User Manual

# Thiophilic Resin User Manual

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# I. Introduction

Thiophilic Adsorption Chromatography (TAC) was developed in 1984 by Porath et al. TAC is a group-specific, salt-dependent purification technique with distinct adsorption affinity towards immunoglobulins and $\alpha_2$-macroglobulins. The term "thiophilic" refers to an affinity for sulfone groups that lie in close proximity to thioether groups (Porath et al., 1985). TAC has proven to be an easy and economical method for purification of many types of proteins, especially immunoglobulins.

With TAC purification methods, thiophilic proteins are absorbed to the resin in the presence of salt and then eluted by decreasing the salt concentration and thus the thiophilic interaction. One example of this method is given in Figure 1 which illustrates how step-wise reduction of the salt concentration was effective in separating IgG (peak II) from other proteins in whole rabbit serum.

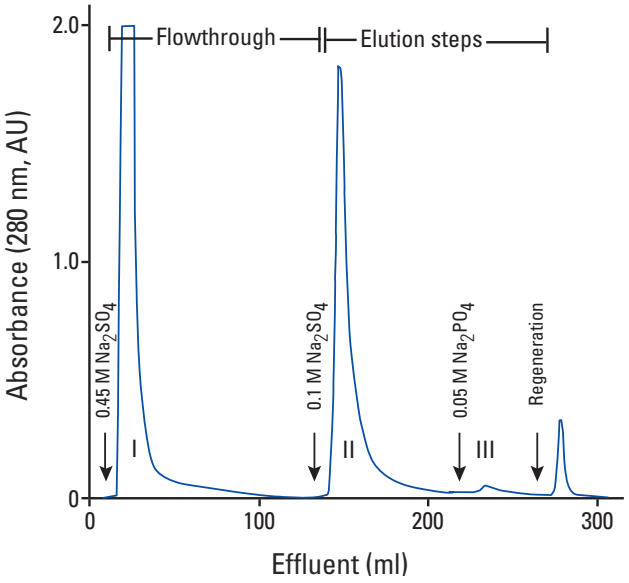


**Figure 1. Thiophilic-Uniflow Resin purifies IgG from whole rabbit serum.** Serum was saturated to 0.45 M $Na_2SO_4$ at pH 7.0, and was loaded on the equilibrated 5.0 X 1.6 cm column at 2 ml/min. Peak I is the unbound serum proteins that flow through the column. The IgG fraction elutes when the salt concentration is lowered to 0.1 M $Na_2SO_4$ (peak II). Only a small amount of protein eluted with the absence of sulfate salt (peak III) and with Regeneration Buffer 2.

# I. Introduction, continued

**TABLE I: PROPERTIES OF THIOPHILIC-UNIFLOW & -SUPERFLOW**

| Feature | Thiophilic-Uniflow | Thiophilic-Superflow |
|---|---|---|
| Batch/gravity | yes | yes |
| FPLC use | no | yes |
| Scale | Analytical | Analytical |
| Capacity (mg protein/ml adsorbent) | 20 | 25 |
| Matrix<br>linked agarose | | Cross-linked agarose Cross- |
| Maximum linear flow rate (cm/min) | 2 | 5 |
| Maximum volumetric flow rate (ml/min)<br>5 x 1 cm internal diameter column | 1.6 | 4 |
| pH stability | 2-10 | 2-10 |
| Supplied as | bulk/slurry<br>50% in 25% ethanol | bulk/slurry<br>50% in 25% ethanol |
| Storage | 4°C<br>Do not freeze | 4°C<br>Do not freeze |

**Thiophilic-Uniflow Resin** was prepared using Uniflow 4 agarose beads which permit linear flow rates as high as 2 cm/min. **Thiophilic-Superflow Resin** was prepared using Superflow 6 agarose cross-linked beads, which permit linear flow rates as high as 5 cm/min. In both cases, the agarose was activated with divinylsulfone and coupled to mercaptoethanol. See Figure 2 for a schematic depiction of the thiophilic resin ligand. Thiophilic-Uniflow and -Superflow can be regenerated and reused without detrimental effects on specificity and capacity.

