

Section 4C

Purification by Affinity Chromatography

Overview of technique

In immunoaffinity chromatography, an antibody against the epitope-tagged portion of the fusion protein is covalently attached to a support resin. Affinity chromatography (Figure 4C.1) allows purification of large amounts of fusion protein from cell lysates and supernatants (Templeton, 1992; Field *et al.*, 1988).

Affinity chromatography offers the following advantages over other chromatography methods:

- ▶ Saves time by providing a highly purified epitope-tagged protein in a single step
- ▶ Preserves protein activity by using gentle elution conditions
- ▶ Produces highly purified tagged protein

The advantages of the immunoaffinity method often offset the initial high cost and labor involved in making your own immunoaffinity resin.

Note: For detailed, step-by-step affinity chromatography procedures, see Hermanson, Mallia and Smith (1992).

Getting started: Procedures for affinity chromatography

We describe below general guidelines for making and using your own immunoaffinity resin, that is, for attaching any tag-specific antibody to a support resin and purifying the corresponding epitope-tagged protein (Figure 4C.2).

Caution: The guidelines given in these procedures are only a starting point for developing an affinity purification system and have not been optimized for a particular epitope tag or tag-specific antibody. Optimize these procedures experimentally to account for the variables introduced by different tagged proteins, antibodies, and chromatography methods.

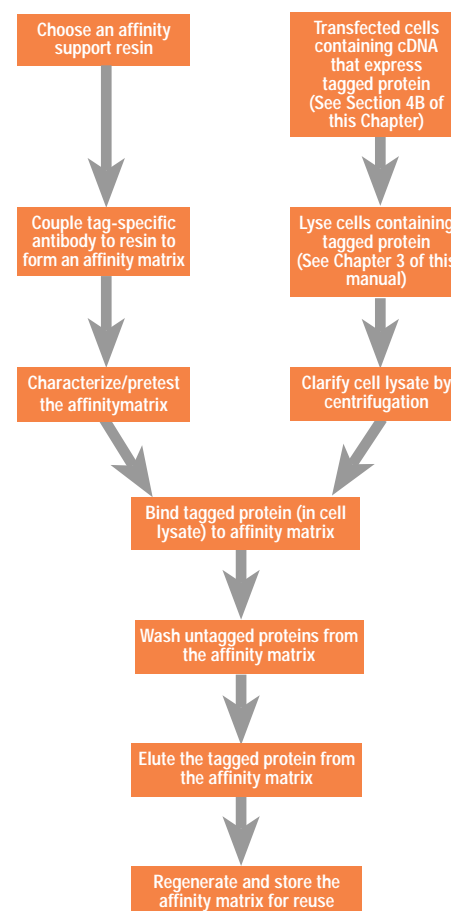


Figure 4C.2: Flow diagram for developing and using an affinity purification system for an epitope-tagged protein. Details are given in the text.

Note: The products printed in *colored type* are available from Boehringer Mannheim. For detailed ordering information on these and related products, see the Boehringer Mannheim Product Ordering Guide, Section 5B of this manual.

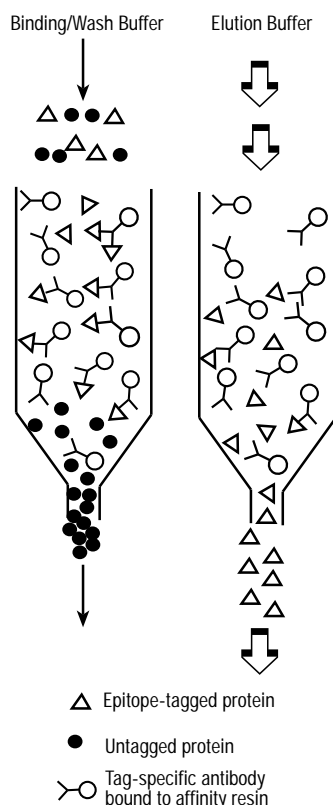


Figure 4C.1: How affinity chromatography works. A mixture of proteins in a Binding/Wash Buffer is passed over affinity resin beads that contain covalently bound tag-specific antibody. Untagged proteins (●) pass through the column, while tagged proteins (Δ) are captured by the tag-specific antibody bound to the beads. An Elution Buffer releases the tagged protein from the beads.

