Affi-Gel® Hz Immunoaffinity Kit

Instruction Manual

Catalog Number 153-6060

For Technical Service
Call Your Local Bio-Rad Office or
in the U.S. Call **1-800-4BIORAD**
(1-800-424-6723)

BIO-RAD

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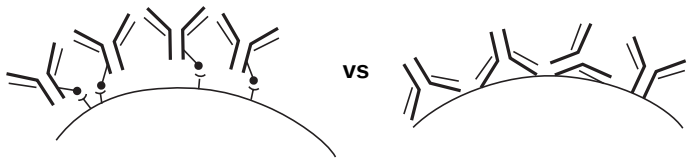
Section 1
Introduction

The Affi-Gel Hz immunoaffinity kit is a unique approach to IgG coupling to an agarose support matrix for affinity purification. This kit achieves a more uniform orientation of coupled antibody than currently available activated supports which couple via primary amines. Affi-Gel Hz hydrazide gel is an agarose support which reacts with the aldehydes of oxidized carbohydrates to form stable, covalent hydrazone bonds. Immunoglobulin G is a glycoprotein which contains approximately 3% carbohydrate localized on the Fc region (heavy chain) of the antibody. Periodate oxidation of vicinal hydroxyls of the sugars of these carbohydrates forms aldehyde groups for specific coupling to Affi-Gel Hz gel. This coupling through the carbohydrate eliminates the loss of antibody activity experienced in primary amino coupling at or near the antigen binding site by allowing the correct orientation of the antibody.

1.1 Immunoaffinity Coupling and Usefulness of the Immobilized Antibody



Oriented coupling results in higher antibody activity for greater antigen binding capacity per coupled IgG. IgG immobilized on Affi-Gel HZ hydrazide gel through the carbohydrate of the Fc region.

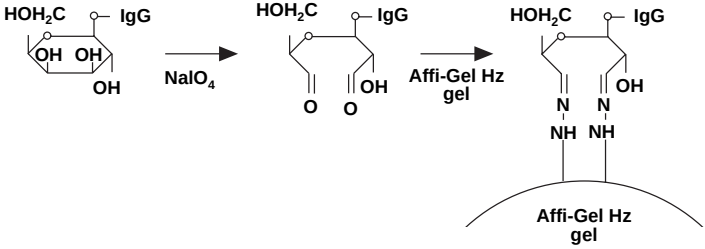
Random coupling yields low antigen binding due to attachment at or near the binding side of the antibody. IgG coupling directly to agarose support via primary amine.

Carbohydrate moieties on Fc region of IgG.

Hydrazide functional group on gel spacer arm.

Immunoglobulin G.

1.2 Hydrazide Coupling Chemistry

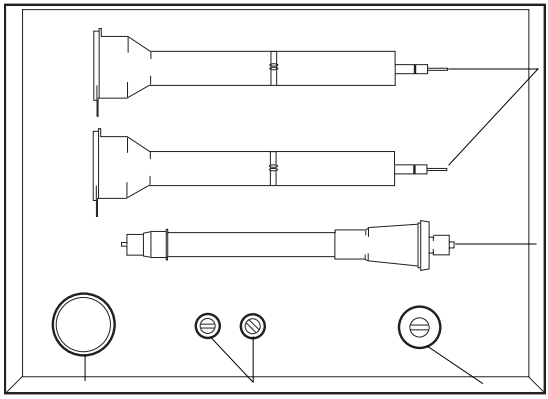


Sugar residue of carbohydrate on the Fc region of IgG.

Periodate oxidation of vicinal hydroxyls to form aldehydes.

Oxidized IgG specific coupling to Affi-Gel Hz hydrazide gel.

