Activated Immunoaffinity Supports

Catalog Numbers

153-6046 **Affi-Gel® 10 Gel**

153-6052 **Affi-Gel 15 Gel**

153-6098 **Affi-Gel 10 and 15 Gel**

BIO-RAD

Table of Contents

Section 1
Introduction

Affi-Gel 10 and Affi-Gel 15 affinity supports are activated immunoaffinity supports that offer rapid, high efficiency coupling for all ligands with a primary amino group, including proteins throughout the entire range of pIs and low molecular weight compounds such as peptides.[1] Both Affi-Gel 10 and 15 supports are N-hydroxysuccinimide esters of a derivatized crosslinked agarose gel bead support, and both couple to ligands spontaneously in aqueous or non-aqueous solution.

The Affi-Gel 10 support, which contains a neutral 10-atom spacer arm, has been used to couple a variety of materials in affinity chromatography, immunoadsorption, and other techniques. The Affi-Gel 15 support contains a cationic charge in its 15-atom spacer arm which significantly enhances coupling efficiency for acidic proteins at

physiological pH. Both Affi-Gel 10 and Affi-Gel 15 supports offer the following advantages:

- Covalent amide bonds couple the protein to the terminal carboxyl of the spacer arm
- Highly stable in chaotropic agents and from pH 2-11
- Rapid, gentle coupling within 4 hours
- Easy to use
- High capacity of up to 35 mg protein per ml

Section 2
Coupling Chemistry

Ligands with free alkyl or aryl amino groups will couple spontaneously with Affi-Gel 10 or 15 supports in aqueous or non-aqueous solution (refer to Figure 1). Upon addition of ligand, the N-hydroxysuccinimide is displaced, and a stable amide bond is formed. Since the reactive ester immobilized on the gel is highly selective for primary amino groups, spurious side reactions with the ligand (i.e., cross-linking or other modifications in free solution) are eliminated. Free sulfhydryls are among functional groups other than primary amines known to compete for coupling.

Affi-Gel 10 and Affi-Gel 15 supports are well suited for coupling low molecular weight ligands. This can be done in aqueous solution or, when solubility of the ligand permits, in organic solvent.

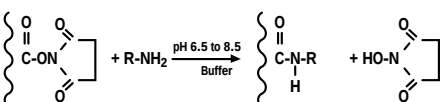
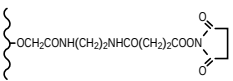
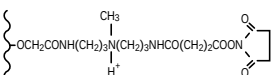


Fig. 1. Coupling reaction of Affi-Gel supports with ligand containing free amino groups.

Product Description for Affi-Gel 10 and 15 gels

Matrix	Bio-Gel A-5m agarose gel
Exclusion limit (M_r)	5,000,000
Bead size	75-300 µm (50-200 mesh)
Spacer arm	
Affi-Gel 10	
Affi-Gel 15	
Shipping medium	100% isopropanol
Capacity	
Chemical capacity	15 µmoles/ml of gel
Protein capacity	35 mg/ml
Stability of unreacted support	
Temperature	-70 to 0 °C
pH range	3-10
Organic solvents	stable in alcohols, DMSO, dioxane, formamide
Storage	-20 °C 1 year
	-70 °C 1.5 years

Section 3
General Coupling Conditions

3.1 pH Dependence

A major advantage of Affi-Gel 10 and 15 supports is the mild conditions which will permit coupling. This is particularly advantageous in applications which involve sensitive enzymes or other proteins that irreversibly lose biological activity when exposed to conditions outside their physiological range. Coupling can be achieved with Affi-Gel 10 and 15 supports between pH 3.0 to 10.0.

In order to maintain pH control, a minimum buffer strength of 10 millimolar is recommended. Suitable buffers include MES, MOPS, HEPES, POPSO, acetate, and bicarbonate. Do not use buffers such as Tris or glycine. They contain primary amino groups which will couple to the gel, as will any primary amine-containing compound which contaminates the ligand preparation.

