# Coupling protein to CNBr activated Sepharose Beads.

**Practical points:**

a) Your protein MUST be pure - the coupling reaction is non-selective almost anything will end-up covalently attached to the resin.

b) You MUST accurately determine the concentration of your protein solution so you will know how much to add to what amount of resin.

c) It is not uncommon for a protein to lose (or retain) biological activity after coupling to CNBr Sepharose. Since you cannot predict a priori what will happen if activity is important you will have to make sure you have an appropriate assay.

d) The beads are larger than the hole at the end of a yellow pipet tip. So cut it off with a razor blade so that you have a hole about 0.5 mm in diameter.

1. Weigh out the required amount of CNBr activated Sepharose. Typically .06 grams dry will make around 0.2 mls of hydrated resin and will have a binding capacity of about one milligram of protein.
2. Wash the beads 10 times with 1 ml of 1mM HCl (1.3 microliters of concentrated Hcl diluted to 15 mls. with 18 megohm water. Vortex with your finger or very briefly on a vortexer and spin down in a microfuge (use a low speed setting ie 6000 rpm for about 5 sec.). Aspirate the supernatant and repeat 10 times.
3. Add 0.5 ml of 0.1M sodium phosphate buffer pH 6.5-7.5 (the exact pH depends on the lot number and the length of time the resin has been sitting around - the better the resin the lower the pH required to initiate coupling). Spin down the resin immediately and remove supernatant and add the protein solution to be coupled (also must be around pH 7.0) and vortex as in 1. *** This step must be performed rapidly as the resin begins to react with water as soon as the pH is increased. Use an old microcentrifuge that has a disabled interlock - you must be shown how to do this safely the first time!
4. Place on the rotating platform in the cold room for 6 hours or overnight.
5. Spin down the resin as in 1. and SAVE THE SUPERNATANT to assay for uncoupled protein using the Napthol Blue Black Nitrocellulose staining method.
6. Add 0.4 ml of 1M Monoethanolamine ( NH2(CH2)2OH ) pH 7.5 (adjusting the pH is important) and rotate for 6 hours or overnight. This step blocks any reactive sites that have not bound proteins or hydrolysed in water.
7. Test for residual protein in the supernatant that has not coupled by spotting 1, 2, and 5 microliters of your sample onto a small strip of nitrocellulose and staining with Napthol Blue Black. (see protocol in Western Blotting section). Uncoupled protein can be recoupled to new resin by starting over. If there is significant protein in the sup you had better measure it accurately (Bradford or something) and determine your coupling efficiency. I do this by assuming that 5 mgs protein per ml of resin is 100%. If the coupling efficiency is less than 25% you have a problem! Maybe the resin if it has been open a long time, more likely cockpit error.

8. Spin down your resin, remove the supernatant (monoethanolamine) and wash with a buffer suitable to your purpose in making the resin. For example if you have coupled an antibody to the resin for use in immunoprecipitation reactions then it is logical to wash the resin in 1X TXSWB (1% Triton X-100, 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, 1mM PMSF (fresh)) THREE times. For example for 200 microliters of resin wash 3 times with 1.0 ml. Staining Nitrocellulose with Napthol Blue Black.

This is an incredibly rapid and easy way to stain a nitrocellulose blot for total protein. If you have put the blot in blocking buffer it will not work because the blocking buffer is full of protein!

**Napthol Blue Black Sigma N3005**

1. Stain Filter 2-3 min. in 0.1% amido black in methanol:acetic acid:water (45:10:45 v/v). Use enough stain to nicely cover your blot - you do not need it to be swimming around (10-15 mls for a BioRad miniblot is lots) The time can be as little as 15 seconds if you have a lot of protein.
2. Rinse filter for 30 sec. in water with gentle mixing. (20-30 mls for a miniblot)
3. Destain in methanol:acetic acid:water (90:2:8 v/v). (3 time 20-30 mls for a miniblot)
4. Rinse in water to remove excess destain.

   To Quantify a protein from the blot (before drying):
5. Cut out the band of interest
6. Place in a tube with 0.6ml 25 mM NaOH/0.05 mM EDTA in 50% ethanol. Gentle swirling should be sufficient to get efficient elution of the dye.
7. Read A630 after elution is complete.

Ref. Anal. Biochem. (1973) 56:502-514. This is called a Schaffer-Weismann Protein Assay.