I. Preparing your own affinity column matrix

IA. Choosing the affinity support resin

Choose an affinity support resin and a coupling chemistry for attaching an antibody to the support resin. For guidelines, study the chromatography literature and resin manufacturer's technical information. Acceptable support resins include:

- ▶ Activated affinity **POROS® media** for Perfusion Chromatography® available in 20 and 50 µm particle sizes for analytical and preparative separations

Example: **POROS 20 HY** allows coupling of the carbohydrates on the Fc region of an antibody to support resin via hydrazide chemistry.

- ▶ Other suitable activated POROS media, with different coupling chemistries (epoxy, aldehyde, hydroxyl) are available individually or as a methods investigation kit
- ▶ Other commercially available activated resins
Example: **Affi-Gel™ 10 matrix** (Bio-Rad) allows attachment of an antibody through the amino groups (with N-Hydroxysuccinimide chemistry) to a 10-carbon spacer arm.
- ▶ POROS-based Protein A and Protein G and Protein A-agarose or Protein G-agarose crosslinked to a tag-specific antibody with DMP (dimethylpimelimidate)

IB. Coupling the antibody to the resin

- 1 Use the resin manufacturer's guidelines to determine how much tag-specific antibody to bind to the resin.
- 2 Depending upon the resin chosen, use one of the following methods to couple the tag-specific antibody to the support resin:
 - ▶ **For hydrazide coupling to POROS 20 HY:** Follow the general procedures given in the POROS 20 HY pack insert to oxidize the carbohydrate portion of the antibody to aldehydes, then couple the activated antibody to the POROS 20 HY resin.
 - ▶ **For coupling to other POROS activated affinity resins:** Follow the general procedures given in the POROS pack inserts.
 - ▶ **For coupling to other commercially available activated resins:** Either follow published methods (Field *et al.*, 1988; Frost *et al.*, 1981; O'Shannessy and Hoffman, 1987; Prickett, Amberg and Hopp, 1989) or the resin manufacturer's procedures to cross-link the antibody to a preactivated resin.

- ▶ **For coupling to POROS-based Protein A and Protein G and Protein A- or Protein G-agarose:** Expose the antibody to Protein A- (or Protein G-) agarose, then add a cross-linker to covalently attach the antibody to Protein A (or Protein G) (Templeton, 1992; Zhou *et al.*, 1992).

Caution: While coupling the antibody to the resin, monitor the A280 (protein content) of the antibody solution. This absorbance should drop significantly after the solution is exposed to the coupling resin. If absorbance does not decrease after coupling, then the antibody did not bind to the resin; repeat the coupling procedure at a different pH or in a different buffer.

Note: Sometimes, it is a good idea to couple a second, control antibody to the resin in parallel. If both antibodies do not bind, the affinity resin may not be properly activated. If the control antibody binds, but the tag-specific antibody does not, the resin is activated, but the conditions for binding the tag-specific antibody may not be optimal.

IC. Preparing the coupled resin for use or storage

- 1 Equilibrate the prepared affinity resin with at least 3 column volumes of Binding Buffer, that is the buffer to be used for sample binding and/or initial washes.
Note: Binding Buffer is a low ionic strength buffer containing components that will keep the tagged protein stable. For many tagged proteins, we recommend phosphate buffered saline (PBS), pH 7.4, as Binding Buffer.
- 2 Store the resin as a slurry in Binding Buffer + 0.1% Azide (Binding Buffer containing 0.1 g sodium azide per 100 ml) at 4°C until it is used.
Caution: Never allow prepared affinity resin to dehydrate after it has been hydrated. Do not freeze.

