Affi-Prep® Hz Hydrazide Support

Instruction Manual

Catalog Numbers
156-0015
156-0016
156-0017

BIO-RAD

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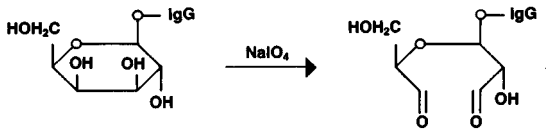
Section 1
Introduction

Affi-Prep Hz support consists of hydrazide groups covalently bound to a macroporous polymeric support via a 10-atom spacer arm. The hydrazide groups react with oxidized carbohydrates to form stable, covalent hydrazone bonds. Immunoglobulins and other glycoproteins can be coupled to this support through their carbohydrate moieties. Immunoglobulins are glycoproteins which contain carbohydrate moieties localized on the Fc region of the antibody. Periodate oxidation of vicinal hydroxyls of the sugars of these carbohydrates forms aldehyde groups for site-specific coupling to the Affi-Prep Hz support.[1] This site-specific coupling of antibody with the antigen binding sites oriented outward can result in higher antigen binding capacities than achievable using currently available activated supports which couple via primary amines.[2]

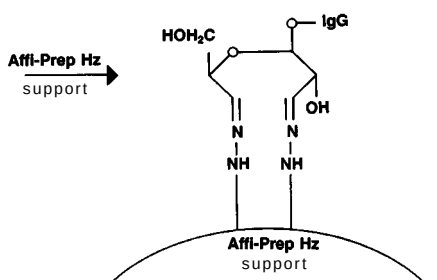
The Affi-Prep Hz support has the ability to couple more than 10 mg antibody per milliliter of gel, although coupling between the 1-5 mg antibody/ml gel is recommended for optimal column efficiency. A larger

concentration of antibody coupled to the gel does not necessarily translate into a proportionally higher antigen binding capacity.

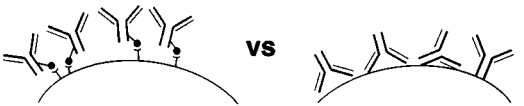


Sugar residue of carbohydrate on the Fc region of IgG.

Periodate oxidation of vicinal hydroxyls to form aldehydes.



Oxidized IgG specific coupling to Affi-Prep Hz hydrazide support.



IgG immobilized on Affi-Prep Hz support through the carbohydrate of the Fc region. Oriented coupling results in higher antibody activity with greater antigen binding capacity per coupled IgG.

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

Carbohydrate moieties on Fc region of IgG.

Hydrazide functional group on gel spacer arm.

Immunoglobulin G.

1.1 Materials Required for Use with the Affi-Prep Hz Support

- Chemicals: Sodium acetate, sodium chloride, sodium sulfate, sodium m-periodate, glycerol.
- Dialysis membrane, Econo-Pac® 10 empty columns (catalog number 732-1010), Bio-Gel® P-6DG desalting gel (catalog number 150-0738) for buffer exchange steps.

- UV/VIS spectrophotometer.
- pH meter.
- Rotating mixer.

Section 2
Preparation of Buffers

1. Recommended oxidation buffer: We recommend the use of 0.02 M sodium acetate, pH 5.0, containing 0.15 M sodium chloride.
2. Recommended coupling buffer: We recommend the use of either
   A. 0.1 M sodium acetate, pH 4.5, with 0.5 M sodium sulfate.
   B. 0.1 M sodium acetate, pH 4.5, with 1.0 M sodium chloride.

Note that buffer (A) is not soluble at 4 °C, and should be kept at room temperature. This limited solubility should be taken into account for the steps in the procedure where it may be preferred to work at 4 °C.

Do not store antibodies in either buffer.

Section 3
Antibody Sample Preparation

Prior to coupling to the Affi-Prep Hz support, the antibody must be at least partially purified. A high degree of purity will insure that higher concentrations of antibody are available for oxidation and immobilization. In addition, the higher the purity of the antibody, the greater the potential for maximum coupling efficiency. There are many approaches to antibody purification; only a few will be presented here.

Ascites and Serum Samples

IgG from serum or ascites fluid may be purified by DEAE Affi-Gel® blue chromatography or protein A chromatography with Affi-Gel or Affi-Prep protein A supports. DEAE Affi-Gel blue gel removes all serum components except IgG and transferrin. With DEAE Affi-Gel blue gel, the IgG fraction passes through in the void volume and the serum albumin and proteases are strongly bound. Protein A binds specifically to the Fc region of many mammalian IgG species.