The Affi-Gel 10 support couples proteins best at a pH near or below their isoelectric point, and the Affi-Gel 15 support couples proteins best near or above their isoelectric point. Therefore, when coupling at neutral pH (6.5-7.5), the Affi-Gel 10 support is recommended for proteins with isoelectric points of 6.5 to 11 (neutral or basic proteins), and the Affi-Gel 15 support is recommended for proteins with isoelectric points below 6.5 (acidic proteins). See Table 1.

The difference in coupling efficiency of the Affi-Gel 10 and Affi-Gel 15 supports for acidic and basic proteins can be attributed to interactions between the charge on the protein and charge on the gel. Hydrolysis of some of the active esters during aqueous coupling will impart a slight negative charge to the Affi-Gel 10 support. This negative charge will attract positively charged proteins (proteins buffered at a pH below their isoelectric point) and enhance their coupling efficiency. Conversely, the negative charge will repel negatively charged proteins (proteins buffered at a pH above their isoelectric point) and lower their coupling efficiency. The Affi-Gel 15 support, due to the tertiary amine incorporated into its arm, has a slight overall positive charge, and the effects are reversed.

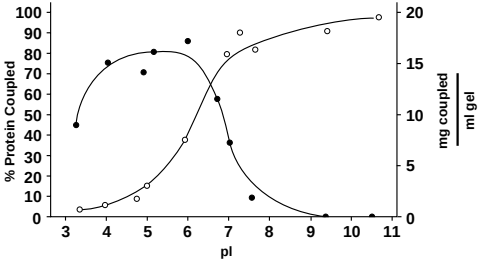


Fig. 2. Protein coupling with Affi-Gel and Affi-Gel 15 supports. Coupling conditions: Each protein solution (3 ml 0.1 M MOPS, pH 7.5, containing 40 mg protein) was combined with 2 ml of Affi-Gel media. The gel slurry was mixed at 4 °C for 2 hours, and then stripped with 7 M urea containing 1 M NaCl. The uncoupled protein was determined, using published $E^{1\,cm}_{280}$, by dilution of an aliquot of the urea effluent into 0.1 M HCl and measurement of the absorbance at 280 nm (●—● Affi-Gel 15 gel; ○—○ Affi-Gel 10 gel).

Table 1. Protein Coupling to Affi-Gel 10 and Affi-Gel 15 Support

Protein	pI	Coupling Efficiency (%) Affi-Gel 15 Gel	Affi-Gel 10 Gel
1. Fetuin	3.3	43	3.0
2. Alpha-1-antitrypsin	4.0	76	5.0
3. Ovalbumin	4.7	70	8.5
4. Bovine serum albumin	4.9	80	14
5. Human transferrin	5.9	87	36
6. Bovine hemoglobin	6.8	59	83
7. Human globulin	5.8-7.3	39	90
8. Myoglobin	6.8-7.8	10	85
9. Cytochrome c	9.3	0	90
10. Lysozyme	10-11	1	95

In addition to its effect on coupling, the slight charge associated with each gel may sometimes be exploited in the affinity separation itself, for example, it may be used to enhance binding of weakly sorbed material, or elution of strongly absorbed materials. In such cases, it may be preferable to use the Affi-Gel 10 support to couple an acidic protein, or the Affi-Gel 15 support to couple a basic protein. Coupling efficiency can then be enhanced by manipulating the coupling conditions in either of two ways. Select the coupling pH so that the protein has a charge opposite that of the gel, or add salt to the coupling buffer to minimize charge interaction (80 mM $CaCl_2$ may be useful for coupling acidic proteins to the Affi-Gel 10 support and 0.3 M NaCl may be useful when coupling basic proteins to the Affi-Gel 15 support).[2] Examples of these manipulations are shown in Table 2. The more basic or more acidic the protein the larger the observed effects will be.

