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Description

The CBIND Resins and Buffer Kits are used for rapid affinity purification of CBD fusion proteins such as those produced from Novagen's pET or pBAC™ vectors. These vectors encode a cellulose binding domain (CBD) that can be expressed as part of the target protein. All of the CBD•Tag™ domains have affinity for cellulose matrices under similar conditions. The interaction between CBDs and cellulose is driven by hydrophobic interaction at neutral pH; efficient elution is performed with ethylene glycol, which is innocuous to most proteins. Some CBD_{cex}, CBD_{cenA}, and CBD_{cenD} fusion proteins are eluted at low ionic strength (< 5 mM NaCl).

Four formats of CBIND Resin provide a range of protein purification options from analytical scale to batch or column processing:

CBIND Cartridges are designed for quick, syringe-driven purifications at extremely low cost. The CBIND 300 and 900 cartridges have a capacity of up to 1.5 mg and 4.5 mg of CBD fusion protein, respectively. The cartridges have Luer fittings on both ends, allowing quick transitions from loading to washing to elution. The cartridges are also compatible with the Vacuum Manifold enabling processing of up to 12 samples in parallel.

CBIND ReadyRun Columns are designed for use with Novagen's Vacuum Manifold, which enables processing of up to 12 columns simultaneously. The columns consist of pre-poured cellulose matrix packed between two high porosity frits to provide a convenient, ready-to-run affinity resin for CBD fusion proteins. ReadyRun columns have excellent flow rates, and the built-in column reservoir was designed for vacuum manifold processing. Each ReadyRun Column will purify up to 10 mg of protein per run.

CBIND 100 Resin is designed for scale-up of batch purifications of CBD•Tag fusion proteins. The CBD•Tag fusion proteins are purified from clarified crude extracts or solubilized inclusion bodies by batch binding to CBIND 100 Resin, removing unbound proteins by stringent washing, and eluting the target protein under mild, non-denaturing conditions. Batch purification of up to 40 mg protein/gram resin is possible. The resin is supplied as a dry powder; one gram CBIND 100 Resin makes approximately 3 ml bed volume upon hydration.

CBIND 200 Resin is recommended for scaled-up column purification. Very high flow rates are possible with minimal back pressures when running aqueous solvents through CBIND 200 Resin. Test columns with a 15-cm bed height and 2.5-cm diameter have been run at flow rates in excess of 50 ml/min without significant back pressure. This is equivalent to > 600 ml•cm^2/h, which is 20–60 times greater than the allowable flow rates for agarose and cross-linked agarose-based resins. The capacity of CBIND 200 Resin is approximately 5 mg protein/gram resin and it is supplied as a dry powder. One gram of CBIND 200 Resin makes approximately 3-ml bed volume upon hydration.

The **CBIND Buffer Kit** is designed for use with Novagen's CBIND Resins for convenient, rapid, one-step affinity purification of proteins containing CBD•Tag sequences.

CBIND Buffer Kit Components

The kit provides the necessary solutions to run a minimum of ten 2.5 ml columns or the batch equivalent.

- 100 ml 10X CBIND Binding Buffer (200 mM Tris-HCl, pH 7.5)
- 200 ml 5X CBIND Wash Buffer (100 mM Tris-HCl, pH 7.5, 4 M NaCl)
- 100 ml 1X CBIND Elute Reagent (100% ethylene glycol)

Related products	Size	Cat. No.
CBD_{clos}•Tag Antibody	50 µl	70119-3
CBD_{cenA}•Tag Antibody	500 µl	70157-3
CBD_{cex}•Tag Antibody	500 µl	70158-3
Chromatography Column, 5 ml polypropylene, with closures	pkg/4	69673-3
Vacuum Manifold		70147-3
Perfect Protein™ Markers, 10–225 kDa	100 lanes	69079-3
Protease Inhibitor Cocktail Set II (with EDTA)	5 x 1 ml	539132
Protease Inhibitor Cocktail Set III (without EDTA)	5 x 1 ml	539134
Protein Refolding Kit		70123-3
Thrombin, Restriction Grade	50 U	69671-3
Biotinylated Thrombin	50 U	69672-3
Thrombin Cleavage Capture Kit		69022-3
Streptavidin Agarose	5 ml	69203-3
Recombinant Enterokinase	50 U	69066-3
Enterokinase Cleavage Capture Kit		69067-3
EKapture™ Agarose	1.5 ml	69068-3
Factor Xa, Restriction Grade	400 U	69036-3
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Xarrest™ Agarose	5 ml	69038-3

Purifying CBD Fusion Proteins with CBIND Resins

The protocols described here begin with a crude or partially fractionated cell lysate or media fraction that has been clarified by centrifugation or filtration, or with a refolded CBD•Tag fusion protein. Procedures for cloning and expression of target proteins in pET CBD Vectors, as well as cell fractionation, are found in the pET System Manual, TB055. Procedures for cloning and expression in insect cells using pBAC™ CBD vectors are found in the BacVector™ Transfection Kits protocol, TB216. Note that binding to cellulose requires proper folding of the CBD moiety of the fusion protein; reagents and procedures for solubilization and refolding fusion proteins are found in the Protein Refolding Kit and protocol (TB234). All of these protocols are available on request from Novagen or on-line at www.novagen.com.