1.3 Diagram of Kit Components



Econo-Pac® 10DG Desalting Columns

1 x 10 cm Econo-Column® Chromatography Column

Affi-Gel Hz 10 x Coupling Buffer, 25 ml

Affi-Gel Hz Oxidizer, 25 mg $NaIO_4$ each

Affi-Gel Hz Hydrazide Gel, 5 ml

Materials and Equipment Not Included in this Kit

Essential

15 ml screw cap test tubes - polypropylene or polystyrene, leak-free

End-over-end rotating mixer

Transfer pipets

pH meter

UV-visible spectrophotometer

Optional

Bio-Rad Protein Assay Kit I (catalog number 500-0001)

Section 2
Monoclonal Antibodies

Due to the unique specificity of monoclonal antibodies, coupling to Affi-Gel Hz gel may not be optimal for all monoclonal antibodies. Loss of activity may occur depending on the individual monoclonal. To minimize loss of valuable antibody preparations, Bio-Rad highly recommends that a sample coupling experiment be performed to determine the efficiency of the coupling reaction. After the results of the sample coupling are determined, the remaining antibody can be coupled to the Affi-Gel Hz gel. If results are less than adequate with the Affi-Gel Hz gel, use Affi-Gel 10 gel to immobilize the monoclonal antibody.

Section 3
Antibody Purification

Prior to IgG coupling to Affi-Gel Hz hydrazide gel, the antibody must be at least partially purified. A high degree of purity will insure that higher IgG concentrations are available for oxidation and immobilization to the affinity support. This increased purity of IgG will maximize coupling capacity and purification potential. There are many approaches to antibody purification, and only a few will be presented here.

For use with this kit, the purified antibody should be in a total protein concentration of 1-5 mg/ml in a total maximum volume of 5 ml prior to buffer exchange. This recommended volume is necessary for rapid desalting and buffer exchange with the Econo-Pac® 10DG columns. Regardless of the purification method(s) chosen, it will be necessary to buffer exchange the purified antibody into the Affi-Gel Hz coupling buffer prior to oxidation and coupling following the procedure in this manual. See Buffer Exchange, Section 4.1.

IgG from serum or ascites can be purified by using a combination of ion exchange with the Macro-Prep® high S support and hydrophobic interaction chromatography with the Macro-Prep t-butyl support (or a combination of affinity chromatography with CM Affi-Gel blue gel and hydroxyapatite chromatography with Macro-Prep CHT, type I support). DEAE Affi-Gel blue chro-

matography may also be used to remove most serum components except transferrin.

IgG in ascites fluid and serum may be purified by Affi-Gel protein A agarose (MAPS® II kit) low pressure chromatography, or by application to Affi-Prep® protein A medium to high pressure polymeric matrix. Protein A, a surface protein from *Staphylococcus aureus*, binds the Fc region of many mammalian IgG species. Protein A purification will yield highly purified IgG for coupling to Affi-Gel Hz gel.

Hybridoma tissue culture supernatant containing dilute antibody (µg/ml concentrations) will necessitate a scheme to concentrate and purify the immunoglobulin. Ammonium sulfate precipitation followed by dialysis and ion exchange chromatography, Bio-Gel® HT hydroxyapatite, or Affi-Gel protein A gel are options that will yield highly purified, concentrated antibody.

Some immunoglobulins are sensitive to high ammonium sulfate concentrations or low pH and, as a result, total antibody activity can be irreversibly reduced prior to coupling to Affi-Gel Hz gel. Care must be taken to minimize the duration of antibody exposure to these conditions to retain activity essential to the applications of the immunoaffinity matrix.

Section 4
Immobilization Protocol

4.1 Buffer Exchange

Prior to coupling to Affi-Gel Hz gel, it is necessary to exchange the buffer in which the purified antibody appears. The Affi-Gel Hz coupling buffer is optimized for antibody oxidation and immobilization to Affi-Gel Hz hydrazide gel. This buffer exchange can be easily performed using the Econo-Pac 10DG desalting columns provided.

Antibody immobilization to Affi-Gel Hz gel consists of buffer exchange prior to the oxidation of the carbohydrate moieties on the Fc region, desalting, and coupling. This oxidation forms aldehydes for binding to the hydrazide functional group on the gel. Since the carbohydrate constitutes a small percentage of the total glycoprotein weight, the coupling reaction will progress slower than activated, self-coupling gels. The bond formed is a stable hydrazone linkage that is chemically resistant to many common elution conditions employed

in affinity chromatography.

4.1A Dilution of Affi-Gel Hz 10x Coupling Buffer

1. Dilute Affi-Gel Hz 10x coupling buffer 1:10 with distilled, deionized water and mix well.

2. Check the pH of the diluted coupling buffer with a pH meter. The pH of the diluted buffer should be 5.5. If it is necessary to correct the pH of the diluted Affi-Gel Hz coupling buffer, use 1.0 M acetic acid or 1.0 M NaOH to bring the pH to 5.5.

