INSTRUCTIONS

UltraLink® Biosupport Medium

53110 53111 53112

PIERCE Biotechnology
PERBIO
3747 N. Meridian Road
P.O. Box 117
Rockford, IL 61105

0678.1

Number	Description
53110	**UltraLink® Biosupport Medium**
	Contents:
	UltraLink® Biosupport Medium, 1.25 g (approximately 10 ml of gel)
	Disposable Column Trial Pack, includes the following:
	Disposable Polystyrene Columns, 0.5-2.0 ml, 2 each
	Disposable Polypropylene Columns, 1.0-5.0 ml, 2 each
	Disposable Polypropylene Columns, 2.0-10.0 ml, 2 each
	Accessory Pack, 1 each
53111	**UltraLink® Biosupport Medium**
	Contents:
	UltraLink® Biosupport Medium, 6.25 g (approximately 50 ml of gel)
	Disposable Column Trial Pack, includes the following:
	Disposable Polystyrene Columns, 0.5-2.0 ml, 2 each
	Disposable Polypropylene Columns, 1.0-5.0 ml, 2 each
	Disposable Polypropylene Columns, 2.0-10.0 ml, 2 each
	Accessory Pack, 1 each
53112	**UltraLink® Biosupport Medium,** 0.25 g (approximately 2 ml of gel)
	Storage: Upon receipt products should be stored at room temperature. Products are shipped at ambient temperature. After opening, store the support at 4°C with desiccant. Rehydrated support should be stored at 4°C.
	Products are guaranteed for one year from the date of purchase when handled and stored properly.

Introduction

Pierce UltraLink® Biosupport Medium is a preactivated, ready-to-use bead designed for use in affinity chromatography. These beads are composed of a *bis*-acrylamide/azlactone copolymer that is slightly hydrophobic in its active form and highly cross-linked. Because the azlactone functionality is copolymerized with the matrix material, the binding capacity is an integral part of the bead. This results in a high level of functionality throughout the porous bead matrix.

The support is provided in dry form and reacts rapidly with nucleophiles, which allows coupling to a wide range of proteins and small molecules. The beads average 50-80 microns in diameter with a very open architecture that provides both high surface area and high pore volume. Due to the rigid polymeric nature of this support, it has excellent utility in medium-to-low pressure chromatography applications. These beads are further described in US Patent No. 4,871,824 and European Patent EP0392,735 B1.

For most ligands, the addition of a chaotropic salt can greatly increase the coupling efficiency (Figure 7, Coupling Buffer Information, Appendix G). Other factors that can impact coupling efficiency are also described.

Procedure Summary

1. Add protein solution to dry beads and mix for 1 hour.
2. Add quench solution and mix for 2.5 hours.
3. Wash beads 5 times.
4. Resuspend beads in buffer of choice.

Important Product Information

Chemistry

The azlactone functionality of the UltraLink® Biosupport Medium couples nucleophiles on ligands via a ring opening reaction to attach the ligand to the support through stable covalent linkages. For example, amino-functional ligands will form stable amide bonds. Due to the unique azlactone chemistry, there is no leaving group or toxic chemical byproduct as a result of the coupling reaction, therefore this coupling chemistry is extremely safe and easy to use.

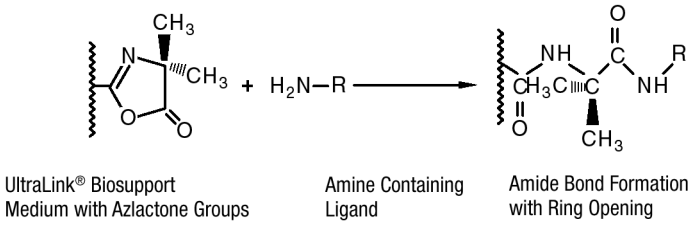

Figure 1. Reaction of azlactone ring on UltraLink® Biosupport Medium with amine-containing ligand.