Different salts can promote adsorption of target proteins. The most commonly used salts are potassium sulfate, sodium sulfate, and ammonium sulfate. In addition, salt concentration can differentially affect the adsorption kinetics of IgG, IgM, IgA, Fab and Fc fragments, and complement factors C3 and C4 (Lutomski et al., 1995; Oscarsson et al., 1992; Schulze et al., 1994; Yurov et al., 1994). The technique of using salt concentration to promote adsorption works with whole serum and ascite fluid (Belew, et al. 1987; Bog-Hansen, 1995; Juronen, et al., 1991; Yurov, et al., 1994), as well as other sources (Goubran-Botros, et al., 1998; Schulze, et al., 1994).

The use of TAC has advantages over other methods for purifying immunoglobulins. When compared to Protein A affinity chromatography, TAC is a more economical technique for purifying immunoglobulins from whole serum and tissue cultures (Porath & Belew, 1987; Scoble & Scopes, 1997). The main difference between these methods is that with TAC immunoglobulins are eluted at neutral pH, but with Protein-A elution requires a low pH that may affect the antibodies' structure. In comparison to Protein-A-based immunoadsorbents, thiophilic adsorbents have broader affinity towards

## I. Introduction, continued

immunoglobulins (Hutchens & Porath, 1986). For example, chicken antibodies harvested from yolk do not bind well to Protein A, but bind well to Thiophilic Resin (Hansen, et al., 1998). The salt-based purification is a straightforward and common technique used in protein purification. Furthermore, >99% of total proteins are recovered using a thiophilic adsorbent in comparison to less than 92% for phenyl and 75% for octyl agarose adsorbents (Oscarsson et al., 1995).

Thiophilic adsorbents can also purify other types of proteins such as horseradish peroxidase (Chaga et al., 1992), allergens (Goubran-Botros et al., 1998) glutathione peroxidase (Huang et al., 1994), procollagen (Pedersen & Bonde, 1994), acetolactate synthase (Poulsen & Stougaard, 1989), insect hemolymph proteins (Samaraweera et al., 1992), serpins (Rosenkrands et al., 1994), lactate dehydrogenase (Kminkova & Kucera, 1998), and tuberculosis antigen proteins (Rosenkrands et al.,1998).

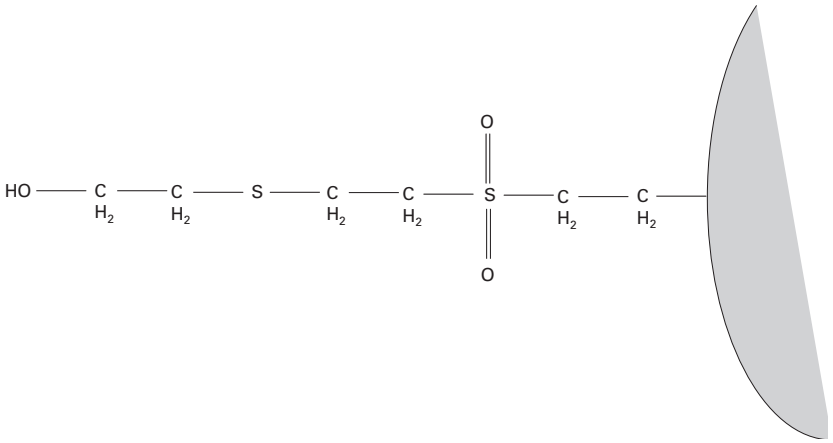


**Figure 2. Thiophilic Ligand.** Chemical structure of the thiophilic ligand is shown above. Agarose cross-linked beads (right) were activated with divinylsulfone and coupled to mercaptoethanol to produce this structure.

## II. List of Components

Store all resins at 4°C.

**Thiophilic-Uniflow Resin**

| Catalog No. | Size |
|---|---|
| 635614 | 100 ml |

**Thiophilic-Superflow Resin**

| Catalog No. | Size |
|---|---|
| 635616 | 10 ml |
| 635617 | 100 ml |

## III. Additional Materials Required

The following reagents are required, but not supplied.

