Published in final edited form as:

Methods Enzymol. 2014 ; 541: 27–34. doi:10.1016/B978-0-12-420119-4.00003-3.

Coupling Antibody to Cyanogen Bromide-Activated Sepharose

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Abstract

Antibodies will be immobilized on a cyanogen bromide-activated Sepharose for subsequent use in pull-down assays or immunoaffinity purification (see Immunoaffinity purification of proteins).

1. THEORY

Cyanogen bromide (CNBr)-activated Sepharose is a readily available commercial product. Proteins are coupled to the resin through primary amines. While other coupling chemistries are available, the CNBr-based resin is a good choice because of the mild reaction conditions and broad applicability to different types of proteins.

2. EQUIPMENT

Centrifuge
Nutator mixer or rocking platform mixer
UV/vis spectrophotometer
Magnetic stir plate
Beaker, 1 l
Magnetic stir bars
Dialysis tubing or Slide-A-Lyzer dialysis units
Amicon protein concentrators (optional)

3. MATERIALS

Purified monoclonal antibody
CNBr-Activated Sepharose 4 Fast Flow (GE Healthcare)
Hydrochloric acid (HCl)
Sodium bicarbonate (NaHCO₃)
Sodium chloride (NaCl)

Tris base
 Sodium acetate (NaOAc)
 Potassium chloride (KCl)
 Sodium phosphate monobasic (NaH₂PO₄)
 Potassium phosphate, dibasic (K₂HPO₄)
 Sodium carbonate (Na₂CO₃)

3.1. Solutions & buffers

Step 1 Activation buffer.

Dilute 4 μ l HCl in 50 ml water for a final concentration of 1 mM

Coupling buffer.

Component	Final concentration	Stock	Amount
NaHCO ₃ , pH 8.3	100 mM	1 M	100 ml
NaCl	500 mM	5 M	100 ml

Add water to 1 l

Step 3 Quenching buffer.

Dilute 5 ml of 1 M Tris-HCl, pH 8.0 in 45 ml water for a final concentration of 100 Mm

Step 4 High pH wash buffer.

Component	Final concentration	Stock	Amount
Tris-HCl, pH 8.0	100 mM	1 M	25 ml
NaCl	500 mM	5 M	25 ml

Add water to 250 ml

Low pH wash buffer.

Component	Final concentration	Stock	Amount
NaOAc, pH 4.0	100 mM	1 M	25 ml
NaCl	500 mM	5 M	25 ml

Add water to 250 ml

Storage buffer (PBS, pH 7.4)

Component	Final concentration	Stock	Amount
NaCl	137 mM	5 M	1.37 ml
KCl	2.7 mM	4 M	33.8 μ l
NaH ₂ PO ₄	10 mM	0.5 M	1 ml
K ₂ HPO ₄	2 mM	0.5 M	0.2 ml

Add water to 50 ml

4. PROTOCOL

4.1. Duration

Preparation	Variable
Protocol	2 days

4.2. Preparation

Obtain purified monoclonal antibody. The antibody can be purchased commercially or purified from either ascites fluid or media from hybridoma cell lines.

See Fig. 3.1 for the flowchart of the complete protocol.

5. STEP 1 PREPARATION OF ANTIBODY AND RESIN

5.1. Overview

Dry resin is swelled and activated. The antibody is dialyzed into a buffer compatible for coupling.

5.2. Duration

4–5 h

- 1.1 Dialyze the antibody into cold Coupling Buffer at 4 °C. Change to fresh buffer after 2 h and continue dialyzing for another 2 h.
- 1.2 Measure the absorbance at 280 nm of the final antibody solution and calculate its concentration.
- 1.3 Concentrate the antibody to 1–2 mg ml⁻¹ if it is too dilute.
- 1.4 Determine the amount of resin needed. Approximately 2 mg of antibody can be coupled to 1 ml of swollen resin.
- 1.5 Weigh out 0.25 g of dry resin for every 1 ml of hydrated resin needed.
- 1.6 Add 5-column volumes of cold Activation Buffer to resin.
- 1.7 Incubate on a nutator or platform rocker for 2 h at 4°C.

