Affinity CBP Fusion Protein Detection Kit

INSTRUCTION MANUAL

Catalog #200370

Revision A

For In Vitro Use Only

200370-12

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Affinity CBP Fusion Protein Detection Kit

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Affinity CBP Fusion Protein Detection Kit

MATERIALS PROVIDED

Material provided [a,b]	Concentration	Quantity
Biotinylated calmodulin (bio-CaM)	100 µg/ml	10 µg
Nitroblue tetrazolium (NBT)	75 mg/ml in 70% (v/v) *N,N*-dimethylformamide (DMF)	2.0 ml
5-Bromo-4-chloro-3-indilyl phosphate (BCIP)	50 mg/ml in 100% (v/v) DMF	1.5 ml
Polyoxyethenesorbitan monolaurate (Tween® 20)		5.0 ml
Streptavidin alkaline phosphatase (AP)		0.1 ml

[a] Sufficient materials are provided in the Affinity CBP fusion protein detection kit for ~10 reactions using nitrocellulose membranes of ~8 × 6 cm.

[b] The components of the Affinity CBP fusion protein detection kit are shipped at –20°C.

STORAGE CONDITIONS

Biotinylated calmodulin (bio-CaM): –20°C
All other components: 4°C

Revision A

ADDITIONAL MATERIALS REQUIRED

Western Blotting and Biotinylated Calmodulin Probing of Electroblots

Laemmli sample buffer (1×)§
Nitrocellulose membranes
Transfer buffer (1×)§
Blocking solution§ or bovine lacto transfer technique optimizer (BLOTTO)§
Tris-buffered saline calcium chloride and Tween® 20 (TBSCT)§
Tris-buffered saline calcium chloride (TBSC)§
Plastic wrap

Color Development Reaction

Note *Prepare the color substrate solutions just prior to use in the color development reaction and store the substrate solutions protected from light.*

Color development solution§
Whatman® 3MM paper
Tris-buffered saline calcium chloride (TBSC)
Stop solution§

Dot Blot Assay

Nitrocellulose membrane strip (8 × 6 cm)
Biotinylated calmodulin (bio-CaM) dilution and streptavidin AP dilution
Blocking solution or BLOTTO
TBSCT
TBSC
Plastic wrap
Color development solution
Whatman 3MM paper
Stop solution

§ See Preparation of Media and Reagents

INTRODUCTION

The Affinity CBP fusion protein detection kit[1,2] provides rapid and sensitive detection of as little as 10 ng of calmodulin-binding peptide (CBP) affinity-tagged fusion protein. The detection method is based on an immunoblotting format using biotinylated calmodulin (bio-CaM)[3] as the primary probe and streptavidin alkaline phosphatase (AP) as the detection reagent. The Affinity CBP fusion protein detection kit can be used to monitor expression levels of CBP fusion proteins in culture or crude lysates, to asses the stability of fusion proteins, to detect fusion proteins without the need for specific antibodies, and to study CaM-mediated signal transduction pathways. The Affinity CBP fusion protein detection kit is ideal for use with the Stratagene Affinity protein expression and purification system. Figure 1 illustrates the powerful specificity of bio-CaM in tag protein detection.