   Sodium azide, at a concentration of 0.02%, can be added to the diluted coupling buffer for long-term storage. However, it is best to dilute only the amount of 10x concentrate required.

4.1B Buffer Exchange with Econo-Pac 10DG Desalting Columns

Buffer exchange with Econo-Pac 10DG columns gives a minimum dilution of the purified antibody. Sample volumes may vary, but only 3.0 ml should be run at a time. These columns can be regenerated and reused if adequately washed after protein collection.

1. Remove the upper cap from the Econo-Pac 10DG column and pour off the excess buffer above the top frit.

2. Add 20 ml of the diluted Hz coupling buffer, pH 5.5 (fill to the 30 ml mark), and snap off the bottom tip to start the column flowing.

3. Allow the buffer to drain to the top frit. The column will not run dry. Flow will stop when the buffer level reaches the top frit.

4. Add up to 3.0 ml of purified IgG sample to the Econo-Pac 10DG column. Allow the sample to run completely into the column. If applying a sample of less than 3.0 ml, add the difference in diluted coupling buffer to the column, allowing it to run completely into the column (3.0 ml sample volume). Discard the first 3.0 ml eluted from the column.

5. Add pH 5.5 coupling buffer (120% of the starting sample volume) to the top of the Econo-Pac 10DG column and collect 0.5 ml fractions. Monitor the absorbance of the fractions at 280 nm. Pool the fractions containing the purified antibody in Affi-Gel Hz coupling buffer.

6. The Econo-Pac 10DG column should be washed with at least 20 ml of diluted coupling buffer, pH 5.5, to regenerate for desalting use after oxidation.

7. Place the yellow end cap over the column tip snugly to prevent leakage. Add 5 ml of diluted coupling buffer with 0.02% sodium azide to the column and replace the top cap for column storage.

8. The antibody is now ready for sodium periodate oxidation.

4.2 Oxidation of IgG

The oxidation of purified IgG will require the use of sodium m-periodate ($NaIO_4$).

Warning: Sodium periodate is a powerful oxidant. Avoid contact and inhalation. May be harmful if swallowed. May react violently with reducing agents, hydrides, and finely powdered metals. Use only as directed in this manual. Wear gloves and eye protection.

Affi-Gel Hz oxidizer is used as a stock solution and added to IgG as required. Since sample volumes will vary, it is necessary that the ratio of stock solution to sample be given, rather than actual volumes. Sodium periodate is light sensitive; therefore, oxidation must be performed in the dark. Maximum storage of the diluted sodium periodate stock solution is 1 week at 4 °C, in the dark glass vial provided. At the recommended sodium periodate concentrations, oxidation of carbohydrate moieties is specific and does not alter IgG activity.

1. Add 1.2 ml of distilled, deionized water to the sodium periodate vial (25 mg), replace stopper, and vortex until dissolved.

2. Place the purified IgG sample (in diluted coupling buffer) in a screw cap, polypropylene, or polystyrene tube. Make certain that the tube selected will not leak.

3. Add the sodium periodate stock solution at one-tenth the volume of purified IgG sample (*e.g.*, 400 µ1 of $NaIO_4$ stock solution to 4.0 ml of purified IgG). For rabbit IgG, add the sodium periodate at three-tenths the volume of purified IgG (*e.g.*, 1.2 ml $NaIO_4$ to 4.0 ml purified IgG).

4. Secure cap and cover the tube with foil.

5. Rotate the antibody/sodium periodate mixture end-over-end for l hour at room temperature.

6. Immediately proceed to desalting, Section 4.3.

4.3 Desalting Procedure

Immediately after the 1 hour oxidation, it is necessary to remove the sodium periodate from the IgG solution. Sodium periodate remaining in the IgG sample will adversely affect coupling efficiency. This desalting procedure is the same as that for buffer exchange (Section 4.1). It is important not to collect and pool fractions beyond the protein peak, since this will result in sodium periodate contamination of the oxidized IgG sample.

1. The Econo-Pac 10DG column(s) used in the buffer exchange should be washed and equilibrated in diluted coupling buffer, pH 5.5.