Chromatography on Affi-Gel or Affi-Prep protein A supports will yield a highly purified antibody.

Tissue Culture Supernatant Samples

Hybridoma tissue culture supernatant containing dilute antibody (mg/ml concentrations) may require a combination of concentration and purification steps prior to oxidation. Ammonium sulfate precipitation followed by dialysis and Macro-Prep® ion exchange chromatography, Macro-Prep ceramic hydroxyapatite, or Affi-Gel or Affi-Prep protein A are options that will yield highly purified, concentrated antibody.[3,4]

For more information regarding antibody purification, request bulletins 1092, 1099, 1107, and 1424.

3.1 Buffer Exchange of the Antibody

Prior to oxidation of the IgG, it is necessary to buffer exchange the purified antibody into the oxidation buffer (see Section 2). It is important that the pH be between 4.5 and 5.5 for complete oxidation to occur.

Buffer exchange can be accomplished either by dialysis, by the use of the prepacked Econo-Pac 10DG desalting columns, or with Bio-Gel P-6DG desalting gel. If dialysis is chosen for buffer exchange, dialyze the purified antibody in 1,000-fold excess oxidation buffer, overnight at 4 °C. To achieve 1,000-fold dialysis economically, it will be necessary to change the dialysis buffer at least once.

Section 4
Immobilization Procedure

4.1 Oxidation of IgG

1. Oxidation of purified IgG requires the use of sodium periodate ($NaIO_4$). Make a sodium periodate stock solution (0.5 M) by weighing out 107 mg/1.0 ml of distilled, deionized water. Mix the $NaIO_4$ stock solution well. Weigh out only as much sodium periodate as needed. Store the $NaIO_4$ salt at 4 °C, in the dark.

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.

2. Add 1/50 volume of periodate stock solution to the purified antibody (e.g., 40 µl stock solution to 2 ml antibody).
3. Perform the IgG oxidation in a leak-proof glass tube or container covered with foil. Mix gently for 30-60 minutes at room temperature.
4. Immediately after the oxidation, add 1/20 volume of glycerol (e.g., at a final concentration of 100 mM or 100 µl to 2 ml) and mix for 10 minutes. The addition of glycerol stops the oxidation reaction.
5. Buffer exchange the oxidized IgG (see Section 3.1) into coupling buffer to remove sodium periodate and prepare the oxidized antibody for coupling. Sodium periodate remaining in the IgG sample could adversely affect coupling efficiency and should be removed.

Note 1: If coupling buffer A (containing sodium sulfate) is the coupling buffer of choice, it may be convenient to dialyze the oxidized antibody against oxidation buffer at this step to avoid solubility problems at 4 °C. The sodium sulfate concentration of the sample after dialysis can be adjusted by adding 1 M sodium sulfate to a final concentration of 0.5 M.

Note 2: The aldehydes which form during oxidation are fairly stable. The oxidized antibody can be left in the dialysis buffer for up to 36 hours or over the weekend.

Note 3: An alternative to dialysis at this point is ammonium sulfate precipitation and desalting on a Bio-Gel P-6DG column. This will permit rapid sodium periodate removal, concentration of IgG, and use a minimal volume of coupling buffer.

a. After the 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.
b. Centrifuge the precipitated antibody and remove the supernatant. Resuspend the pellet in coupling buffer, pH 4.5.

c. Pass the IgG over a desalting column. Collect and pool the protein fractions. The IgG will elute in the void volume of the column. 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.

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

4.2 Preparation of the Affi-Prep Hz Support

The Affi-Prep Hz support 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, the Affi-Prep Hz support must be exchanged into coupling buffer. This can be accomplished using a sintered glass funnel or centrifuge tube.

1. Transfer the desired amount of support from the bottle and allow to settle. Remove the isopropanol supernatant and wash with 10 volumes of distilled, deionized water. Repeat.
2. Wash support with 10 volumes coupling buffer, pH 4.5. Repeat.
3. Add an equal volume of the buffer to the support, mix, and transfer the slurry to a leak-proof reaction container. Allow support to settle. Remove the buffer above the settled support before coupling.