As mentioned above, optimal results with column or cartridge-based purification will be obtained using clarified lysates that are free of suspended aggregates or debris. Viscous lysates containing high molecular weight chromosomal DNA should be avoided. DNA can be sheared by sonication or degraded by brief treatment at room temperature with 1 µg/ml DNase I in the presence of 5 mM Mg^{2+}. Nucleic acids in crude lysates can also be removed by precipitation with polyethyleneimine (Polymin P; Sigma) or streptomycin sulfate, as described in the pET System Manual, TB055.

All of the CBD•Tag domains have affinity for cellulose matrices under similar conditions. The interaction between CBD domains and cellulose appears to be driven by hydrophobic interaction at neutral pH. Various methods have been tested for elution of CBD_{clos}, CBD_{cex} and CBD_{cenA} proteins from cellulose (Seeboth et al., 1992; Tomme et al., 1994), but the best general method for elution of all CBD•Tag forms is using low polarity solvents such as ethlylene glycol. Ethylene glycol gently elutes fusion proteins while maintaining solubility and can be easily removed by dialysis. For some fusion proteins tested in our laboratory, particularly fusions to CBD_{cex} and CBD_{cenA}, greater than 50% of cellulose bound protein could be eluted with water, low salt solution (≤ 5 mM NaCl), or 20 mM Tris HCl pH ≥ 9; and the remainder eluted with ethylene glycol. In contrast, nearly 100% elution is possible with ethylene glycol in a single step. Note that water elution may be an option for CBD_{cex} and CBD_{cenA} fusions.

Use of Ethylene Glycol

The purified proteins obtained using the CBIND resins and protocols are in a final solution containing a high concentration of ethylene glycol. There are some important considerations when working with this reagent.

Caution: *Ethylene glycol constitutes a hazard if ingested and even small amounts can cause renal damage. The lethal dosage in humans in approximately 1.4 ml/kg. Wear gloves and appropriate laboratory apparel when working with this reagent. See the MSDS for additional information.*

Ethylene glycol can give a false positive signal in some commonly used Coomassie dye-binding protein assays, such as Coomassie Plus (Pierce Chemical Co.). The recommended protein assay is the BCA assay (Pierce Chemical Co.). Protein standards for this assay should be diluted in ethylene glycol for maximum accuracy, although ethylene glycol at concentrations of up to 10% (final) appears to have no effect on the signal. Eluted protein fractions can be dialyzed to remove ethylene glycol prior to assay if the Bradford assay is preferred.

Water or Low Salt Elution

When eluting CBD•Tag fusions with water or low salt solutions, rinse the cellulose with 1X CBIND Binding Buffer prior to elution. The 1X CBIND Binding Buffer rinse removes the salt present in the 1X CBIND Wash Buffer.

Purifying CBD Fusion Proteins on CBIND Cartridges

1. Remove the plunger from a 30 cc syringe and attach the barrel to the Luer fitting on the CBIND Cartridge.
2. Prepare 10 ml of 1X CBIND Binding Buffer by adding 1 ml 10X CBIND Binding Buffer to 9 ml deionized water.

3. Fill the barrel with approximately 5 ml of 1X CBIND Binding Buffer. Replace the plunger and gently push the buffer through the cartridge to equilibrate the resin. Avoid pushing large volumes of air through the resin.
4. Detach the syringe, remove the plunger, and reattach the barrel to the cartridge. Fill the barrel with crude protein sample.
5. Replace the plunger and gently push the sample through the cartridge. Save the flow through fraction for later assay.
6. Wash the loaded cartridge with 5 ml of 1X CBIND Binding Buffer.
7. Prepare 5 ml of 1X CBIND Wash Buffer (add 1 ml 5X CBIND Wash Buffer to 4 ml deionized water) and push through the cartridge.
8. Elute bound CBD fusion protein from the cartridge with 1 ml (CBIND 300) or 3 ml (CBIND 900) 1X CBIND Elute Buffer.