II. Characterizing and pretesting the affinity matrix

- 1 (Optional) Determine the binding capacity of the affinity matrix, as follows:
 - ▶ In a centrifuge tube or other suitable container, mix small amounts of the resin with measured amounts of radioactively or nonradioactively labeled epitope-tagged protein (either the target epitope-tagged protein or a model epitope-tagged protein that resembles the target protein).

- ▶ Incubate the protein and resin together, with gentle agitation, for a minimum of 15 min at 4°C.

Caution: Longer periods of incubation (up to 2 h) may be needed to insure maximum binding.

- ▶ Pellet the resin by centrifugation and save the supernatant for later analysis.
- ▶ Wash the resin several times with Binding Buffer and save the supernatants.
- ▶ Determine the amount of labeled protein remaining in the supernatant and washes.
- ▶ Determine the amount of labeled protein remains bound to the resin.

Note: ([starting label] – [label in supernatant/washes] = [label on resin])

Note: For an alternative using unlabeled protein, determine the capacity of the affinity matrix by preparing a small column of the resin, then deliberately overloading it, as follows. Add aliquots of the sample to the column, collect the effluent from the column after each sample application, and test the effluents for epitope-tagged protein. The column is overloaded when the concentration of tagged protein in the effluents begins to rise.

- 2 Prepare a variety of possible elution buffers for removing the bound protein from the affinity resin. Possible elution buffers include:

- ▶ Specific peptide or protein containing the amino acid sequence of the epitope tag
- Note:** We recommend tag peptide, at 1 mg/ml, for initial experiment. Determine optimal concentration as outlined in the next step of this procedure.

Note: Boehringer Mannheim sells peptides containing the epitopes for which we have detecting antibodies. These are potentially effective and non-denaturing eluants.

- ▶ Low or high pH (for example 100 mM glycine, pH 3.0, or 100 mM triethanolamine, pH 11.0)
- ▶ High salt (5M LiCl at neutral pH)
- ▶ Ionic detergents (1% SDS)
- ▶ Dissociating agents (2 to 8 M urea)
- ▶ Chaotropic agents (3M thiocyanate)
- ▶ Combination of above components

Caution: Some detergents, dissociating agents, or chaotropic agents may irreversibly damage the affinity resin, and reduce its performance.

- 3 Determine the most effective elution buffer by incubating potential elution buffers (from Step 2 above) separately with affinity resin, as follows:

- ▶ In a centrifuge tube or other suitable container, mix an aliquot of the affinity resin containing bound, tagged protein (from Step 1 above) with one column volume of an elution buffer.

Note: One column volume is an amount of elution buffer equivalent to the packed volume of the affinity resin aliquot.

- ▶ Incubate the elution buffer and resin together, with gentle agitation, for 15 min at 4°C.

- ▶ Pellet the resin in a centrifuge and remove the supernatant (first eluate).

- ▶ Resuspend the resin in a second column volume of elution buffer, then repeat the incubation and centrifugation steps to obtain a second eluate.

- ▶ Pool the first and second eluates and determine the amount of tagged protein released from the column into the eluates.

- ▶ After performing the above steps with each possible elution buffer, choose the buffer that released the most tagged protein from the affinity resin, and designate as Elution Buffer (for use in Procedure IIIC below).

Note: If any of the possible elution buffers released no tagged protein from the resin, consider using that buffer as a Second Wash Buffer in Wash Procedure IIIB below.

- 4 Test the affinity resin by the chromatography method you intend to use:

- ▶ Pack the affinity resin into an appropriate column.

- ▶ Determine the optimal column flow rate for binding the tagged protein to the packed resin.

- ▶ Determine other relevant physical performance characteristics of the packed resin.

Note: Manufacturers offer a wide variety of specialized chromatography systems, including Perfusion Chromatography, high pressure liquid chromatography (HPLC), and other variations of liquid chromatography. Each has a different set of relevant performance characteristics. Follow the recommendations from the manufacturers of the resin and column to determine these characteristics.

- 5 Before using resin in a purification, treat the resin with the Elution Buffer (and Second Wash Buffer, if applicable) to be used in the purification, then re-equilibrate with Binding Buffer.