4.3 Coupling the Oxidized IgG to the Affi-Prep Hz Support

For optimal coupling to the Affi-Prep Hz support, the IgG concentration should be between 1-5 mg/ml and the volume of the antibody sample should be minimized (sample volume should be less than three times the volume of support to be used). IgG has been coupled to

Affi-Prep Hz support at concentrations greater than 10 mg/ml, however, no binding capacity studies were performed at this level of coupled antibody.[5] A larger quantity of antibody coupled to the support does not necessarily translate into a proportionally higher antigen binding ability.[6]

1. Add oxidized, buffer exchanged IgG to the washed Affi-Prep Hz support.
2. Couple 10-24 hours at room temperature or 4 °C with adequate, but gentle, mixing. **Do not use a magnetic stir bar.**
3. After the coupling reaction is complete, pour Affi-Prep Hz/IgG slurry into a column, collect the eluant, measure the volume, and assay for protein content (post coupling sol'n A in mg).
4. Wash the column with 3 column volumes of a buffer containing 0.5 M NaCl (e.g., 20 mM sodium phosphate, 0.5 M NaCl, pH 7.0). Collect the column eluant, save for efficiency determination, and assay for protein content (post coupling sol'n B in mg).
5. Store the column with buffer containing 0.02% sodium azide at 4 °C until ready for use.

4.4 Calculation of IgG Coupling Efficiency

The efficiency of IgG coupling to the Affi-Prep Hz support 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.

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.

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

$$(\text{Total coupled protein}) = (\text{Total protein before coupling in mg}) - (\text{post coupling sol'n A in mg}) - (\text{post coupling sol'n B in mg})$$

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

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 less purity.

The calculation of coupling efficiency for Affi-Prep Hz support is a measure of IgG with the optimal orientation for affinity purification. IgG not coupled to the gel may be used for subsequent coupling or immunoassays.

Section 5
Immunoaffinity Applications

5.1 Conditioning the Immunoaffinity Column

The immunoaffinity column must be conditioned before the sample mixture is applied.

1. Add 2-4 bed volumes of the buffer chosen for antigen elution to the affinity column (see Section 5.3).

2. Regenerate the column with at least 5 bed volumes of application buffer (e.g., PBS, etc.). The immunoaffinity column is now ready for sample application.

5.2 Anitgen 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 the 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 Anitgen Elution Suggestions

The elution conditions necessary to break the antibody-antigen bond will 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 the Affi-Prep Hz support, and affect column performance. Start with conservative rather than severe conditions and optimize elution with slight modifications from run to run. Request bulletin 1099 for a discussion of elution schemes.

The following elution strategies are commonly used:

1. Neutral pH elution: The use of 4 M $MgCl_2$ in 20 mM sodium phosphate is often useful for elution in the neutral pH range (e.g. pH 7.0).
2. Acid elution (pH 2-3.5) is common. This may cause inactivity of some proteins and can reduce solubility of IgG. Examples are 0.2 M glycine-HCl , pH 2.5, 0.1 M acetic acid, or 0.15 M sodium citrate, pH 3.0. If acid elution is used, do not allow the support to sit in the elution buffer. Immediately wash the support with a neutral buffer.
3. Chaotropic salts (3-5 M NaSCN, 3-6 M guanidine-HCl, 6 M urea) are often effective. These are usually used at neutral pH to low pH.

Section 6
Product Information

6.1 Product Performance

Matrix	Hydrophilic, macroporous (1,000 Å pore size) polymeric beads
Particle size range	Nominal 40-60 µm
Pressure limit	1,000 psi
Max. linear flow rate	2,000 cm/hr
IgG capacity	1-10 mg antibody/ml support
Elution pH operating range	pH 2-10
Shelf life uncoupled gel	1 year at 2-8 °C in isopropyl alcohol
Storage after coupling	PBS with 0.02% NaN_3, at 2-8 °C
Shipping buffer	Isopropyl alcohol

6.2 Ordering Information

Catalog Number	Product Description
156-0015	**Affi-Prep Hz Hydrazide Support,** 5 ml
156-0016	**Affi-Prep Hz Hydrazide Support,** 25 ml
156-0017	**Affi-Prep Hz Hydrazide Support,** 500 ml
153-6055	**Affi-Gel Hz Oxidizer,** 250 mg

6.3 References

1. O'Shannessey, D. J. and Quarles, R. H., *J. Immun. Methods*, **99**, 153-161 (1987).
2. Little, M., Siebert, C. and Matson, R., *BioChromatography*, **3**, 156-159 (1988).
3. Zola, H. and Neoh, S. H., *BioTechniques*, **7**, 806-809 (1989).
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5. Morehead, H.W., O'Shannessy, D. J. and Siebert, C. J., *J. Chromatog.*, **587**, 171-176 (1991).
6. Matson, R. S. and Little, M. C., *J. Chromatog.*, **458**, 67-77 (1988).