Batch Purification with CBIND 100 Resin

1. Determine the amount of CBIND 100 Resin required for purification of your target protein. CBIND 100 Resin has a capacity of up to 40 µg protein/mg dry resin. To ensure that you are using a saturating amount of resin, assume that all of your target protein is correctly folded and functional for cellulose binding. An alternative is to perform small scale purification with a range of resin:protein mass ratios and scale up at the ratio where the best results are observed.
2. Add the calculated amount of dry CBIND 100 resin directly to your protein sample. For purification of CBD•Tag fusions from culture medium, the dry resin can be added directly to the media. Vortex gently to distribute the resin and incubate with gentle mixing or rotation for 30 min at room temperature.
3. Centrifuge sample at 7,500 × g for 10 min.
4. Carefully decant the supernatant into a clean container for analysis.
5. Wash the pellet with 10 pellet volumes of 1X CBIND Bind Buffer with gentle vortexing.
6. Centrifuge as in step 3 and retain pellet.
7. Wash pellet with 1X CBIND Wash Buffer and centrifuge again as in step 3.
8. Carefully decant the supernatant and remove as much residual liquid as possible from the pellet.
9. Add 1 resin volume of 1X CBIND Elute Buffer. Mix gently and incubate at room temperature for 10 min.
10. Centrifuge as in step 3 and carefully transfer the supernatant, which contains the target protein, to a fresh tube.
11. Repeat steps 9 and 10 twice more. Pool the supernatants to recover the eluted protein.

Column Purification with CBIND 200 Resin

1. Determine the amount of CBIND 200 Resin required for purification of your target protein. CBIND 200 Resin has a capacity of up to 5 µg protein/mg dry resin. To ensure that you are using a saturating amount of resin, assume that all of your target protein is correctly folded and functional for cellulose binding. An alternative is to perform small scale purification with a range of resin:protein mass ratios and scale up at the ratio where the best results are observed.
2. Weigh out the desired amount of resin and transfer to a clean beaker. Hydrate by adding approximately 10 volumes of deionized water. Place column in column clamp, on a ring stand fitted with a stopcock on the column outlet. Swirl the resin slurry until evenly suspended and pour into the column. Open the stopcock and allow the column to flow. Rinse the beaker with additional water and pour into the column. Allow the water to flow through the column until it is a few centimeters above the settled resin and then close the stopcock.
3. Prepare 15 column volumes of 1X CBIND Binding Buffer by diluting the 10X stock in deionized water. Equilibrate the resin with 5 column volumes of 1X CBIND Bind Buffer. Allow the buffer to flow down to the top of the resin.

4. Carefully apply the protein sample, either by directly pipetting onto the column or loading by siphon. For use with an FPLC (Pharmacia Biotech) or similar system, use moderate flow rates (< 10 ml/min) to prevent resin compression.
5. Wash the column with 10 column volumes of 1X CBIND Bind Buffer.
6. Prepare 10 column volumes of 1X CBIND Wash Buffer.
7. Wash the column with 10 column volumes of 1X CBIND Wash Buffer.
8. Elute the target protein with 3 column volumes of 1X CBIND Elute Buffer.

Multiple Sample Processing with the Vacuum Manifold

The Novagen Vacuum Manifold enables simultaneous processing of up to 12 samples. Loading, washing and elution steps can be performed rapidly, and all fractions can be collected in individual tubes because of the unique rack design used for the vacuum chamber. Fractions can be collected in either 1.5–2.0 ml tubes or in 15 ml conical tubes. Your vacuum source and a pressure release valve attached to the Vacuum Manifold control the vacuum level; individual stopcocks for each port enable single column control. The rugged glass chamber and polypropylene lid are rated to withstand vacuum levels of up to 20 inches Hg. The vacuum manifold is compatible with the CBIND Cartridges, but maximum flow rates are obtained with the CBIND ReadyRun Columns. In addition, because the ReadyRun Columns have a built in reservoir, additional syringe barrels are not required. When using the CBIND Cartridges, each cartridge must be fitted with a syringe barrel to allow buffer addition when using the manifold.