Table 2. Coupling Efficiency of Acidic and Neutral-to-Slightly-Basic Protein Under Various Coupling Conditions

Coupling Buffer	Affi-Gel 10 Coupling Efficiency (%)	Affi-Gel 15 Coupling Efficiency (%)
Bovine Serum Albumin, pI 4.9		
0.1 M MOPS, pH 7.5	14	80
0.1 M MOPS, pH 7.5 + 80 mM CaCl	90	——
0.1 M MOPS, pH 7.5 + 0.3 M NaCl	22	47
0.1 M MES, pH 4.8	90	38
Human Globulin, pI 7.0 (average)		
0.1 M MOPS, pH 7.5	83	40
0.1 M MOPS, pH 7.5 + 0.3 M NaCl	69	70
0.1 M $NaHCO_3$, pH 8.5	80	70

3.2 Temperature

Coupling at 4 °C is recommended whenever possible. The slower reaction rate at this temperature will afford a greater measure of control, and many ligands will have a greater stability at 4 °C than at 20 °C.

3.3 Time

Coupling to the Affi-Gel 10 and 15 supports is rapid. As shown in Figure 3, for the Affi-Gel support, about 75% of the maximum binding achieved with gamma globulin at pH 7.5 takes place within 30 minutes at 4 °C. Ninety percent of the maximal coupling is achieved in an hour, and within 4 hours the reaction is complete.

3.4 Ligand Concentration

The amount of protein coupled is proportional to the amount of protein added to the gel up to about 30 mg protein coupled per ml of gel (Figure 4). The efficiency of coupling will vary with the protein and conditions of coupling (Figure 1). Greater than 30 mg protein/ml gel may be coupled, but efficiency will taper off. When maximum total capacity is desired, a high concentration of ligand should be chosen (50 to 60 mg protein per ml of gel). When maximum efficiency is the goal, as would be the case with ligand preparations of limited quantity, the ligand concentration should be in the range of up to 25 mg protein per ml of gel. Unbound sample may be recovered and reused without further treatment. Optimum coupling efficiency is achieved when the total reaction volume is between 1.5 and 4.5 ml per ml of gel bed.

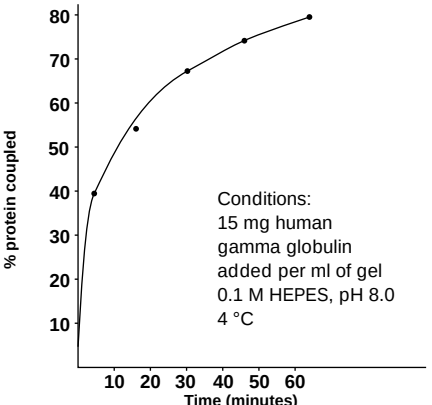


Fig. 3. Effect of time on protein coupling to Affi-Gel 10 gel. A similar relationship is observed with Affi-Gel 15 gel.

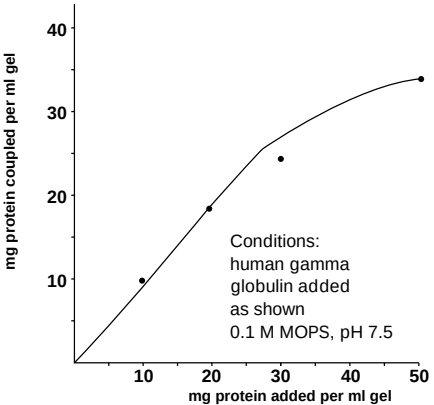


Fig. 4. Effect of amount of protein added on protein coupling to Affi-Gel 10 gel. A similar coupling capacity is observed with Affi-Gel 15 gel.

Section 4
Recommended Storage Conditions

If Affi-Gel 10 gel is stored at -20 °C it retains at least 80% of original activity for at least 1 year. Storing at -70 °C will extend the shelf life further.

Section 5
General Instructions

5.1 Aqueous Coupling

Material and equipment required for coupling ligands to Affi-Gel 10 and Affi-Gel 15 gel under aqueous conditions.