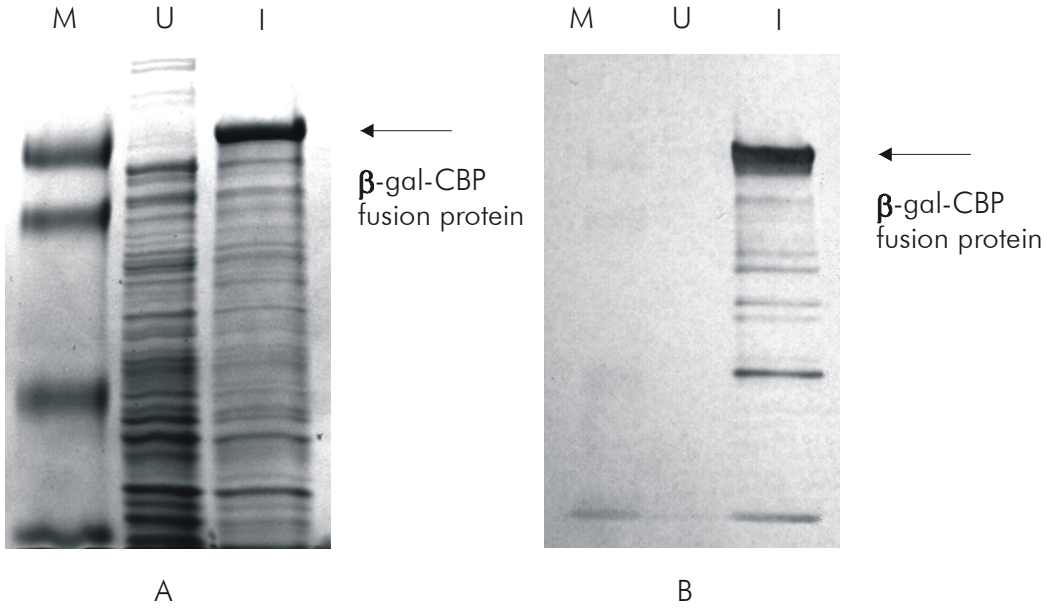

Figure 1 Detection of CBP fusion protein using bio-CaM. A log-phase culture of BL21 (DE3) pLysS competent cells was induced for 3 hours following removal of a portion of the culture for an uninduced control. These competent cells harbor a derivative of the pCAL-c vector, which expresses a β-galactosidase-CBP (β-gal-CBP) fusion protein. A 5-µl aliquot of each culture was loaded in duplicate onto a 4–20% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Following electrophoresis, half of the gel was stained with Coomassie® Brilliant Blue (A). The other half was electrophoretically transferred to a nitrocellulose membrane and probed with bio-CaM from this kit (B). Lane M shows a prestained molecular weight standard with fragment sizes of 106, 80, 49, and 32 kDa. Lane U shows an uninduced culture and Lane I shows an induced culture. The arrows indicate the position of the β-gal-CBP fusion protein, demonstrating the kit's strong specificity for tagged protein.

PROTOCOL GUIDELINES

Biotinylated Calmodulin

- After first use, aliquot into convenient volumes and store at –20°C.
- Avoid repeated freeze-thaw cycles.

Endogenous Biotinylated *Escherichia coli* Protein

- There are no endogenous *E. coli* proteins that will bind to the bio-CaM probe; however, there is a single biotinylated protein that is recognized by the streptavidin AP detection reagent, which may appear as a 22.5 kDa background band on the developed nitrocellulose blot. If the predicted molecular weight of the target CBP fusion protein is close to 22.5 kDa, care should be taken to use the appropriate acrylamide concentrations for SDS-PAGE analysis to allow resolution of the target and background proteins when analyzing crude lysates. In addition, uninduced controls should be run. The intensity of the background band will remain constant between uninduced and induced cultures, whereas the target protein should give little or no detectable signal in the uninduced culture.

Preparation of Reagents

- Use freshly prepared reagents. Use deionized, double-distilled water (ddH_2O) or the equivalent for all stock solutions and serial dilutions.

- Use sterile, concentrated stock solutions for the preparation of all reagents. The stock solutions necessary for all reagent preparations in this kit are listed in the following table:

Stock Solution	Sterilization method or storage conditions
1 M Tris-HCl (pH 7.5)	Autoclave
1 M Tris-HCl (pH 9.5)	Autoclave
20 mM Tris-HCl (pH 2.9)	Autoclave
5 M NaCl	Autoclave
1 M $CaCl_2$	Autoclave
1 M $MgCl_2$	Filter sterilize
1 M isopropyl-1-thio-β-D-galactopyranoside (IPTG) in water	Store at –20°C

WESTERN BLOTTING[4] AND BIOTINYLATED CALMODULIN PROBING OF ELECTROBLOTS

Note *Unless otherwise indicated, all procedures are conducted at room temperature on a shaker. If a lower temperature is required, increase the incubation times accordingly.*

1. Boil the protein samples in 1× Laemmli sample buffer for 2 minutes and load the samples on a 4–20% (w/v) gel for SDS-PAGE analysis. Include a molecular weight marker on the gel and electrophorese.

2. Presoak a nitrocellulose membrane in 1× transfer buffer for 20 minutes.

3. Electroblot the proteins onto a nitrocellulose membrane for 1 hour at 200 mA (30 V) in 1× transfer buffer.

4. Following electrophoretic transfer of the proteins to the nitrocellulose membrane, block the nitrocellulose membrane by incubating the membrane in blocking solution or in BLOTTO (use at least 25 ml of blocking solution or BLOTTO for an 8- × 6-cm membrane).

   Note *Blocking may also be performed overnight at 4°C.*

5. Wash the nitrocellulose membrane twice with TBSCT for a minimum of 5 minutes/wash, followed by a single wash with TBSC.

