodium borate, pH 8.2

Column Accessories, porous discs (6), white tips (5), resin separators (2) and column extender

Storage: Upon receipt store at 4°C. Store the Disuccinimidyl Suberate (DSS) desiccated at 4°C. Product is shipped at ambient temperature.

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Introduction

The Thermo Scientific Pierce IgG Plus Orientation Kit uses recombinant Protein G agarose and DSS crosslinker to prepare an affinity column for antigen purification. Protein G binds to most mammalian IgGs through their Fc regions, but some binding also occurs through the Fab region. However, since most binding does occur through the Fc region, this support immobilizes and orients the antibody so the antigen-binding sites remain available for efficient antigen purification. The homobifunctional NHS-ester crosslinker DSS covalently couples the bound and oriented antibody to the immobilized Protein G (Figure 1). Crude sample is then incubated with the immobilized antibody, allowing the immune complex to form. Unwanted material is washed from the affinity column, and then the bound antigen is dissociated. This method is advantageous because the antibody does not contaminate the final antigen preparation and the immobilized antibody support is preserved for additional use. Depending on the stability of the immobilized antibody, the prepared affinity support may be used 2-10 times, which conserves precious antibody. For antibodies that do not have high affinities to recombinant Protein G (e.g., dog and guinea pig), a similar kit is available with Protein A Columns (Product No. 44893).

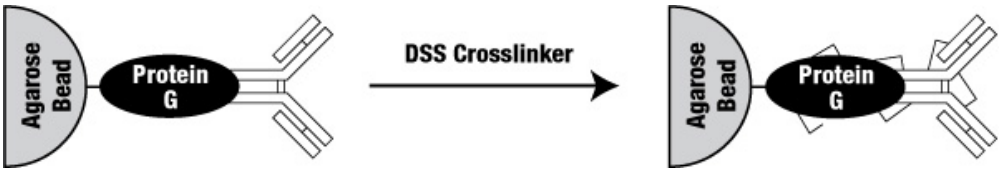

Figure 1. DSS covalently crosslinks affinity-bound antibody to Protein G agarose.

Additional Materials Required

- Dimethylformamide (DMF, Product No. 20672) or Dimethylsulfoxide (DMSO, Product No. 20684)
- Ultrapure water
- 16 × 125mm test tubes
- Sodium azide for column storage

Procedure for Crosslinking an Antibody to Protein G Agarose

A. Antibody Preparation

Note: For optimal results, use affinity-purified antibody. Although serum may be used, the antibody that is specific for the antigen of interest may comprise only 2-5% of the total IgG in the serum sample and will result in low antigen yields.

- **Lyophilized antibody:** For optimal results, use an amount of antibody equal to 80% of the binding capacity of the Protein G Column. For example, because the binding capacity for human IgG is approximately 20mg/mL of resin, dissolve 16mg of human IgG in 2mL of Antibody Binding/Wash Buffer. The approximate binding capacity for rabbit IgG is 8-10mg/mL and 6-8mg/mL for mouse IgG.

  Note: Lyophilization buffer must not contain primary amines such as glycine or Tris. Remove amines by dialysis or resin filtration.

- **Antibody in solution** (samples in buffers, IgG fractions, etc.): Remove primary amines by resin filtration or dialyze against a phosphate buffer (e.g., Product No. 28372, 0.1M phosphate, 0.15M NaCl) because primary amines such as Tris or glycine are not compatible with the crosslinker DSS. Dilute antibody 1:1 (total volume 2-4mL) with Antibody Binding/Wash Buffer. See note with previous bullet point concerning lyophilized antibody for antibody amounts to use.

B. Column Preparation and Equilibration

Note: Do not allow resin bed to become dry. Equilibrate columns and buffers to room temperature before use.

1. Remove top cap and twist off bottom tab from a Protein G column. Place column in a test tube and allow storage solution to drain. If resin has settled along the column sides during shipment or storage, gently suspend resin by stirring with a Pasteur pipette before draining column. This procedure will ensure that a proper chromatographic bed is maintained as the storage solution passes through the column.
2. Equilibrate column with 5mL of Antibody Binding/Wash Buffer. Place bottom cap (a supplied white tip) on column when solution reaches top of resin bed.