- **Sample Buffer:**
  50 mM sodium phosphate; 0.55 M sodium sulfate (pH 7.0)
- **Equilibration Buffer:**
  50 mM sodium phosphate; 0.5 M sodium sulfate (pH 7.0)
- **Elution Buffer:**
  20 mM sodium phosphate, 20% glycerol, pH 7.0
- **Regeneration Buffers:**
  Buffer 1: Same as Elution Buffer (above)
  Buffer 2: 35 mM sodium phosphate; 30% n-propanol (pH 7.0)
- **Sodium sulfate**
- **TALON 2-ml Disposable Gravity Column** (Cat. No. 635606)
- **50-ml filtration bottle**
- **Deionized water**
- **25% Ethanol**
- **0.45-µm filter**
- **Low-Pressure Chromatography Column**
- **Peristaltic pump** (Must provide flow rates from 0.1–5 ml/min)
- **Fraction collector**
- **FPLC column** (for FPLC purification using Thiophilic-Superflow Resin)
- **UV spectrophotometer**
- **Chart recorder**

# IV. Purification of IgG from Whole Serum

**PLEASE READ THE ENTIRE PROTOCOL BEFORE BEGINNING**

Before starting, prepare buffers as specified in Additional Materials (Section III).

The following protocol is optimized for purifying IgG from whole serum or tissue culture supernatant using Low-Pressure or Batch/Gravity Flow Chromatography. For FPLC purification, see Section V. For purifying other immunoglobulins, use the following methods as a general starting point to determine optimal purification conditions. Section VI provides a general protocol for purifying other types of proteins.

## A. Sample Preparation

Below are two methods for preparing samples from whole serum or tissue culture supernatant. The **Dilution Method** is recommended for use with small sample volumes and 1-ml columns because sample dilution is not practical when scaling-up the procedures. The **Saturation Method** is recommended for use with larger sample volumes and columns ≥5 ml.

### 1. Dilution Method

a. Dilute 1 ml of whole serum or tissue culture supernatnant with 9 ml of Sample Buffer.

b. Filter sample through a 0.45-µm filter to avoid clogging and extend the life of the thiophilic resin.

c. Store the sample on ice to diminish degradation of the sample.

### 2. Saturation Method

a. Add 355 mg of anhydrous sodium sulfate to 5 ml of whole serum or tissue culture supernatant.

b. Dissolve the salt crystals by gently inverting the container.

c. To extend the life of the adsorbent, filter sample through a 0.45-µm filter before loading on the column.

d. Store on ice.

# IV. Purification of IgG from Whole Serum continued

## B. Low-Pressure Column Chromatography

The following protocol provides general guidelines for packing a 5-ml column. Once packed, a column can be reused several times when washed or regenerated between uses. **Use 1 ml of Thiophilic Resin per 1–3 ml of whole serum or tissue culture supernatant.**

Consult the manufacturer's instructions when assembling and packing a column for valuable clues concerning column operation, and pressures.

### 1. Column packing and equilibration

**Note:** Use deionized water to wet the bottom filter of the column. To decrease air bubble formation, leave ~0.5 cm of water inside the column before adding Thiophilic Resin.

a. Thoroughly resuspend Thiophilic Resin.
b. Transfer 10 ml of resin suspension to a 50-ml filtration bottle, and allow resin to settle.
c. Remove supernatant, and add 5 ml of deionized $H_2O$.
d. Thoroughly resuspend resin.
e. Degas resin by applying a vacuum to the filtration bottle.
f. Gently resuspend resin.
g. Immediately transfer 10 ml of resin suspension to a low-pressure chromatography column.
h. Fill column with deionized water.
i. Allow resin to settle.
j. To avoid trapping air between the top adaptor and the liquid surface, add adaptor at a 45° angle. Push adaptor gently down to the surface of the resin bed.
k. Run the pump at a flow rate of 0.5 ml/min to avoid trapping air inside the column pump and tubing during assembly. Fill peristaltic pump tube with Equilibration Buffer, and connect tube to column inlet.
l. Equilibrate column with ≥10 column volumes of Equilibration Buffer. Use flow rates ≤2 cm/min; therefore, for a column with an internal diameter of 1 cm, use a maximum flow rate of 1.56 ml/min.
m. Check pH (7.0) of the flow-through buffer to verify the column is equilibrated.