   Notes *If nitrocellulose membranes are combined during the wash steps, less of the recommended quantity of wash solution can be used; separate the nitrocellulose membranes with forceps at each change to ensure adequate exposure of each membrane. Never touch the nitrocellulose membranes with bare hands; always use gloved hands or forceps. It is also important to not allow the nitrocellulose membranes to dry during any step.*

6. Probe the nitrocellulose membrane with bio-CaM as outlined in the following steps:

   a. Pipet 1.0 ml of TBSC containing 300 ng of bio-CaM onto a sheet of wrinkle-free plastic wrap spread on a clean, flat surface.

   b. Lay the blot, protein-side down, in the pool of TBSC/bio-CaM. Make sure that no air bubbles form between the nitrocellulose membrane and the solution.

   c. Wrap the blot in plastic wrap and incubate the blot for 1 hour at room temperature under slight pressure (e.g., place the blot underneath an Eppendorf® rack).

Note *Probing may also be performed overnight at 4°C.*

7. Wash the nitrocellulose membrane as described in step 5.

8. Gently rock the nitrocellulose membrane in 1:2000 dilution of streptavidin AP in TBSC (use at least 30 ml for an 8- × 6-cm nitrocellulose membrane) for a minimum of 60 minutes at room temperature.

   Note *Probing may also be performed overnight at 4°C.*

9. Wash the nitrocellulose membrane again as described in step 5 and proceed to the color development reaction.

COLOR DEVELOPMENT REACTION

Notes *In addition to the components provided in the Affinity CBP fusion protein detection kit, a number of color substrate solutions are required to perform the color development reaction (see* the Color Development Reaction *section of* Additional Materials Required *for a complete listing).*

Prepare the color substrate solutions just prior to use and store the substrate solutions protected from light.

Nitroblue Tetrazolium and 5-Bromo-4-chloro-3-indilyl Phosphate (BCIP) Color Development Solution Preparation

1. Dilute the NBT in 20 ml of color development solution to a final concentration of 0.3 mg/ml (for a standard 8- × 6-cm nitrocellulose membrane).

2. Add BCIP dropwise to a final concentration of 0.15 mg/ml.

   Note *If a precipitate forms, warm the NBT-BCIP color development solution to 37°C and pass the color development solution through a 0.2-μm filter.*

Color Development on the Nitrocellulose Membrane

1. Remove the nitrocellulose membrane from the final TBSC wash (see step 9 of *Western Blotting and Biotinylated Calmodulin Probing of Electroblots*) and blot off any excess moisture with Whatman 3MM paper.

2. Immerse the nitrocellulose membrane in the NBT-BCIP color development solution (see *Nitroblue Tetrazolium and 5-Bromo-4-chloro-3-indilyl Phosphate (BCIP) Color Development Solution Preparation*). Allow the color development reaction to proceed in the dark until the positive reactions are clearly visible (usually within minutes) or incubate overnight.

   Notes *If extended color development times are required (1–5 hours), store the nitrocellulose membranes in the dark and check the membranes periodically to minimize the background.*

   If a visible precipitate forms that is not associated with specific dots or bands, the color development solution should be replaced with a fresh color development solution and the color development reaction should be allowed to continue.

3. Remove the color development solution from the nitrocellulose membrane and rinse the membrane with TBSC.

4. Terminate the color development reaction by immersing the nitrocellulose membrane in stop solution.

5. Air dry the nitrocellulose membrane and store the membrane protected from light.

If desired, the dot blot assay may be performed to quickly and simply determine if the fusion protein has been induced.

DOT BLOT ASSAY

Note *In addition to the components provided in the Affinity CBP fusion protein detection kit, a number of additional materials are required to perform the dot blot assay (see* Dot Blot Assay *subsection of* Additional Materials Required *for a complete list).*

The dot blot assay assesses the ability of the bio-CaM dilution and the streptavidin AP dilution to detect different levels of fusion protein and also checks the reaction of the NBT and BCIP with the streptavidin AP.

1. Cut out a nitrocellulose membrane strip and mark a grid pattern on the membrane strip using a soft pencil.

2. Prepare the nitrocellulose membrane strip with serial protein dilutions, bio-CaM, and streptavidin AP as indicated below:

    a. Spot 1 µl of each of the following serial protein dilutions onto the nitrocellulose membrane strip: 1 µg/µl, 100 ng/µl, 10 ng/µl and 1 ng/µl.

    b. Spot 1 µl of bio-CaM at a concentration of 10 ng/µl onto the nitrocellulose membrane strip.

    c. Spot 1 µl of a 1:2000 dilution of streptavidin AP on the nitrocellulose membrane strip.