III. Using the affinity matrix to purify an epitope-tagged protein

Note: We recommend dedicating a single batch of affinity resin to the purification of one specific tagged protein that was produced in one specific expression system. This strategy will minimize cross-contamination of samples.

Note: The guidelines given below were developed for simple column chromatography systems (for example, glass or plastic columns, elution by pumped or gravity flow). They will need to be modified for specialized chromatography systems such as HPLC and Perfusion Chromatography. Use these guidelines and the recommendations from the manufacturer of the specialized system to determine the optimal purification scheme.

Note: To realize the full benefits of Perfusion Chromatography, follow the detailed protocols contained with each POROS product.

IIIA. Binding the tagged protein to the affinity matrix

Use either the column method (Procedure IIIA.1) or the batch method (Procedure IIIA.2) to bind the sample containing the epitope-tagged proteins to the affinity resin.

Caution: If needed, keep sample cold throughout binding, washing, and elution steps. If sample is easily degraded, low temperature can slow the rate of degradation and improve results.

IIIA.1 Column binding method

- 1 Work at 4°C throughout sample loading. Chill all buffers to 4°C before use.
- 2 Resuspend the prepared affinity resin in fresh Binding Buffer (from Procedure IC) and pour the resin into a column of suitable size, using published guidelines (Cuatrecasas, Wilchek and Anfinsen, 1968; Harlow and Lane, 1988; Hermanson, Mallia and Smith, 1992; Wilchek, Miron and Kohn, 1984).
Note: If possible, make the width of the poured column greater than its height.
- 3 Prepare the sample containing the epitope-tagged protein as follows:
 - ▶ Remove large, particulate matter from the sample by centrifugation.
 - ▶ (Optional) To ensure optimal binding of the tagged protein to the affinity resin, use dialysis or other buffer exchange

methods to transfer the sample into Binding Buffer.

- 4 Apply the chilled (4°C) sample containing the epitope-tagged proteins slowly to the affinity column.
Caution: Optional flow rate must be determined for each tagged protein.
- 5 (Optional) If there is a quick, convenient assay for the epitope-tagged protein (for instance, native fluorescence or an enzymatic activity assay), determine if all the epitope-tagged protein in the first aliquot bound to the column.
Note: Save all column flow-through samples until the end of the purification procedure. Assay flow-through samples for the presence of the epitope-tagged protein before discarding them.
- 6 Go to Wash Procedure IIIB below.

IIIA.2 Batch binding method

- 1 Work at 4°C throughout sample loading. Chill all buffers to 4°C before use.
- 2 Prepare a slurry of the chilled affinity resin in fresh Binding Buffer (from Procedure IC).
- 3 Prepare the sample containing the epitope-tagged protein as follows:
 - ▶ Remove large, particulate matter from the sample by centrifugation.
 - ▶ (Optional) To ensure optimal binding of the tagged protein to the affinity resin, use dialysis or other buffer exchange methods to transfer the sample containing the epitope-tagged protein into Binding Buffer.
- 4 In a suitable container, mix the entire chilled sample with enough resin slurry to bind all the epitope-tagged protein in the sample.
Caution: Do not exceed a ratio of 5 volumes sample to 1 volume resin. If necessary, concentrate the sample before mixing it with the resin.
- 5 Incubate the sample and the resin for 2 h on a rocking platform at 4°C.
Note: We recommend a minimum of 2 hours incubation to insure maximum binding and recovery of the tagged protein. If convenient, you may continue the incubation overnight at 4°C. Use shorter incubation periods only if the tagged protein is unstable.
Caution: Do not use a magnetic stir plate, since the stirring process can damage the affinity resin.

- 6 Depending on the size and number of samples you are processing, do one of the following:

If you are	Then go to
Processing many small volume samples simultaneously	Procedure VI (purification by centrifugation)
Processing a few large volume samples	Step 7 of this procedure (purification by column)

- 7 Pour the entire slurry into a column or a sintered glass filter of appropriate size and let the unbound sample components drain through the column resin by gravity flow.
Note: Save all flow-through samples until the end of the purification procedure. Assay the flow-through for the presence of the epitope-tagged protein before discarding them.