2. Follow the buffer exchange procedure in Section 4.1. Remember to limit the sample volume to 3.0 ml per column, per run. If a larger sample volume requires multiple desalting runs, wash each Econo-Pac 10DG column with at least 20 ml diluted coupling buffer after each desalting run.

3. Reserve a small aliquot to determine starting IgG concentration. This aliquot will be used to calculate IgG coupling efficiency. Measure the volume of oxidized IgG to be coupled.

4.4 Coupling of Oxidized IgG to Affi-Gel Hz Hydrazide Gel

4.4A Washing Affi-Gel Hz Hydrazide Gel

Affi-Gel Hz hydrazide gel is supplied in isopropanol.

Warning: Isopropanol is poisonous and flammable. Keep away from heat, sparks, and open flame. May cause eye burns and skin irritation. Avoid breathing vapor as it irritates eyes, nose, and throat. Wear gloves and eye protection.

Just prior to coupling, Affi-Gel Hz gel must be washed with diluted coupling buffer, pH 5.5, to remove isopropanol.

1. With a pipet, transfer the gel/isopropanol slurry to a clear 15 ml tube and allow the gel to settle.

2. Remove isopropanol supernatant, add 10 ml of diluted coupling buffer, pH 5.5, and mix well. Allow the gel to settle. Repeat.

3. Remove the supernatant above the gel. Add 5 ml of diluted coupling buffer, pH 5.5.

4. Transfer the gel buffer slurry to a coupling reaction tube. If less than 5 ml of gel is to be used for coupling, wash only the volume of gel to be coupled. Unused gel should remain in isopropanol and be stored at 4 °C.

4.4B IgG Coupling

As previously recommended, the IgG concentration should be between 1-5 mg/ml of gel. The total IgG sample volume limitation of 5 ml is suggested to facilitate buffer exchange and desalting in the Econo-Pac 10DG columns. Slightly larger sample volumes will exist at the time of coupling to Affi-Gel Hz gel.

1. Add oxidized, desalted IgG sample to gel in the reaction tube. Cap securely and rotate end-over-end for 10-24 hours at room temperature.

2. After coupling reaction is complete, pour gel/IgG slurry into the 1.0 x 10 cm Econo-Column® chromatography column provided. Collect the column eluant and measure the volume.

3. Wash the Affi-Gel Hz immunoaffinity column with 1 column volume of a suitable buffer containing 0.5 M NaCl (*e.g.*, PBS 0.5 M NaCl, pH 7.0). Collect the column eluant and save for efficiency determination.

4. Wash the column with an application buffer containing 0.02% sodium azide. If buffers other than PBS 0.5 M NaCl are to be used, equilibrate the column in 10 volumes of this buffer. Place yellow end cap securely onto bottom of the Econo-Column chromatography column. Replace top cap and store column with buffer above the gel bed at 4 °C until ready to use.

4.4C Calculation of IgG Coupling Efficiency

The efficiency of IgG coupling to Affi-Gel Hz gel can be calculated indirectly. Quantitation of the difference in IgG present before and after coupling will enable efficiency determination. IgG coupling can be calculated accurately for samples of high purity. For samples contaminated with other glycoproteins, the percentage of IgG coupled cannot be calculated by total protein determination.

For Sample of High Purity

Measure the absorbance at 280 nm in a quartz cuvette against an appropriate buffer blank. Dilute the IgG sample to obtain absorbance values between 0.1 and 1.0.

$$\frac{\text{Abs. @ 280 nm}}{1.4} = \text{(mg IgG/ml) x dilution factor x sample volume = total IgG}$$

[total protein before coupling] - [total uncoupled protein (eluant + 0.5 M NaCl wash)]

$$= \text{[total coupled protein]}$$

$$\frac{\text{(total coupled protein)}}{\text{(total protein before coupling)}} \text{ x 100 = \% protein coupled}$$

The starting and final IgG solutions and 0.5 M NaCl wash can be analyzed with the Bio-Rad Protein Assay to determine total protein as an alternative to the absorbance method or for IgG samples of less purity.