3. Air dry the nitrocellulose membrane strip for 5 minutes.

4. Immerse the nitrocellulose membrane strip in blocking solution or BLOTTO for 1 hour at room temperature to block nonspecific protein-binding sites.

5. Wash the nitrocellulose membrane strip two to three times for 5 minutes each in 50 ml of TBSCT. Follow with a single wash with TBSC.

6. Prepare a 1-ml dilution of bio-CaM at a concentration of 300 ng/ml in TBSC. Transfer the 1-ml bio-CaM dilution to a piece of plastic wrap and place the nitrocellulose membrane strip face down in the dilution, making sure that no air bubbles form between the nitrocellulose membrane strip and the plastic wrap. Incubate the nitrocellulose membrane strip on the bio-CaM dilution for 1 hour at room temperature.

7. Wash the nitrocellulose membrane strip as described in step 5.

8. Gently rock the nitrocellulose membrane strip in 10 ml of diluted streptavidin AP in TBSC (1:2000 dilution) for a minimum of 60 minutes at room temperature.

9. Wash the nitrocellulose membrane strip as described in step 5.

10. Prepare the color substrate solutions:

    a. Dilute the NBT in 10–20 ml of color development solution to a final concentration of 0.3 mg/ml.

    b. Add BCIP dropwise to a final concentration of 0.15 mg/ml.

    Note *If a precipitate forms, warm the NBT-BCIP color development solution to 37°C and pass the solution through a 0.2-µm filter.*

11. Remove the nitrocellulose membrane strip from the final TBSCT wash and blot off any excess moisture with Whatman 3MM paper.

12. Immerse the nitrocellulose membrane strip in color development solution. Allow the color development reaction to proceed in the dark until positive reactions are clearly visible. Check the color development reaction every minute for a positive reaction.

13. Rinse the residual precipitated dye from the nitrocellulose membrane strip with TBSC.

14. Immerse the nitrocellulose membrane strip in stop solution.

15. Air dry the nitrocellulose membrane strip and store the strip protected from light.

TROUBLESHOOTING

Western Blotting, Biotinylated Calmodulin Probing of Electroblots, and Color Development Reaction

Observation	Suggestion(s)
Nothing is visible on the nitrocellulose membrane	The streptavidin AP or color development solution may not be working or is improperly prepared. ♦ Ensure the Streptavidin AP or color development solution is fresh and is properly prepared ♦ To test the activity of the working dilution of streptavidin AP, add 1.0 ml of the color development solution to 1.0 ml of the streptavidin AP diluted in TBST as indicated on the Certificate of Analysis. If no colored material precipitates in 5–10 minutes, repeat this test with a fresh dilution of streptavidin AP. If colored material does precipitate, proceed to the Dot Blot Assay to check the protein, bio-CaM, and streptavidin AP probe
Excessive background on the nitrocellulose membrane	Verify that the blocking solution or BLOTTO was used at the proper concentration, that the Tween 20 is added during the wash steps and that the nitrocellulose membrane is completely washed Ensure that the concentration of the bio-CaM and/or the streptavidin AP is not too high and that the streptavidin AP was diluted in TBSC
Appearance of a 22.5-kDa background protein on bio-CaM blots containing crude lysates	There is a single endogenous *E.Coli* protein that is naturally biotinylated and thus recognized by the streptavidin AP detection reagent. To verify that the band is due to the endogenous *E.Coli* protein and not derived from the pCAL vector, control samples from uninduced cultures, cultures containing plasmid without insert, or cultures without plasmid may be run alongside the induced culture sample. The background band should have an equivalent intensity in all of these samples. If further verification is desired, blots can be probed with streptavidin AP directly without first probing with bio-CaM; in this case, only the biotinylated protein will be detected. If the target CBP fusion protein has a molecular weight that is close to 22.5 kDa, the acrylamide concentration of the analytical SDS–PAGE gel should be adjusted to allow maximum resolution in this size range. Use a polyacrylamide concentration of 18%, with an acrylamide:bis-acrylamide ratio of 36.5:1. If the target protein comigrates with the endogenous protein under these conditions, an alternative detection method should be considered