## IV. Purification of IgG from Whole Serum continued

**2. Chromatography**

a. Decrease flow rate to ~1 cm/min (e.g., 0.78 ml/min for a column with an internal diameter of 1 cm).

b. Load 1–3 ml of sample per 1 ml of Thiophilic Resin.

c. Extensively wash out nonadsorbed proteins with Equilibration Buffer. Collect fractions, and measure their absorbance at 280 nm. When the absorbance decreases to ~0.030 AU, switch to Elution Buffer, and collect fractions. In general, ≤10 column volumes of Elution Buffer are needed to efficiently elute IgG.

d. If residual salt affects the activity or the functional assay of the eluted protein, dialysis may be required. Concentrations of ammonium sulfate over 1.0 M will affect electrophoretic analyses.

### C. Batch/Gravity Flow Chromatography

**1. Column packing and equilibration**

The following protocol provides guidelines for packing a 2-ml gravity flow column.

a. Thoroughly resuspend Thiophilic Resin.

b. Ensure column is plugged with a stopper. Immediately transfer 2 ml of resin suspension to a TALON® 2-ml Disposable Gravity Column.

c. Allow resin to settle.

d. Wash column with five bed volumes of deionized water.

e. Equilibrate column by washing with ≥10 column volumes of Equilibration Buffer.

f. Check pH (7.0) of the flow-through buffer to verify that the column is equilibrated.

**2. Chromatography**

a. Load 1–3 ml of whole serum per 1 ml of Thiophilic Resin.

b. Extensively wash out nonadsorbed proteins with Equilibration Buffer. Collect fractions, and measure their absorbance at 280 nm. When the absorbance decreases to ~0.030 AU, switch to Elution Buffer, and collect fractions. In general, ≤10 column volumes of Elution Buffer are needed to efficiently elute IgG.

c. If residual salt affects the activity or the functional assay of the eluted protein, dialysis may be required.

## IV. Purification of IgG from Whole Serum continued

### D. Column Regeneration

1. Wash column with five column volumes of Regeneration Buffer 1.
2. Wash column with five column volumes of Regeneration Buffer 2.
3 Store column in 25% ethanol at room temperature for 1–2 weeks; alternatively, store column at 4°C for >2 weeks.

## V. FPLC Purification using Thiophilic-Superflow Resin

**PLEASE READ THE ENTIRE PROTOCOL BEFORE BEGINNING**

Before starting, prepare buffers as specified in Additional Materials (Section III). For low-pressure or batch/gravity-flow purification, see Section IV.

### A. Sample Preparation

Below are two methods for preparing starting samples. The **Dilution Method** is recommended for use with small sample volumes and 1-ml columns. The **Saturation Method** is recommended for use with larger sample volumes and columns ≥5 ml.

#### 1. Dilution Method

a. Dilute 1 ml of whole serum with 9 ml of Sample Buffer.
b. To extend the life of the resin and avoid clogging of the column, filter sample through a 0.45-µm filter before loading sample.
c. Store on ice to discourage degradation in the sample.

#### 2. Saturation Method

a. Add 355 mg of anhydrous sodium sulfate to 5 ml of whole serum.
b. Dissolve the salt crystals by gently inverting the container.
c. To extend the life of the adsorbent, filter sample through a 0.45-µm filter.
d. Store on ice.

### B. Preparation of Thiophilic-Superflow Resin for FPLC Purification

1. We recommend a column whose internal diameter is at least 1 cm. Columns similar to Pharmacia's HR 10/2 or HR 10/10 are convenient because a volumetric flow rate of 0.78 ml/min can be used during loading. We recommend a bed length of at least 3 cm.
2. Pack the column according to its manufacturer's specifications. We recommend a linear flow rate of at least 5 cm/min for packing. The linear flow rate is the volumetric flow rate, in ml/min, divided by the area of the cross-section of the column ($\pi r^2$ where r is the column radius in cm.)

## V. FPLC Purification continued

3. Due to the diffusion constraints of antibodies inside the pores of the resin, a relatively low flow rate must be used during loading. The flow rate for washing and eluting can be increased significantly, thus, reducing purification time and increasing yield. At a loading linear flow rate of 0.5 to 1 cm/min, the capacity for antibodies from whole serum or filtered raw ascite fluid is approximately 20–25 mg per ml of resin. Equilibration with Equilibration Buffer can be performed at the same flow rate.