Section 5
Applications of Affi-Gel Hz Immobilized IgG

In this section, suggestions for sample application and elution are presented. Conditions listed are commonly employed for elution in affinity purification. These are by no means the only elution conditions which may be used. When choosing elution conditions for your application, refer to Section 5.3 and Section 6 on precautions and product specifications. Elution conditions should facilitate satisfactory purification without damaging the affinity column or product.

5.1 Conditioning the Immunoaffinity Column

It is necessary to condition the column prior to applying the sample mixture.

1. Remove column from 4 °C and allow it to reach room temperature. Add 2-4 bed volumes of the buffer chosen for antigen elution to the affinity column.
2. Regenerate the column with at least 5 bed volumes of application buffer (such as PBS, pH 7.0). The immunoaffinity column is now ready for sample application.

5.2 Sample Application

1. Sample is applied to the immobilized IgG column. Samples should be free of particulates. Complex samples should be diluted in application buffer and filtered if necessary. This will enhance specific binding to the immobilized IgG and prolong column life.
2. Wash column with 2 bed volumes of 0.5 M NaCl in application buffer to remove any unbound protein.
3. Wash column with 1-2 bed volumes of application buffer of lower NaCl concentration. The column is now ready for elution of bound antigen.

5.3 Elution Suggestions

The elution conditions necessary to break the antibody-antigen bond vary according to bond strength. Elution conditions listed are suggestions, and optimal conditions should be determined empirically. When choosing elution schemes for affinity purification, select conditions which give satisfactory purification without damaging the matrix or the product. Very harsh conditions may denature the antibody coupled to Affi-Gel Hz gel and affect column performance. Start with conservative rather than severe conditions and optimize elution with slight modifications from run to run. In general, elution should be carried out quickly. Request bulletin 1099 for further discussion of elution schemes.

1. Add 2 bed volumes of eluant to affinity column.
2. Collect fractions and/or monitor elution profile with UV-visible detector. The eluted antigen should be neutralized if eluted in low pH, or precipitation may occur.
3. Allow the elution buffer to reach the top of the gel bed. Quickly regenerate the column in application buffer containing 0.02% sodium azide, and store at 4 °C until next use.
4. Quantitate purification yield.

Acid Elution

0.2 M glycine-HCl, pH 2.5

0.1 M acetic acid

0.15 M sodium citrate, pH 3.0

0.5 M formic acid

Chaotropic Elution

4 M NaSCN

6 M urea

5 M guanidine-HC1

Elution Strategies

The preceding eluants generally do not denature antibodies.* Eluant must be compatible with the antigen. Several methods should be tried in the following order:

1. Acid (pH 2-3.5) is common. May cause inactivity of some proteins. Can reduce solubility of IgG.
2. Chaotropic salts (3.5 M NaSCN, 3-6 M GuHCl) are often effective. Usually used at neutral pH. SCN should not be used with low pH.

*Minimize the exposure to harsh conditions by neutralizing acid quickly and diluting chaotropes.

Section 6
Production Information

6.1 Affi-Gel Hz Hydrazide Gel Product Specifications

Operating temperature range	2-30 °C
Elution pH operating range	2-11
Particle size range	75-300 µm hydrated

6.2 Ordering Information

Catalog Number	Product Description
153-6060	**Affi-Gel Hz Immunoaffinity Kit**
153-6047	**Affi-Gel Hz Hydrazide Gel,** 25 ml
153-6054	**Affi-Gel Hz 10x Coupling Buffer,** 500 ml
153-6055	**Affi-Gel Hz Oxidizer ($NaIO_4$),** 250 mg
732-2010	**Econo-Pac 10DG Desalting Columns,** 30
153-7307	**DEAE Affi-Gel Blue Gel,** 100 ml
153-6159	**Affi-Gel Protein A MAPS II Kit**
156-0006	**Affi-Prep Protein A Support,** 5 ml
156-0005	**Affi-Prep Protein A Support,** 25 ml

Catalog Number	Product Description
130-0150	**Bio-Gel HT Hydroxyapatite,** 250 ml
156-0030	**Macro-Prep High S Support,** 100ml
156-0090	**Macro-Prep t-Butyl HIC Support,** 100ml
157-0040	**Macro-Prep Ceramic Hydroxyapatite,** type I, 100g
153-7304	**CM Affi-Gel Blue Gel,** 100ml

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