Product Characteristics

A. Stability

- pH stability of matrix: 1-13
- Temperature stability: 4-40°C
- Reusability: Over 100 cycles with 99% capacity (Refer to Reusability Information, Appendix H for additional details)

B. Coupling Capacities

- Protein A: 240 mg/g beads = 30 mg/ml gel
- IgG: 88 mg/g beads = 11 mg/ml gel
- Bovine Serum Albumin (BSA): 120 mg/g beads = 15 mg/ml gel

B. Specifications

- Average Particle Size 50-80 microns
- Functionality: >250 µmoles/g = >31.25 µmoles/ml gel = >0.25 mEq/g = >0.03 mEq/ml gel
- Molecular Exclusion Limit: >2,000,000 daltons
- Average Surface Area : >250 m^2/g
- Average Pore Volume: >1.2 cc/g (60% of bead volume)

- Pore Size: 1,000 Å
- Swell Volume: 8-10 ml gel/g beads*
- Maximum Pressure: 100 psi (6.9 bar)
- Maximum Linear Velocity: 3,000 cm/hour
- Endotoxin Level (by LAL): <0.62 EU/ml gel

*Check label for specific lot swell volume value

Additional Materials Required

- Amine-containing ligand to be coupled in appropriate buffer (see Coupling Buffer Information, Appendix G)
- Vortex
- Rocking platform or rotating device
- Quench solution (3.0 M ethanolamine, pH 9.0)
- Centrifuge, filtration device with < 25 micron filters or Handee™ Spin Cup Columns (Product No. 69700) for small volume samples
- Wash solutions, such as phosphate buffered saline (PBS) [BupH™ Phosphate Buffered Saline Packs, 0.1 M phophate, 0.15 M NaCl; pH 7.2, Product No. 28372] and 1.0 M sodium chloride (NaCl).

General Coupling Instructions

Note: For best results, please read this entire instruction booklet prior to using this product.

Note: The entire coupling reaction, including all incubation times, can be completed within 6 hours.

1. Weigh out sufficient beads for the column. (Refer to the individual lot swell volume value to determine the quantity of beads needed for the column.) Coupling can be accomplished in a centrifuge tube or beaker.
2. Add protein solution directly to the dry beads. There is no need to swell the beads prior to use; the coupling buffer in the sample will swell the beads.

   Note: For most ligands, the addition of a chaotropic salt can greatly increase the coupling efficiency (See Figure 7, Coupling Buffer Information, Appendix G).
3. Vortex sample at medium speed for a few seconds to suspend beads in the coupling solution. Gently rock or rotate for 1 hour at RT. Coupling can also be done at 4°C or 37°C if ligand is susceptible at RT.

   Note: DO NOT USE MAGNETIC STIRBARS. They may damage the beads.
4. Centrifuge sample at 1,200 x *g* at RT for 5-10 minutes or until beads are pelleted. Alternatively, filtration using a 25 micron frit or, for small volumes, Handee™ Spin Cup Columns may be used to separate the protein solution from the beads.
5. Remove supernatant by aspiration or decanting. Use care to retain beads in tube. This supernatant may be used for determination of the amount of ligand not coupled to the beads.

   Note: Due to the use of Triton® X-100 surfactant in the production of the beads, there may be interference with an A_{280} measurement of the uncoupled protein. Therefore, the Pierce BCA Protein Assay (Product No. 23225) is recommended.
6. Add quench solution to the beads at 10 times bead volume to block unreacted azlactone sites. Refer to Quenching and Quench Solutions, Appendix F, for additional details.
7. Vortex and gently rock or rotate sample for 2.5 hours.
8. Centrifuge sample at 1,200 x *g* for 5-10 minutes or until beads are pelleted. Remove and discard supernatant after centrifugation.

9. Resuspend beads in 10 times the bead volume of PBS.
10. Vortex sample at medium speed for a few seconds to resuspend beads in the wash solution and gently rock or rotate sample for 15 minutes.
11. Centrifuge samples as described in Step #8.
12. Resuspend bead pellet in 1.0 M NaCl. The use of a high-salt wash solution, such as 1.0 M NaCl, will remove non-specifically attached protein.
13. Vortex and gently rock or rotate sample for 15 minutes as in Step #10.
14. Centrifuge samples as in Step #8.
15. Resuspend bead pellet in PBS for 15 minutes as in Step #9.
16. Vortex and gently rock or rotate sample for 15 minutes as in Step #10.
17. Centrifuge beads as in Step #8.
18. Repeat PBS wash as in Steps #15-17.
19. Resuspend beads in final buffer of choice.