C. Binding the Antibody to the Protein G Column

Below are two methods for column loading. To obtain a uniform antibody loading, the first method is optimal. However, for volumes greater than 4mL of dilute antibody solutions (10-100µg/mL), it may be more practical to use the second method.

Method 1: Column loading for antibody concentrations >100µg/mL

1. With bottom cap in place, add 2-4mL of antibody solution to the column.
2. Place top cap on column and suspend resin by repeated inversion and tapping the column.
3. Continue mixing by inversion for 30 minutes at room temperature.
4. Remove top and bottom caps sequentially. Place column in a test tube.

5. Allow resin to settle while remaining solution passes through column. Reserve this solution. It contains unbound antibody and may be analyzed to determine binding efficiency by measuring the absorbance at 280nm or with a protein assay such as BCA (Product No 23227).
6. When the antibody solution has passed through column, transfer the column to a new tube and wash resin with 2 × 5mL of Antibody Binding/Wash Buffer.

   Note: If desired, collect wash fractions in new test tubes to recover any unbound antibody.
7. Upon completing the wash, replace bottom cap.

Method 2: Column loading for antibody concentrations of 10-100µg/mL

1. Place an equilibrated column into a test tube and pass the diluted antibody through resin. If a large volume is to be applied, several 5mL aliquots may be added sequentially and the column moved to new tubes as needed. Reserve the flow-through; this contains unbound antibody and may be analyzed to determine binding efficiency.
2. When the antibody solution has passed through column, transfer the column to a new tube and wash resin with 2 × 5mL of Antibody Binding/Wash Buffer.
3. Replace bottom cap.

D. Crosslinking the Bound Antibody

Note: DSS is moisture-sensitive. Store DSS refrigerated and desiccated. To avoid moisture condensation onto the product, equilibrate vial to room temperature before opening. Dissolve DSS in DMSO or DMF immediately before use.

1. Prepare Crosslinking Buffer by reconstituting contents of Phosphate Buffered Saline Pack with 500mL ultrapure water.
2. Dissolve contents of one vial of DSS in 1mL of DMSO or DMF. Transfer dissolved DSS to a new test tube and add 1.5mL of Crosslinking Buffer.

   Note: Because DSS is a hydrophobic molecule, a microprecipitate may form when it is added to the aqueous medium, resulting in a cloudy appearance. Nevertheless, the reaction will proceed efficiently and the microprecipitate may disappear during conjugation.
3. Immediately pipette the solution onto the center of the antibody-bound column.
4. Place the top cap on the column and suspend the resin by repeated inversions and by tapping the column.
5. Continue mixing by inversion for one hour at room temperature.
6. After reaction is complete, remove top cap and then the bottom cap. Place column in a test tube.
7. Allow resin to settle while remaining solution passes through column. Do not allow resin bed to become dry.
8. Wash resin with 5mL of Crosslinking Buffer and replace bottom cap when wash solution has reached the level of the resin bed.

E. Blocking Remaining Active Sites

1. Add 2mL of Blocking Buffer to column to block any remaining nonreacted NHS-ester (DSS) groups.
2. Replace top cap and suspend resin by repeated gentle inversions and by tapping the resin-bed end of the column.
3. Continue mixing by gentle inversion for 10 minutes.
4. Remove top and bottom caps sequentially. Place column in a test tube.
5. Allow resin to settle while remaining solution passes through column.
6. Replace bottom cap and add 5mL of IgG Elution Buffer. Replace top cap and invert column at least 10 times to thoroughly mix contents.
7. Remove top and bottom caps sequentially and allow solution to flow through column. Wash column with an additional 2mL of Elution Buffer. This buffer will elute IgG that is not covalently attached to the Protein G. The flow-though may be assayed for antibody to determine crosslinking efficiency.

8. Wash resin two times with 5mL of Antibody Binding/Wash Buffer. The top porous disc may be placed in the column before the second wash. This may be accomplished using the following steps:
   a. Replace bottom cap and apply the second 5mL wash.
   b. Float a porous disc on the buffer surface above the resin.
   c. Use an inverted serum separator (the narrow end) to push the porous disc into the column to within 1mm of the resin bed surface. Avoid compressing the resin bed.
   d. Remove bottom cap and allow the second wash solution to pass through resin. When the buffer reaches the level of the top disc, the flow will stop automatically.