1. Attach a CBIND ReadyRun Column or a CBIND Cartridge fitted with a 10 cc syringe barrel to the Luer stopcock on the manifold lid. Place the reservoir in the chamber to catch the equilibration buffer.
2. Fill each column with approximately 5 ml of 1X CBIND Binding Buffer. Apply vacuum to the chamber, close the main vacuum valve and allow the vacuum level to build and stabilize. Open the stopcocks for each column and allow the buffer to flow through. When the buffer has been drawn through, close each stopcock.
3. Shut off the vacuum source and open the main valve. Remove the manifold lid and replace the reservoir with the rack fitted with the required number of tubes in the correct positions for the columns being run. Replace the manifold lid, close the main valve and reapply the vacuum.
4. Apply the protein samples to the columns and open the stopcocks. Allow the flow through fraction to collect in the rack of tubes.
5. Shut off the vacuum as in step 3 and replace the tube rack with the reservoir. (Replace the tubes in the rack if you wish to retain the wash fractions.) Wash each column with 3 volumes (approximately 15 ml) of 1X CBIND Binding Buffer.
6. Wash each column with 3 volumes of 1X CBIND Wash Buffer (15 ml).
7. Replace the reservoir with a rack of clean tubes for the elution step. Add 3 ml 1X Elute Buffer to each ReadyRun Column, 1 ml for CBIND 300 Cartridge, or 3 ml for CBIND 900 Cartridge.
8. Apply vacuum and collect the elution fractions.

Processing the Sample after Elution

You can change the buffer of the purified sample or concentrate the sample by one of several methods; of these, dialysis is the simplest (see below). Three alternative procedures are as follows:

1. Dialyze into the buffer of choice. After dialysis, the sample may be concentrated by sprinkling solid polyethylene glycol (15,000–20,000 molecular weight) or Sephadex G-50 (Pharmacia) on the dialysis tubing. Use dialysis tubing with an exclusion limit of 6,000 MW or less, and leave the solid in contact with the tubing until the desired volume is reached, replacing it with fresh solid as necessary.
2. Use plastic disposable microconcentrator units (e.g., Centricon; Amicon) as directed by the manufacturer to both desalt and concentrate the sample by ultrafiltration.
3. Desalt the sample by gel filtration on Sephadex (G-10, G-25, G-50; Pharmacia) or Bio-Gel (P6DG, P-10, P-30; Bio-Rad).

Cellulose-Based Dialysis and Filtration Devices

Purification of fusion proteins may involve the use of dialysis membranes and filtration devices. Some membranes and filters are cellulose-based (e.g., cellulose acetate) and therefore are candidate substrates for binding by CBD fusion proteins. However, in typical applications, any binding of the fusion protein results in negligible losses. For example, the CBD binding capacity of a cellulose acetate dialysis membrane is only 10–20 µg per square centimeter (O. Shoseyov, personal communication). Significant loss of a CBD fusion protein during dialysis would only be observed when the protein was very dilute and/or present in very small amounts. The same would hold true for the filtration of small volumes of dilute CBD fusions. In the case of filtration, alternatives such as nylon or polyethersulfone membranes (PES, Nalgene) are available.

Proteolytic Cleavage and Removal of CBD•Tag Sequences

CBIND purified CBD•Tag fusion proteins can be proteolytically cleaved with Factor Xa (Cat. no. 69037-3), recombinant enterokinase (rEK, Cat. No. 69066-3) or thrombin (Cat. No. 69671-3). The Factor Xa Cleavage Capture Kit, the Thrombin Cleavage Capture Kit, and the Enterokinase Cleavage Capture Kit, enable removal of Factor Xa, thrombin or rEK, respectively, from the cleavage reaction by affinity capture. In addition, the CBD fusion partner can be removed from the reaction by passing the cleavage reaction products over CBIND Cartridges or ReadyRun Columns, or by batch absorption to CBIND 100 Resin or column capture with CBIND 200 Resin (depending on the scale required). The standard cleavage conditions for Novagen's rEK, thrombin, and Factor Xa are compatible with CBD capture using any of the CBIND Resins.

CBD Vector from which the CBD fusion protein is derived	Molecular weight of CBD•Tag polypeptide after cleavage with the following proteases:		
	Thrombin	Enterokinase	Factor Xa
pET-34b(+)	17.75 kDa	21.17 kDa	NA
pET-35 b(+)	17.75 kDa	NA	21.35 kDa
pET-36 b(+)	12.58 kDa*	16.0 kDa*	NA
pET-37 b(+)	12.58 kDa*	NA	16.18 kDa*
pET-38 b(+)	13.14 kDa*	NA	NA
pBAC-7, pBACgus-7	17.75 kDa	21.17 kDa	NA
pBAC-8, pBACgus-8	17.75 kDa	NA	21.35 kDa
pBAC-9, pBACgus-9	12.58 kDa	NA	NA
pBAC-10, pBACgus-10	11.85 kDa*	14.7 kDa*	NA
pBAC-11, pBACgus-11	11.85 kDa*	14.7 kDa*	NA

* = Assumes signal sequence (2.24 or 4.11 kDa) already removed.

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