Related Pierce Products

28372	**BupH™ Phosphate Buffered Saline**, 0.1 M phosphate, 1.5 M NaCl, pH 7.2
23225	**BCA Protein Assay***
69700	**Handee™ Spin Cup Columns**
21004	**ImmunoPure® IgG Elution Buffer,** 1 L
21009	**ImmunoPure® IgG Elution Buffer,** 3.75 L
29920	**Disposable Polystyrene Columns,** 0.5-2.0 ml, 100/pkg
29922	**Disposable Polypropylene Columns,** 1.0-5.0 ml, 100/pkg
29924	**Disposable Polypropylene Columns,** 2.0-10.0 ml, 100/pkg
29925	**Disposable Column Trial Pack,** 2 columns each plus accessories

Appendix

A. Pressure-Flow Relationship

UltraLink® Biosupport Medium is ideal for low- and medium- pressure chromatography. The following graph illustrates the relationship between the differential pressure on the beads and the linear flow velocity of fluid through the column.

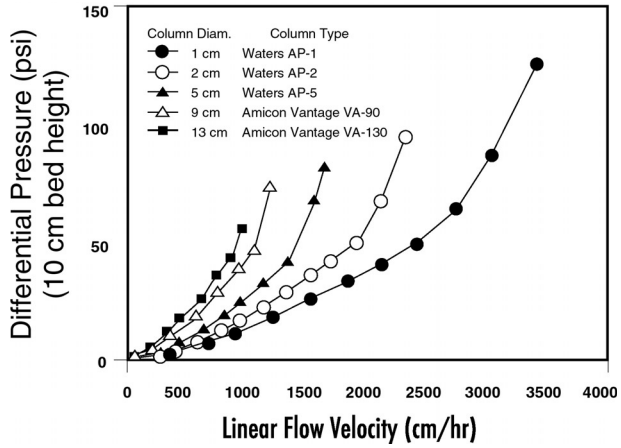

Figure 2. Pressure-flow relationship across UltraLink® Biosupport Medium.

B. Effect of Time and Temperature on Protein Coupling

Ligands couple rapidly and with high efficiency to UltraLink® Biosupport Medium at various temperatures including room temperature (RT), 4°C or 37°C. Recommended conditions for coupling are 1 hour at RT. These conditions may be adjusted as necessary for the specific ligand.

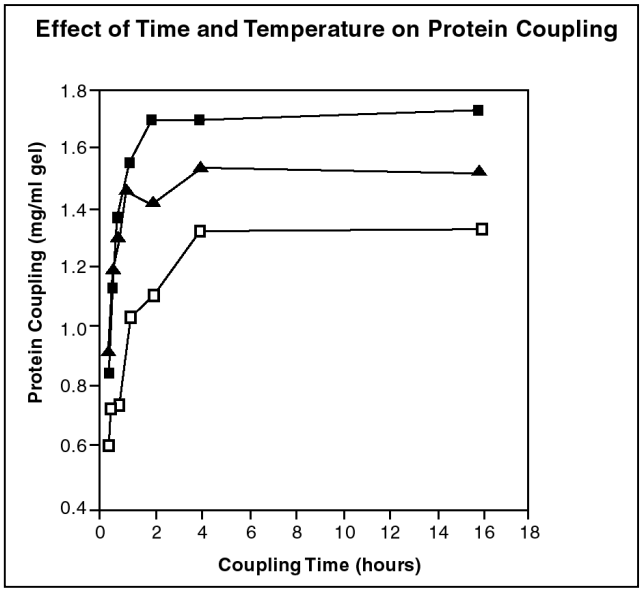

Figure 3. Effect of time and temperature on protein coupling to UltraLink® Biosupport Medium.

Experimental Conditions: Goat anti-rabbit antibody was coupled to UltraLink® beads using 10 mg beads + 1 mg protein/ml in 0.6 M sodium citrate, 0.1 M MOPS; pH 7.5 at 4°C (□), RT (■)and 37°C (▲) for varying periods of time. Coupled ligand was radiometrically determined.