1. Cold distilled water.
2. Coupling buffer of your choice without primary amines or sulfhydryl groups; 10-100 mM HEPES, MOPS, POPSO, acetate, or bicarbonate for proteins.

3. Closed container which holds at least four times the volume of gel.
4. (Optional) rotating shaker.
5. (Optional) Buchner funnel.

Shake the vial, and make sure that you have a uniform suspension. Transfer the desired quantity of slurry to a Buchner funnel or glass fritted funnel. Drain the supernatant solvent, and wash the gel with three bed volumes of cold 10 mM sodium acetate, pH 4.5, or cold (4 °C) deionized water. The wash can be facilitated, particularly when working with larger amounts of gel, by applying a vacuum. Care should be taken, however, not to allow the gel bed to go dry. For optimum coupling of ligands, the washing procedure should be completed and the gel combined with the ligand solution within 20 minutes.

Transfer the moist gel cake to a test tube or flask and add the cold ligand solution. Add at least 0.5 ml of ligand

solution per ml of gel, and agitate sufficiently to make a uniform suspension. Continue gentle agitation of the gel slurry on a rocker, shaker, or wheel for 1 hour at room temperature or 4 hours at 4 °C.

If the coupling time is short, or if the gel is to be used immediately, we recommend a precautionary blocking of any active esters. This can be accomplished by adding 0.1 ml 1 M glycine ethyl ester (pH 8) or 0.1 ml 1 M ethanolamine HCl (pH 8) per ml gel. Allow 1 hour for completion of the blocking reaction. Transfer the gel to a column and wash with water or coupling buffer until the gel is free of reactants, as detected by $O.D._{280}$. Wash the gel with other solvents that will be used subsequently to elute substances specifically bound to the column. The column is now ready for equilibration with starting buffer and application of sample. When not in use, store the columns at 4 °C, and in a starting buffer containing 0.2% sodium azide.

5.2 Anhydrous Coupling

Coupling under anhydrous condition is the preferred method when the solubility of the ligand permits. It is ideal for peptides. Since there is no hydrolysis of active esters in the absence of water, the only reaction which will take place is the one between the ester and the ligand.

Material and equipment required for anhydrous coupling using Affi-Gel 10 and Affi-Gel 15 gel.

1. Cold (anhydrous) isopropanol.
2. Organic solvent of your choice; alcohol, dimethylsulfoxide (DMSO), dioxane, formamide free from free amines, or mixtures of these solvents.
3. Closed container which holds four times the volume of gel to be used.
4. (Optional) rotating shaker.
5. (Optional) Buchner funnel.

Shake the vial, and make sure you have a uniform suspension. Transfer the desired amount to a Buchner funnel, or glass fritted funnel. Drain the supernatant solvent, and wash the gel with at least five bed volumes of cold isopropanol.

Transfer the moist gel to a test tube or flask, and add the ligand solution. Add at least 0.5 ml of ligand solution per ml of gel, and agitate to make a uniform suspension.

To obtain a quantitatively substituted gel with low molecular weight ligands, it is necessary to add a slight excess of ligand. The gel contains approximately 15 µmoles of active ester per ml of gel. In the absence of hydrolysis, factors like time, concentration, and temperature, are less important. The reaction can be carried out at any convenient volume at room temperature for several hours. When using DMSO, conduct the reaction at 20 °C, to avoid the unfavorable viscosity 4 °C.

Any unreacted groups that remain can be blocked by addition of a slight excess of ethanolamine at the end of the reaction. The resulting support will have the lowest possible residual charge.