5.3. Tip

CNBr-activated Sepharose will react with Tris buffer. It is important to remove any traces of Tris from the antibody solution.

5.4. Tip

Performing the reaction with antibodies in a buffer other than Coupling Buffer will reduce coupling efficiency.

5.5. Tip

Coupling efficiency is maximized when the antibody is at a final concentration of 1–2 mg ml⁻¹. A 1 mg ml⁻¹ solution of antibody usually will have an OD₂₈₀ = 1.4.

5.6. Tip

The ratio of antibody to resin can be varied as needed for downstream applications.

See Fig. 3.2 for the flowchart of Step 1.

6. STEP 2 COUPLING THE ANTIBODY TO THE RESIN**6.1. Overview**

The antibody is chemically coupled to the resin.

6.2. Duration

Overnight

2.1 After swelling the resin, centrifuge it at 1000 × g for 5 min. Decant the supernatant.

2.2 Add dialyzed antibody to the resin and incubate overnight on a nutator at 4 °C.

6.3. Tip

Extra CNBr resin can be prepared and incubated with coupling buffer lacking antibody to generate a negative control in later applications to test nonspecific binding to the CNBr support.

See Fig. 3.3 for the flowchart of Step 2.

7. STEP 3 QUENCH THE REACTION**7.1. Overview**

Wash any unreacted antibody from the resin and then ensure that there are no unreacted CNBr sites remaining.

7.2. Duration

4 h

3.1 Centrifuge the resin at 1000 × g for 5 min.

- 3.2 Remove the supernatant and save.
- 3.3 Measure the OD₂₈₀ of the supernatant.
- 3.4 Add 5 column volumes of Coupling Buffer to the resin.
- 3.5 Incubate on nutator mixer for 30 min at room temperature.
- 3.6 Spin down resin at $1000 \times g$ for 5 min and decant supernatant.
- 3.7 Add 5–10 column volumes of Quenching Buffer.
- 3.8 Incubate on nutator for 2–3 h at room temperature.
- 3.9 Spin down the resin at $1000 \times g$ for 5 min and decant supernatant.

7.3. Tip

The coupling efficiency can be calculated by dividing the total amount of antibody in the supernatant in Step 3 by the total amount of antibody loaded on the column in Step 2. Typical coupling efficiencies are in the range of 70%.

See Fig. 3.4 for the flowchart of Step 3.

8. STEP 4 WASH THE RESIN

8.1. Overview

Remove uncoupled antibody from the resin and prepare resin for long-term storage.

8.2. Duration

4 h

- 4.1 Resuspend the resin in 10 column volumes of High pH Wash Buffer.
- 4.2 Spin down the resin at $1000 \times g$ for 5 min and decant supernatant.
- 4.3 Resuspend the resin in 10 column volumes of Low pH Wash Buffer.
- 4.4 Spin down the resin at $1000 \times g$ for 5 min and decant supernatant.
- 4.5 Repeat Steps 4.1–4.4 two more times.
- 4.6 Resuspend the resin in 5 column volumes of Storage Buffer.
- 4.7 Spin down the resin at $1000 \times g$ for 5 min and decant supernatant.
- 4.8 Add 1 column volume of Storage Buffer and store resin at 4 °C.

See Fig. 3.5 for the flowchart of Step 4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Referenced Protocols in Methods Navigator

1. Immunoaffinity purification of proteins.

Figure 3.1.
Flowchart of the complete protocol, including preparation.

Figure 3.2.
Flowchart of Step 1.

Step 2: Coupling antibody to resin

2.1 Centrifuge swollen resin at 1000 x g, 4°C, 5 min
Decant the supernatant

2.2 Add dialyzed antibody solution to CNBr-activated
Sephacrose
Incubate with rocking at 4°C, O/N

Figure 3.3.
Flowchart of Step 2.

Figure 3.4.
Flowchart of Step 3.

Figure 3.5.
Flowchart of Step 4.