Affi-Gel® Hz Hydrazide Gel Scale-Up Instructions

Introduction

The Affi-Gel Hz hydrazide gel provides a unique method for coupling IgG to an agarose support matrix for affinity purification. This technique achieves a more uniform orientation of coupled antibody than is currently possible with activated supports which couple via primary amines. Affi-Gel Hz 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 hydroxyl groups of the carbohydrates forms aldehydes 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 optimal orientation of the antibody.

Prior to coupling to Affi-Gel Hz gel, the antibody should be at least partially purified. A high degree of purity will insure that higher antibody concentrations are available for oxidation and immobilization to the affinity support, thereby maximizing coupling capacity and purification potential. For information regarding antibody purification, request bulletins 1092 and 1115.

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, perform a sample coupling experiment to determine the efficiency of the coupling reaction. When 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.

Scale-up Procedure

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 diluted buffer should be at pH 5.5. If it is necessary to correct the pH of the diluted Affi-Gel coupling buffer, use acetic acid or NaOH to bring the pH to 5.5.

Sodium azide, at a concentration of 0.02% (w/v), can be added to the diluted coupling buffer for long term storage at 4 ˚C. However, diluting only the amount of 10x concentrate required is recommended.

Dialysis of Purified Antibody in Diluted Coupling Buffer

Dialyze the purified antibody in 1,000-fold excess diluted coupling buffer, pH 5.5, overnight at 4 ˚C. To achieve 1,000-fold dialysis economically, it will be necessary to change the dialysis buffer at least once.

Oxidation of IgG with Sodium Periodate

The oxidation of purified IgG will require the use of sodium 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 these instructions. Wear gloves and eye protection.

1. Make a sodium periodate stock solution by weighing out 25 mg/1.2 ml of distilled, deionized water. Mix the $NaIO_4$ stock solution well. Add 0.1 volume of stock solution to the purified antibody (*e.g.*, 2 ml stock solution to 20 ml antibody). Weigh out only as much sodium periodate as is needed. Store the $NaIO_4$ salt at 4 ˚C, in the dark.
2. Perform the IgG oxidation in a leak-proof polypropylene or polystyrene container covered with foil. Mix gently for 1 hour at room temperature.
3. Immediately after the 1 hour oxidation, add glycerol at a final concentration of 20 mM and allow to mix for 10 minutes.
4. Dialyze the oxidized IgG as outlined above. Sodium periodate remaining in the IgG sample will adversely affect coupling efficiency.

An alternative to dialysis at this point is ammonium sulfate precipitation and desalting on a Bio-Gel® P-6 DG column. This will permit rapid periodate removal, concentration of IgG, and use of a minimal volume of coupling buffer.

1. After the 1 hour oxidation of IgG with sodium periodate, add solid ammonium sulfate to the oxidized antibody to yield 80% saturation (422 g/L) slowly, over 20 minutes.
2. Centrifuge the precipitated antibody and remove supernatant. Resuspend the pellet in diluted coupling buffer, pH 5.5.
3. Pass the IgG over a desalting column. Collect and pool the protein fractions. The IgG sample should not exceed 30% of the gel bed volume. It is important not to pool fractions beyond the protein peak since this will result in sodium periodate contamination of the oxidized IgG sample.

Washing the Affi-Gel Hz Gel

Affi-Gel Hz gel is supplied in isopropanol.

Warning: Isopropanol is poisonous and flammable. Keep away from heat, sparks, and open flame. May cause eye 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. The optimal coupling efficiency is achieved when the total reaction volume is between 1.5 and 4.5 ml per ml of gel bed.

1. Transfer the gel/isopropanol slurry to a container. Let the gel settle. Remove the isopropanol supernatant. Add diluted coupling buffer, pH 5.5, at twice the gel volume. Mix well.
2. Allow the gel to settle. Remove supernatant. Repeat the gel wash.
3. Add an equal volume of the buffer to gel; mix. Transfer the slurry to a leak-proof reaction container. Allow the gel to settle. Remove the buffer above the settled gel.

Coupling of Oxidized IgG to Affi-Gel Hz Hydrazide Gel

For optimal coupling to Affi-Gel Hz gel the IgG concentration should be 1-10 mg/ml. IgG has been coupled to Affi-Gel Hz hydrazide gel at a concentration of 40 mg/ml, however, no binding capacity studies were performed at that level of coupled antibody.