Summary of Coupling Conditions

Concentration of ligand	
Protein	25 mg/ml of gel
Low MW ligand	15-20 µmoles/ml of gel
Optimum pH	
Affi-Gel 10 gel	near or below pI of ligand
Affi-Gel 15 gel	near or above pI of ligand
Aqueous buffers	MES, MOPS, HEPES, POPSO, acetate, bicarbonate (avoid Tris, glycine)
Organic solvents	alcohols, DMSO, dioxane, acetone, formamide
Temperature	4 °C recommended
Reaction time	1 - 4 hours
pH range	3-10
Reaction volume	1.5 - 4.5 ml per ml of gel bed
Other compatible buffer components	reducing agents such as 10 mM DTT or nonionic detergents
Blocking reagent	ethanolamine
Suitable ligand	must have primary amino group

Section 6
Monitoring for Protein Coupling

Soluble (unbound) protein remaining in the coupling and wash buffers can be assayed by the Bio-Rad Protein Assay (catalog number 500-0006) or by measuring $O.D._{280}$. If measuring $O.D._{280}$ is preferred, the pH of the sample should be lowered with 10 mM HCl. N-hydroxysuccinimide released during the coupling will absorb at 280 nm at neutral or basic pH. N-hydroxysuccinimide will also interfere with the Lowry protein assay.

Section 7
Troubleshooting

Occasionally, the ligand will not bind to Affi-Gel 10 or 15 affinity support. If the ligand does not bind, or if you get a low capacity column, there are a number of possible reasons.

- Affi-Gel support is more than 12 months old. Try new material.
- The Affi-Gel support has been stored too warm.
- pH is not optimal. For Affi-Gel 10 gel, pH should be near or below the pI of your ligand. For Affi-Gel 15 gel, it should be near or above the pI. Buffer concentration should be at least 10 mM to maintain optimum pH. If pI is not known, try test coupling at a range of different pHs.
- A primary amino group, other than the ligand is present; avoid Tris or glycine buffers.
- Ligand is not pure. For polyclonal IgG samples, switch to Affi-Gel Hz support. For other samples, increase purity of ligand by chromatography or preparative electrophoretic methods.
- Aqueous coupling conditions provide less control than anhydrous conditions. Switch to anhydrous conditions if ligand solubility permits.

- Concentration of the ligand is too low. Protein concentrations of <25 mg/ml or small MW ligand concentrations of <15 mg/ml of gel will yield less than optimum results.
- Ligand is too diluted. Volume of ligand should not exceed 4.5 ml/ml of gel.
- Primary amino groups on ligand are sterically hindered; add nonionic detergent (up to 1%) or a chaotrophic agent such as 1 M guanidine HCl.
- Ligand has a molecular weight greater than 500,000. Affi-Gel 10 and 15 supports are not suitable for this application. Try the Affi-Prep 10 support, which is more porous.

Section 8
Using the Coupled Support

When the Affi-Gel 10 or 15 activated support has been coupled to the ligand, it is ready to use.

General operating conditions

Flow rate	15-25 cm/h
Pressure limit	15 psi
Minimum buffer concentration	50 mM
Stability	
Organic solvents	alcohols
pH	2-11
Temperature	autoclavable (ligand permitting)
Storage	1 year at 4 °C, add 0.02% NaN_3 or other preservative to application or starting buffer.

Section 9
Immunoaffinity Chromatography with Affinity Supports

9.1 Adsorption of the Sample

Preparing Antiserum for Antibody Purification

If an antibody from serum is to be affinity purified on an immobilized antigen support, partial purification of the antiserum is recommended. DEAE Affi-Gel blue gel and CM Affi-Gel blue gel will remove complement factors which bind immune complexes. They will also remove protease which can destroy valuable antibody during sample storage or decrease column life by destroying the immobilized protein. Antiserum should at least be heat inactivated at 56 °C for 30 minutes to destroy the complement factors.

Optimizing Support and Sample Volumes

Use only the required amount of affinity support. If excess support is used, sample elution becomes more difficult because the sample continues to bind and elute as it passes down the column. Stronger elution conditions become necessary, residence time is longer, the eluted peak is broader, and there is a greater risk of denaturation and poor recovery.

One method to insure that only the required amount of affinity gel is used is to apply the sample to the top of the column and elute using reverse flow (see Figure 5).