C. Coupling of Proteins as a Function of pI and Molecular Weight

The UltraLink® support azlactone-functional beads have the capacity to couple proteins of various isoelectric points (pI) and molecular weights effectively and efficiently.

Table 1. Coupling of proteins as a function of pI and molecular weight.

Protein	Molecular Weight (kD)	Protein Offered (mg/ml)	% Coupling Efficiency	Capacity (mg/ml)
Pepsin, pI 2.9	34	1.60	46	8.6
Fetuin, pI 3.3	49	0.76	87	7.9
BSA, pI 4.9	66	1.82	68	14.3
Apoferritin, pI 5.0-5.5	440	1.47	43	7.4
Human IgG, pI 5.8-7.3	160	1.10	92	11.7
HRP, pI 6.1-7.2	40	1.73	35	6.8
Carbonic anhydrase, pI 6.2	29	1.67	75	14.9
Myoglobin, pI 6.8-7.8	17	1.81	48	9.9
Ribonuclease, pI 9.5	14	1.77	57	11.6
Lysozyme, pI 11.0	14	1.59	56	10.3

Experimental Conditions: The proteins were prepared in 0.8 M sodium citrate, 0.1 M bicarbonate; pH 8.6 as the coupling buffer, (due to solubility, the human IgG was prepared in 0.5 M sodium citrate). The General Coupling Instructions were followed with a 1 hour coupling reaction at RT using approximately 10.5 mg beads per sample. The amount of protein covalently coupled to the UltraLink® beads was determined by difference using the Pierce BCA Protein Assay Reagent (Product No. 23225).

D. Effect of Protein Concentration

Due to the high level of azlactone functionality, proteins couple with high coupling capacity and efficiency (80%) to UltraLink® Biosupport Medium over a wide range of concentrations. With IgG, maximal coupling capacity had not been reached at 11 mg IgG bound per ml gel or 88 mg IgG bound per gram of dry beads.

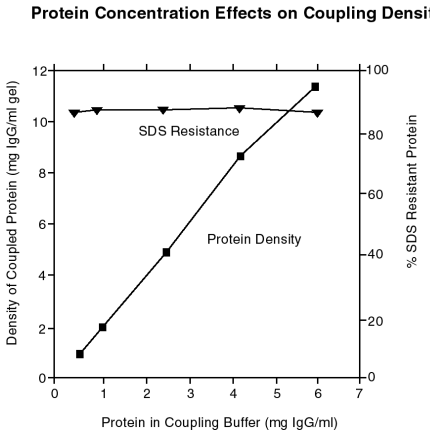

Figure 4. Effect of protein concentration on coupling to UltraLink® Biosupport Medium.

Experimental Conditions: Goat anti-rabbit IgG was prepared in 0.6 M sodium citrate, 0.1 M MOPS; pH 7.5 as the coupling buffer at various protein concentrations. The protein solution was added to the dry UltraLink® beads for coupling following the General Coupling Instructions with a 1 hour coupling reaction at RT, 2.5 hours quenching with 3.0 M ethanolamine, pH 9.0 and washing as per the recommended coupling procedure. To determine the amount of protein covalently coupled to the beads, protein-coupled beads were exposed to 1% sodium dodecyl sulfate (SDS) for 4 hours at 37°C. After incubation, beads were washed three times with 1% SDS for 15 minutes each wash. The amount of SDS resistant or covalently coupled protein was radiometrically determined.

E. Effect of Volume of Coupling Protein Solution

Even though the coupling buffer swells the beads, protein coupling can be accomplished efficiently in concentrated or dilute protein solutions and in varying volumes of coupling buffer. Use of very low or dilute protein solutions, i.e. 0.1 mg/ml may result in a slight decrease in coupling efficiency with a 1 hour coupling time.