Dot Blot Assay

Observation	Suggestion(s)
The protein is not visible on the dot blot membrane, while the bio-CaM probe is visible	The probe is not bound to the protein. The Dot Blot Assay specifically quantitates the protein serial dilutions
	The protein is folded and the tag is located in the interior of the protein molecule and is not making contact with the bio-CaM probe. Perform western blotting. The denaturing characteristics of the gel could cause the protein to unfold, making the tag available for detection by the bio-CaM. Verify the CaM sequence in protein. Ensure that at least 1 mM of $CaCl_2$ is added to all buffers
The bio-CaM probe is not visible on the dot blot membrane, while streptavidin AP is visible	The biotin on the probe is not active or the streptavidin AP is not binding to the bio-CaM probe. Use a freshly prepared dilution of bio-CaM probe or replace the probe completely with a new bio-CaM probe
The streptavidin AP is not visible on the dot blot membrane	The streptavidin AP is not working or is prepared improperly. To test the activity of the working dilution of streptavidin AP, add 1.0 ml of the color development solution to 1.0 ml of the streptavidin AP diluted in TBST as indicated on the Certificate of Analysis. If no colored material precipitates in 5–10 minutes, repeat this test with a fresh dilution of streptavidin AP

PREPARATION OF MEDIA AND REAGENTS

Blocking Solution 1% (w/v) bovine serum albumin (BSA) in TBSC	**BLOTTO** 5 g of nonfat dry milk 1 ml of 1% (w/v) thimerosal–H_2O Adjust volume to 100 ml with TBS Store at 4°C for 7–10 days
Color Development Solution 100 mM Tris-HCl (pH 9.5) 100 mM NaCl 5 mM $MgCl_2$ 1 mM $CaCl_2$	**Laemmli Sample Buffer (1×)** 125 mM Tris-HCl (pH 6.8) 4% (w/v) SDS 20% (v/v) glycerol 0.005% (w/v) bromphenol blue dye 100 mM β-mercaptoethanol
Stop Solution 20 mM Tris-HCl (pH 2.9) 1 mM $CaCl_2$	**TBSC** 20 mM Tris-HCl (pH 7.5) 150 mM NaCl 1 mM $CaCl_2$
TBST TBSC 0.05% (v/v) Tween 20	**Transfer Buffer (1×)** 12 mM Tris-HCl (pH 8.3) 96 mM glycine 20% (v/v) methanol

REFERENCES

1. Vaillancourt, P., Simcox, M. E., Zheng, C. F. and Simcox, T. (1995) *Strategies* 8(2):44.
2. Vaillancourt, P., Simcox, T. G. and Zheng, C. F. (1997) *Biotechniques* 22(3):451-3.
3. Kincaid, R. L., Billingsley, M. L. and Vaughan, M. (1988) *Methods Enzymol* 159:605-26.
4. Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc Natl Acad Sci U S A* 76(9):4350-4.

ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

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Catalog #200370

QUICK-REFERENCE PROTOCOLS

Western Blotting and Biotinylated Calmodulin Probing of Electroblots

- Boil the protein samples in 1× Laemmli sample buffer for 2 minutes and load the samples onto a 4–20% (w/v) gel for SDS-PAGE analysis. Include a molecular-weight marker on the gel and electrophorese
- Presoak a nitrocellulose membrane in 1× transfer buffer for 20 minutes
- Electroblot the proteins on a nitrocellulose membrane for 1 hour at 200 mA (30 V) in 1× transfer buffer
- Block the nitrocellulose membrane by incubating the membrane in blocking solution or BLOTTO (use at least 25 ml for an 8- × 6-cm membrane) with gentle rocking for a minimum of 30 minutes at room temperature or overnight at 4°C
- Wash the nitrocellulose membranes twice with TBSCT for a minimum of 5 minutes/wash, followed be a single wash with TBSC
- Probe the nitrocellulose membrane by incubating the membrane with 300 ng/ml of bio-CaM diluted in TBSC at room temperature for a minimum of 30 minutes.
- Wash the nitrocellulose membranes twice with TBSCT for a minimum of 5 minutes/wash, followed be a single wash with TBSC
- Rock the nitrocellulose membrane in a 1:2000 dilution of streptavidin AP in TBSC for a minimum of 60 minutes at room temperature or overnight at 4°C
- Repeat the wash step twice with TBSCT (see above) and once with TBSC

Color Development Reaction

- Blot off any excess moisture and immerse the nitrocellulose membrane in NBT-BCIP color development solution until the positive reactions are clearly visible (~30 minutes)
- Remove the color development solution from the nitrocellulose membrane and rinse the membrane with TBSC
- Terminate the color development reaction by immersing the nitrocellulose membrane in stop solution
- Air dry the nitrocellulose membrane and store the membrane protected from light.