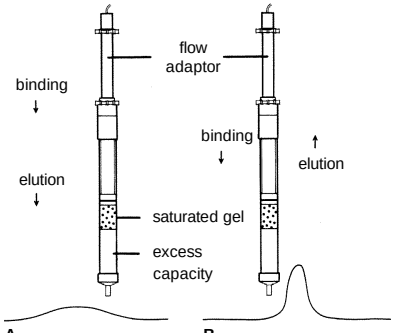


Fig. 5. Use only the required amount of affinity support. A) An excess of affinity support is used. During elution, sample is exposed to excess capacity resulting in great dilution and a broad peak. **B)** The sample is added to the top of the column, then eluted using reverse flow. Only the required capacity is used, resulting in minimal dilution and a sharper peak.

Another method is to titrate the gel with sample, checking the supernatant for unbound sample after each addition. This can be done either in a column or in a batch mode. Continue until the gel is saturated. This method can be used with a small amount of gel and sample to determine the capacity and the amount of gel required for the purification.

9.2 Removal of Unbound Solutes

Proteins or other solutes which are not bound, or are weakly bound by non-specific interactions, must be washed off prior to elution. This can be done by washing with mild chaotropic solutions (1 M NaSCN, 1 M guanidine hydrochloride, 1 M urea), with salts (1 M NaCl), or with detergents (0.5% Triton® X-100). In many cases, the elution buffer can be used, but at a lower concentration. This frequently neglected wash step eliminates proteins which may complicate final elution and helps yield a more highly purified product.

9.3 Elution Strategies

Elution is usually the most difficult step in immunoaffinity chromatography. The objective is to obtain high purity and high recovery of a stable and active product. Attempting to maximize yields, elution conditions which denature the proteins are often chosen.

Antigens and antibodies are bound to each other by a combination of ionic bonding, hydrogen bonding, and hydrophobic interactions.[2] The strength of different antigen-antibody complexes varies widely. Other parameters such as ligand density, steric orientation, and nonspecific interactions can also be important. Many solvents have been used as eluants in immunoaffinity chromatography, and the choice of an effective eluant often appears to be empirical. There is, however, a logical strategy, or sequence of eluants to consider when approaching a new immunoaffinity application.

1. **Specific Elution** with excess antigen or antibody should be considered first, because, in theory, it will always work. It is often impractical due to the cost and availability of the specific eluant. Another disadvantage is that an antigen-antibody complex will be eluted and the dissociation of this complex may be necessary and difficult to achieve.
2. **Acid Elution** is the most commonly employed desorption method and is frequently very effective. Eluants such as glycine-HCl, pH 2.5, 20 mM HCl, and sodium citrate, pH 2.5, can be used to disrupt the antigen-antibody interactions. Acid elution can give low recoveries due to hydrophobic interactions between the antigen and the antibody. An eluant such as 1 M propionic acid, or the addition of 10% dioxane[3] or ethylene glycol to the acid eluant, is more effective in dissociating such complexes.

3. **Base Elution** is less frequently used than acid elution, but, in some cases, it is more effective. Elution with 1 M NH_4OH or with 50 mM diethylamine, pH 11.5, has been shown to be effective with membrane glycoproteins and with certain antigens which precipitate in acid but are stable in base.[3] Organic solvents can also be added to basic eluants as described above with acid elution. An example in which an antibody to dinitrophenyl-bovine serum albumin (DNP-BSA) was coupled to an affinity matrix is described in Reference 5. DNP-BSA could not be eluted at all with acid or with acid plus organic solvents. Base elution gave 60% yield and base plus dioxane gave 95% yield of purified antigen.
4. **Chaotropic Agents** disrupt the tertiary structure of proteins and, therefore, can be used to dissociate antigen-antibody complexes. Chaotropic salts disrupt ionic interactions, hydrogen bonding, and sometimes hydrophobic interactions. Chaotropic anions are effective in the order $SCN^- > ClO^-_4 > I > Br^- > Cl^-$.[6,7] Chaotropic cations are effective in the order guanidine$> Mg^{2+} > K^+ > Na^+$.[7] Eluants such as 8 M urea, 6 M guanidine hydrochloride, and 6 M NaSCN are effective in disrupting most protein-protein interactions. The problem is that these strong chaotropes may destroy the activity of the antigen and/or the antibody. Conditions as mild as possible should always be used.