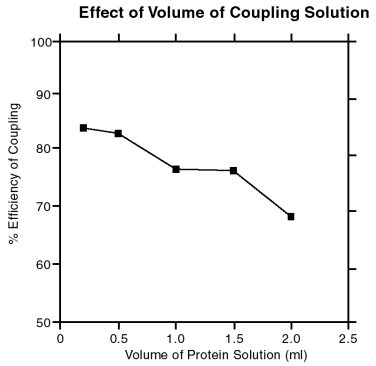

Figure 5. Effect of Volume of Coupling Solution on Efficiency of Coupling to UltraLink® Biosupport Medium

Experimental Conditions: To 10 mg of UltraLink® beads, 200 µg protein (goat anti-rabbit IgG) was added in varying amounts of coupling buffer (0.6 M sodium citrate, 0.1 M MOPS; pH 7.5). Coupling conditions were as outlined in the General Coupling Instructions.

F. Quenching and Quench Solutions

Due to the high level of azlactone functionality in the beads, it is recommended to quench or inactivate excess azlactone groups after protein coupling. The beads can be quenched with 3.0 M ethanolamine, pH 9.0 at RT for a minimum of 2.5 hours. In many cases, the time of quenching can be extended with little, if any, effect on protein coupling or subsequent use for affinity chromatography separations.

Other quench solutions may be used including 1.0 M ethanolamine; pH 9.0, 0.1 M glucosamine in 1.0 M sodium sulfate; pH 8.5, primary amine buffers such as 1.0 M Tris; pH 8.0 and alkylamines; e.g.; butylamine or methylamine.

G. Coupling Buffer Information

Several factors can affect coupling of the ligand to a support, especially the composition of the coupling buffer. Because of the unique coupling reaction with the azlactone functionality of the UltraLink® Biosupport Medium, the coupling buffer solution can be selected and tailored so that it is the most conducive for the ligand and for its coupling to this support. The choice of coupling buffer, including salt concentration and pH, can be easily adjusted without compromising the coupling reaction. Below are a few indicators of the versatility in selection of coupling buffers.

Compatible Coupling Buffers

Due to the chemical nature of the azlactone coupling reaction, a wide range of buffers may be used to covalently couple the ligand to UltralLink® Biosupport Medium. Some of the buffers that have been used to couple proteins are listed below.

Table 2. Coupling buffers compatible with UltraLink® Biosupport Medium

MOPS	CAPS
TAPS	HEPES
TES	TAPSO
CHES	AMPSO
POPSO	MES
Sodium carbonate	Sodium pyrophosphate
Sodium acetate	Sodium bicarbonate
Sodium citrate	Sodium borate
Bicine	Tricine
Phosphate buffered saline	

Important Considerations for Coupling Buffers:

- Use of primary amine buffering systems, such as Tris, allows the primary amine of the buffer to compete with the protein for coupling to the azlactone functionality.
- Sodium azide, phenols, potassium thiocyanate, ß-mercaptoethanol or ammonium sulfate should not be present in the coupling buffer as these chemicals may react with the azlactone functionality of the UltraLink® beads.
- For most ligands, addition of a chaotropic salt will greatly increase the coupling efficiency (see Figure 7: Effects of Salts on Coupling). However, if your ligand is not compatible with high salt, the coupling can be done without the addition of extra salts.

Effect of Coupling Buffer pH on Ligand Binding

Proteins can be efficiently and effectively coupled to UltraLink® beads under a wide pH range. This allows the selection of a coupling buffer that is most compatible with the ligand. An example presented below demonstrates coupling of goat anti-rabbit IgG antibody to the azlactone-functional beads with coupling buffers at varying pH conditions. The most efficient coupling for this antibody was found to be at a pH of 7.5 or greater.

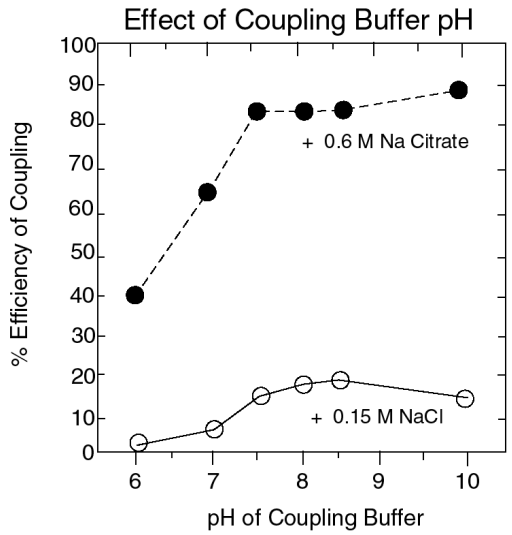

Figure 6. Effect of coupling buffer pH on binding of ligands to UltraLink® Biosupport Medium.