1. Add oxidized, desalted IgG to the washed Affi-Gel Hz hydrazide gel.
2. Couple 10-24 hours at room temperature with adequate mixing. Do not use a magnetic stir bar.
3. After the coupling reaction is complete, pour gel/IgG slurry into a column, collect the eluant and measure the volume. Wash the gel with one column volume of suitable buffer containing 0.5 M NaCl (*e.g.*, 20 mM phosphate, 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 20 mM phosphate, 0.5 M NaCl, pH 7.0, are to be used, equilibrate the column in 10 volumes of this buffer. Store the column with buffer above the gel bed at 4 ˚C until ready for use.

Calculation of IgG Coupling Efficiency

The efficiency of IgG coupling to Affi-Gel Hz gel can be calculated indirectly. Quantitation of the difference in free IgG present before and after coupling will enable efficiency determination. IgG coupling can be calculated accurately by absorbance at 280 nm for samples of high purity. For samples contaminated with other glycoproteins, the percentage of IgG coupled cannot be calculated by total protein determination. The calculation of coupling efficiency for Affi-Gel Hz gel is a measure of IgG with optimal orientation for affinity purification. IgG not coupled to the gel retains biological activity and can be used for subsequent coupling or immunoassays.

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 0.1-1.0.

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

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

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

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 lesser purity.

Conditioning the Immunoaffinity Column

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

1. Remove the column from storage at 4 ˚C and allow it to reach room temperature. Add 2-4 bed volumes of the buffer chosen for anitgen elution to the affinity column. Request bulletin 1099 for a discussion of elution schemes.
2. Regenerate the column with at least 5 bed volumes of application buffer (*e.g.*, PBS, pH 7.0). The immunoaffinity column is now ready for sample application.
3. After column storage, always wash with several bed volumes of buffer prior to performing any binding application.

Affi-Gel Hz Hydrazide Gel Product Specifications

Operating temperature range	2-30 ˚C
Typical IgG coupling range	1-10 mg/ml gel
Elution pH operating range	2-10
Particle size range	75-300 µm hydrated

Ordering Information

Catalog Number	Product Description
Immunoaffinity Kit	
153-6047	**Affi-Gel Hz Hydrazide Gel**, 25 ml
153-6054	**Affi-Gel 10x Coupling Buffer**, 500 ml
153-6055	**Affi-Gel Oxidizer ($NaIO_4$)**, 250 mg
732-2010	**Econo-Pac® 10DG Desalting Columns**, 30
732-8102	**Luer-Lock 2-Way Stopcocks**, 10
150-0738	**Bio-Gel P-6 DG Desalting Gel**, 100 g
500-0001	**Bio-Rad Protein Assay Kit I**
Antibody Purification	
153-7307	**DEAE Affi-Gel Blue Gel,** 100 ml
153-6159	**Affi-Gel Protein A MAPS® II Kit**
156-0005	**Affi-Prep® Protein A Support,** 25 ml
130-0151	**Bio-Gel HT Hydroxyapatite,** 500 ml

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Bio-Rad Laboratories Main Office, *2000 Alfred Nobel Drive, Hercules, California 94547, Ph. (510) 741-1000, Fx. (510) 741-1060*
Eastern Regional Office, *85A Marcus Dr., Melville, New York 11747, Ph. (516) 756-2575, Fx. (516) 756-2594*
Also in: North Ryde, Australia*, Ph. 02-805-5000, Fx. 02-805-1920* ***Wien, Austria****, Ph. (1) 877 89 01, Fx. (1) 876 56 29* ***Nazareth, Belgium****, Ph. 09-385 55 11, Fx. 09-385 65 54*
Mississauga, Canada*, Ph. (905) 712-2771, Fx. (905) 712-2990* ***Beijing, China****, Ph. (01) 2046622, Fx. (01) 2051876* ***Copenhagen, Denmark****, Ph. 39 17 9947, Fx. 39 27 1698*
Espoo, Finland, *Ph. 90 804 2200, Fx. 90 804 1100* ***Ivry sur Seine Cedex, France****, Ph. (1) 49 60 68 34, Fx. (1) 46 71 24 67* ***München, Germany****, Ph. 089 31884-0, Fx. 089 31884-100*
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