F. Storing the Prepared Antibody Column

1. After covalent coupling of the antibody, the affinity column may be washed and stored in an aqueous solution (e.g., phosphate or Tris buffer) containing 0.02% sodium azide.
2. Store the column upright at 4°C and protect from light.

General Procedure for Affinity Purification of an Antigen

This is a general protocol for antigen purification using an antibody column. Because some antigens require more or less stringent conditions for dissociation from an immobilized antibody, optimization of this procedure may be necessary.

A. Preparation of the Immunoaffinity Column

1. Equilibrate the prepared affinity column to room temperature.
2. Remove top cap first to avoid drawing air into resin bed.
3. Remove bottom cap and allow storage solution to drain completely. Upon reaching the level of the top disc, the flow will stop automatically.
4. Equilibrate column with binding buffer (e.g., PBS, 0.1M phosphate, 0.15M NaCl, pH 7.2, Product No. 28372).

B. Sample Application and Elution

1. Dilute antigen sample at least 1:1 with binding buffer.
2. Apply sample to the immunoaffinity column and incubate for ≥1 hour at room temperature or overnight at 4°C.
3. Wash with binding buffer until the flow-through has a baseline absorbance at 280nm.
4. Elute with an elution buffer such as IgG Elution Buffer (Product No. 21004) or 0.1M glycine•HCl, pH 2.8. Collect 1mL fractions and monitor protein content by measuring the absorbance at 280nm.
5. Adjust the pH of the eluted fractions to neutral with a suitable concentrated buffer such as 1M Tris•HCl, pH 9.5 (use approximately 50 µl per ml of fraction collected).

C. Immunoaffinity Column Regeneration

Note: The immunoaffinity column may be regenerated and used again if the immobilized antibody is stable and unaffected by the elution conditions.

1. Wash with 3-4 column volumes of elution buffer.
2. Store in water or a neutral pH buffer (e.g., phosphate or Tris buffer) containing 0.02% sodium azide.

Troubleshooting

Problem	Cause	Solution
Antibody leaching from resin or co-elution of antibody	Too much antibody applied to resin	Wash resin with additional elution buffer until the flow-through has a baseline absorbance at 280nm then re-equilibrate column with Binding/ Wash Buffer and re-apply sample
	Resin was not washed sufficiently with elution buffer before sample was applied	
Antigen does not immunoprecipitate	Antibody not crosslinked to resin	Verify that antibody initially bound to resin by evaluating wash fractions either by A_{280} or with a protein assay. Also verify that the antibody was not eluted during the elution step in crosslinking
	Antibody is sensitive to low pH and was destroyed during wash steps (extremely rare)	Prepare another column and switch to a neutral pH elution buffer such as Gentle Ag/Ab Elution Buffer (Product No. 21027)
	Antibody does not elute using acidic conditions	Use guanidine•HCl, urea, lithium bromide, potassium thiocyanate or non-ionic detergents to elute the antigen **Note:** Using denaturants may cause the immobilized antibody to become inactive

Related Thermo Scientific Products

44893 **Pierce Recombinant Protein A IgG Plus Orientation Kit**

26147 **Pierce Crosslink Immunoprecipitation Kit**

21658 **DSS, No-Weigh™ Format,** 8 × 2mg microtubes

21027 **Pierce Gentle Ag/Ab Elution Buffer,** 500mL

21004 **Pierce IgG Elution Buffer,** 1L

28372 **BupH Phosphate Buffered Saline Packs,** 40 packs, each pack yields 500 ml of 0.1 M phosphate, 0.15M NaCl, pH 7.2 when dissolved in 500mL ultrapure water

23227 **Pierce BCA Protein Assay Kit**

Additional Information Available from Our Website

- 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 buffers for use in affinity and gel filtration columns
- Tech Tip #34: Binding characteristics of Protein A, G, A/G and L

General References

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Horsfall, A.C., *et al.* (1987). Purification of human autoantibodies from cross-linked antigen immunosorbants. *J Immunol Meth* **104:**43-9.

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