Experimental Conditions: The procedures outlined in the General Coupling Instructions were followed using goat anti-rabbit IgG at 1 mg/ml in coupling buffer and 10 mg UltraLink® beads. Coupling buffers with 0.6 M sodium citrate or 0.15 M NaCl include:

pH 6.0 = 0.3 M MES

pH 7.0 = 0.1 M phosphate

pH 7.5 = 0.1 M MOPS

pH 8.0 = 0.1 M TES

pH 8.5 = 0.2 M Tricine

pH 10.0 = 0.2 M carbonate

Effect of Salt Concentrations in the Coupling Buffer

The addition of salts to the coupling buffer may enhance the amount of ligand covalently attached to the Pierce UltraLink® Biosupport Medium, regardless of the pH of the coupling buffer. Addition of salt to the coupling buffer should be based on salt compatibility of the ligand. Goat anti-rabbit IgG was coupled to the UltraLink® beads in three different buffers. To each of these buffers was added three different salts. The efficiency of covalent coupling increased with each buffer as a function of increasing salt concentrations as illustrated in the following graph.

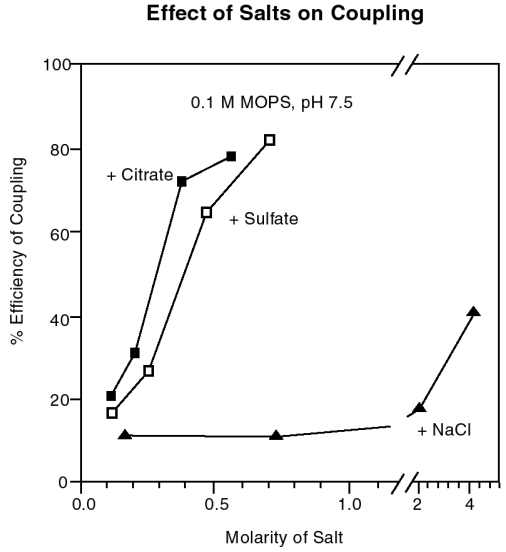

Figure 7. Effect of Salt Concentration in the Coupling Buffer for UltraLink® Biosupport Medium.

H. Reusability Information

UltraLink® Biosupport Medium is an excellent choice for affinity separations, especially when actual chromatography conditions are used and the immobilized ligand needs to be reused. To illustrate this point, rProtein A™ (recombinant Protein A) was coupled to UltraLink® beads and used to recover murine IgG from tissue culture fluid. The specific capacity eluted from this column after greater than 130 cycles remained at 99% of the original capacity.

Table 3. Elution capacity of UltraLink® Biosupport Medium after multiple uses.

Cycle Number	IgG Eluted Capacity (mg/ml)	Area of Eluted Peak ($x10^{-6}$)	% Initial Capacity
1	5.47	1.43	100
20	5.66	1.45	116
50	5.26	1.42	96
100	5.28	1.43	97
139	5.41	1.37	99

Experimental Conditions: A 1.0 x 1.3 cm column was used with 1 ml of rProtein A™ coupled UltraLink® gel. The purification cycle was defined as: Load with 25 ml tissue culture fluid; ATCC HB-124 DB9G8 (murine anti-insulin IgG2ak) cultured in RPM1-1640 + 10% fetal calf serum (Gibco) using an Endotronics Acusyst-P5 hollow fiber reactor. The tissue culture fluid was filtered through a 0.45 micron Millipore Pelicon 6 cassette prior to application to the column. Wash the column with PBS; pH 7.2. Elute with 0.1 M sodium citrate; pH 2.5. Clean with 3.0 M guanidine HCl in 20 mM phosphate;

pH 7.2. Equilibrate with PBS; pH 7.2. The load phase of the cycle was run at 1 ml/minute (60 column volumes [CV]/hour) and all other phases were run at 3 ml/minute (180 CV hour).