When the eluant has been chosen, the elution conditions should be refined by optimizing concentration, time, temperature, and by combining the eluants described above.

It is important to remove the eluted antigen or antibody from the eluant as quickly as possible to minimize the chance of denaturation. If acid or base is used, the

samples should be neutralized immediately following elution. If a chaotrope is used for elution, it can be rapidly removed by desalting (Econo-Pac® 10DG desalting columns, Bio-Gel® P-6 DG desalting gel, Econo-Pac P6 desalting cartridges, or for very small volumes Bio-Spin® columns).

9.4 Special Considerations for Labile Antigens

The stability of some antigens can be a problem. Special considerations may be necessary. The mildest elution conditions possible are desirable, with rapid elution and short exposure times being critical. For labile *immobilized* antigen relatively mild elution conditions can be used, and then a more complete regeneration with a chaotropic salt can be done after every fourth or fifth use of the column. This will increase the life of the column by minimizing exposure to stringent conditions, and strip the column of bound proteins to maintain the capacity.

Alternative procedures have been published for eluting labile antigens from immobilized antibody columns. Deionized water has been reported,[8,9] but yields are generally low. A method of increasing interest is electrophoretic elution.[1] An electrical field is applied and the adsorbed protein is electrophoresed away from the affinity matrix.

9.5 Renaturation of Eluted Proteins

Proteins which have been denatured during elution can often be renatured by the addition of a chaotropic agent such as guanidine-HCl, followed by stepwise dialysis against decreasing concentrations of the chaotrope. The high concentration of guanidine-HCl puts the protein into a random coil configuration. As the chaotrope is slowly removed, the protein will return to its native form.

If you have any questions or suggestions regarding the use of this or any of our other products, contact your local Bio-Rad representative. Inside the United States, call 1-(800)-4BIORAD.

Section 10
Ordering Information

Catalog Number	Product Description	Comments
153-6099	**Affi-Gel 10 Support,** 25 ml	
153-6051	**Affi-Gel 15 Support,** 25 ml	
153-6046	**Affi-Gel 10 Support,** 4 x 25 ml	
153-6052	**Affi-Gel 15 Support,** 4 x 25 ml	
153-6098	**Affi-Gel 10/15 Support,** 25 ml of each	
Purification of antibodies from serum		
153-7304	**CM Affi-Gel Blue Gel,** 100 ml	Cibacron Blue F3GA dye attached to CM Bio-Gel A agarose gel; for rapid purification of IgG from serum.
153-7307	**DEAE Affi-Gel Blue Gel,** 100 ml	Cibacron Blue F3GA dye attached to DEAE Bio-Gel A agarose gel; for rapid purification of IgG from serum.

Catalog Number	Product Description	Comments
Desalting and sample preparation		
150-0738	**Bio-Gel P-6DG Desalting Gel,** 100 g	Rapid protein and peptide desalting
150-0739	**Bio-Gel P-6DG Desalting Gel,** 1 kg	
732-2010	**Econo-Pac 10DG Desalting Columns,** 30 columns of 10 ml	Prepacked Bio-Gel P-6DG; for desalting up to 3.3 ml samples
732-0011	**Econo-Pac P6 Cartridge,** 5 ml	For desalting of up to 2 ml samples
732-6002	**Bio-Spin 6 Chromatography Columns,** 25	For desalting of small sample volumes (<0.1 ml) with minimal dilution). Exclusion limit 6,000.
732-6006	**Bio-Spin 30 Chromatography Columns,** 25	Same as Bio-Spin 6, but with an exclusion limit of 40,000.

Section 11
References

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