### C. FPLC Purification

1. We recommend that you filter your sample through a 0.45-µm filter before FPLC purification. This action will extend the life of the column.
2. During the loading and washing steps, the linear flow rate should not exceed 1 cm/min; therefore, the flow rate should not exceed 0.8 ml/min if your column has an internal diameter of 1 cm. If antibody leakage occurs, the flow rate should be decreased. Once the sample is loaded and the absorbance of the nonadsorbed flowthrough material starts to decrease, you may increase the linear flow rate to 5 cm/min or to 4 ml/min for a column with 1-cm internal diameter. In general, the whole chromatographic purification should not take more than 60 min.
3. Elution can be performed at an elevated flow rate, unless the amount of eluted material is much less than the adsorbed material. Collect 1-ml fractions during chromatography, and store them on ice.
4. Use a bicinchoninic acid (BCA) protein assay (i.e., Lowry) as well as SDS gel-electrophoresis to identify fractions containing your eluted antibody.

### D. Column Regeneration

1. Wash column with five column volumes of Regeneration Buffer 1.
2. Wash column with five column volumes of Regeneration Buffer 2.
3. Store column in 25% ethanol at room temperature for 1–2 weeks; alternatively, store column at 4°C for >2 weeks.

## VI. General Protein Purification

Thiophilic Resin can purify a variety of proteins. The recommendations given below are very general; therefore, you should determine the optimal conditions for each protein.

- It is best to develop an assay or method of detection of a protein, to aid in determining which fractions contain the purified protein of interest.
- Use a portion of the total sample to determine the optimal binding conditions. Then purify the rest of the sample using those parameters.

**Miniscale trial procedure**

1. Obtain a 5 ml sample of cell extract containing a sufficient amount of target protein activity.
2. Divide extract into five aliquots.
3. Saturate each aliquot with increasing salt concentrations. For example, use 0.1, 0.2, 0.3, 0.4, and 0.5 M sodium sulfate.

   **Note:** The optimal salt concentration should have minimal affect on protein activity.
4. Centrifuge samples at 5,000 x g for 25 min to clarify sample.
5. Collect the clear supernatant.
6. Determine residual activity of the target protein. For the chromatography trial, use the supernatant with the highest salt concentration that contains ≥80% of the initial activity.
7. Do a chromatography trial. Test the binding affinity of the target protein of the sample to the resin using the protocol outlined in Section IV.C.

**Protein analysis**

8. Using your assay or detection method, analyze the sample flow-through to determine the distribution of the target protein.
9. **If the target protein adsorbs** to the Thiophilic Resin, optimize the washing and elution conditions.

   Determine the optimal elution conditions by doing a step gradient elution. Decrease the salt concentration of the buffer in steps, washing with at least two column volumes with each decrease in salt concentration. Analyze the target protein distribution in each elution step.

   **If the target protein has not adsorbed to the resin** under any of the starting salt concentrations, switch the salt to ammonium sulfate. With ammonium sulfate, you can adsorb at a higher sulfate salt concentrations (≤4.1 M). Repeat the steps outlined above.
10. If the target protein still does not adsorb, it is possible to obtain significant purification using "negative adsorption"; that is when the majority of unwanted proteins are adsorbed to the resin, allowing the protein of interest to pass through the column unadsorbed (Chaga et al., 1992).

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## Appendix: Reagent Compatibilities

**TABLE II : REAGENT COMPATIBILITY**

| Reagent | Acceptable Concentration |
|---|---|
| β-Mercaptoethanol | - |
| CHAPS | - |
| DTT, DTE | - |
| EDTA, EGTA | 10 mM |
| Ethanol | 30%, Regeneration Only |
| Ethylene glycol | - |
| HEPES | 50 mM |
| Glycerol* | 20%* |
| Guanidinium | - |
| Imidazole | 200 mM at pH 7.0–8.0 |
| KCl | 500 mM |
| MES | 20 mM |
| MOPS | 50 mM |
| NaCl | 1.0 M |
| NP-40 | - |
| Phosphate | 50 mM |
| SDS | - |
| Tris | 50 mM |
| Urea | - |

- = not compatible

* Glycerol is not compatible for sample loading.

# Notes

| Contact Us For Assistance | |
|---|---|
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| Fax: 800.424.1350 (toll-free) | Fax: 650.424.1064 |
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This document has been reviewed and approved by the Clontech Quality Assurance Department.