I. Resin/Column Maintenance Topics

Column Preparation Procedures

Columns can be prepared using either gravity or flow packing procedures. It is recommended that a 75% v/v slurry of beads be used to prepare columns fitted with a < 25 micron frit. When preparing a column for operation at higher flow rates (> 500 cm/hour), it is recommended that the gel be packed at a flow rate 25% higher than the operational flow rate for a minimum of 10 column volumes.

Storage Conditions after Ligand Coupling

Once the ligand has been coupled to the UltraLink® Biosupport Medium, storage of the ligand-coupled support at 4°C is recommended. If desired, a preservative solution may be included with storage at 4°C. Extended storage in the following solutions and subsequent binding in affinity separations has been documented.

Table 4. Effect of storage conditions on binding capacity of immobilized ligand on UltraLink® Biosupport Medium.

Storage Solution (in PBS)	Specific Capacity with Storage at 4°C (mg/ml eluted IgG) at 1 month	Specific Capacity with Storage at 4°C (mg/ml eluted IgG) at 3 months
20% Ethanol	18.5	21.6
0.05% Sodium Azide	20.0	19.1
0.01% Benzyl Alcohol	19.1	20.9
0.05% Thimerosal	19.9	20.2
0.01 N Sodium Hydroxide	19.8	14.6

Experimental Conditions: rProtein A™ was coupled to UltraLink® beads as per the General Coupling Instructions. Before storage, the specific capacity of the rProtein A™ coupled beads was determined using rabbit IgG and the beads allocated to the various storage conditions. 1 ml columns were poured and washed with 40 column volumes of PBS to remove the preservative solution before the 10 ml of 3 mg/ml rabbit IgG was loaded in 10 mM phosphate, pH 7.5. Specific capacities, using 0.1 M glycine, 2% acetic acid elution, pH 2.2, were determined by A_{280} measurements and chromatograms.

Note: It is not recommended that 2% chlorhexidine gluconate in 20% ethanol be used as a preservative as precipitation of this preservative may occur with storage at 4°C.

Cleaning Stability

UltraLink® Biosupport Medium coupled to protein ligands can be successfully exposed to a wide variety of cleaning solutions without loss of specific eluted capacity. A stable protein (rProtein A™) was coupled to the beads, columns prepared, loaded with human IgG and specific capacities determined as outlined in the preceding Experimental Conditions. Each column was exposed to the following cycling conditions for 5 cycles:

1. Equilibration with PBS, pH 7.5
2. IgG loading in PBS, pH 7.5 (10 ml at 3 mg/ml)
3. Elution with: 0.1 M glycine, 2% acetic acid, pH 2.2
4. Cleaning solution for 10 column volumes
5. Column equilibration with PBS, pH 7.5

Generally, high salt concentration and acidic and basic cleaning solutions have no effect on column capacities. Chaotropes can be successfully used at appropriate concentrations.

Table 5. Effect of cleaning solution on binding capacity of immobilized ligand on UltraLink Biosupport Medium.

Cleaning Solution	Specific Capacity (mg IgG/ml gel) Cycle 1	Specific Capacity (mg IgG/ml gel) Cycle 5
4.0 M NaCl in PBS	19.9	19.9
4.0 M NaCl in 0.1 N HCl	18.2	18.9
6.0 M Guanidine-HCl*	19.3	17.6
2.0 M Sodium Thiocyanate	18.2	17.9
8.0 M Urea	18.5	17.7
20% Ethanol in PBS**	19.2	17.5
0.1 N Sodium Hydroxide	17.8	18.4
1.0 N Ammonium Hydroxide	18.6	20.2
0.1 N o-Phosphoric Acid	17.6	19.1

* Refer to the Reusability section for additional information on the effect of column cleaning with guanidine-HCl.

** Refer to Storage section for additional information on the use and effect of 20% ethanol in PBS.

Product References

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General References

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US Patent No. 4,871,824 .

European Patent EP0 392,735 B1.