- 8 Go to Wash Procedure IIIB below.

IIIB. Washing untagged proteins from the affinity matrix

- 1 After applying all the sample to the resin, wash the resin with at least 3 column volumes of chilled Binding Buffer (from Procedure IC) until nonspecifically bound sample proteins are removed from the column.

Caution: Compare the absorbance at 280 nm (A_{280}) of the column eluants to the A_{280} of the Binding Buffer alone. Continue the wash until the absorbance of the washes approaches that of the buffer alone.

Caution: Collect all wash samples and assay for the epitope-tagged protein before discarding them.

- 2 (Optional step) Repeat Step 1 with Second Wash Buffer (as determined in Step 3 of Procedure II above).

Caution: Make sure that Second Buffer will not release tagged protein from the column. Collect all second wash samples and assay for the epitope-tagged protein before discarding them.

- 3 Allow the last wash to drain to the top of the resin.

Note: Allowing wash to drain to the top of the resin bed before adding Elution Buffer will result in higher concentrations of eluted sample in the first Elution Buffer fractions.

IIIC. Eluting the tagged protein from the affinity matrix

- 1 To recover the epitope-tagged protein, apply 2–3 column volumes of chilled Elution Buffer (determined in Procedure II, Step 3) to the resin.

- 2 Collect eluant fractions of convenient volume (for instance, 0.5 column volume each) and assay for the tagged protein.

Caution: Compare the absorbance at 280 nm (A_{280}) of the column eluates to the A_{280} of the Elution Buffer alone. Continue flow of Elution Buffer until the absorbance of the eluates approach that of the buffer alone.

Depending on the Elution Buffer selected and the stability of the tagged protein eluted, the eluted sample may need to be neutralized immediately following elution. For instance, add 1 M Tris (pH 8) to eluants containing pH 3 buffer to bring them to neutral pH.

- 3 Pool fractions that contain the highest concentration of tagged protein.

Note: If the assay for the tagged protein is time-consuming, pool the eluted fractions that have the highest A_{280} , then assay the pooled fractions to confirm the presence of the tagged protein.

IV. Regenerating the affinity matrix

- 1 After all epitope-tagged protein has been eluted, re-equilibrate the affinity resin by passing at least 3 column volumes of Binding Buffer through the column.

Caution: In addition, Elution Buffer contains detergents, dissociating agents, or chaotropic agents, the immunoaffinity matrix may not be reusable. Tag-specific antibody resins have a finite life expectancy that is dependent on the composition of samples processed and the number of process cycles.

- 2 Next, do one of the following:

If you want to	Then go to Procedure
Immediately purify tagged protein from another sample	III
Store the resin for later use	V

V. Storing the affinity matrix

- 1 Pass at least 3 column volumes of Binding Buffer containing 0.1% Azide (0.1 g Sodium azide in 100 ml) through the column.
- 2 Store the column at 4°C.

VI. An alternative centrifugation procedure for small scale purifications

For small scale purifications from multiple samples, you may perform the batch binding, wash and elution steps in a centrifuge tube rather than a column. In that case, do the following:

- 1 Follow Steps 1–5 of the Batch Binding procedure (Procedure IIIA.2) with the resin and sample in a centrifuge tube.
- 2 After Step 5 of the Batch Binding procedure, pellet the resin containing bound protein in a centrifuge and completely aspirate the supernatant.
- 3 Perform the wash steps by resuspending and incubating the resin in the appropriate buffers (that is, Binding Buffer and/or Second Wash Buffer), pelleting the resin after each buffer treatment, and aspirating the supernatant.
Caution: Determine the optimum number and duration of the washes experimentally.
- 4 Perform the elution step by resuspending and incubating the resin in Elution Buffer, pelleting the resin again, and removing the supernatant carefully with a pipette.
- 5 Assay the Elution Buffer supernatants for tagged protein.