

CMC Biosensors Initiative

Hahn Laboratory

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Generation of a Dye-based (MeroCBD) Cdc42 biosensor for activation studies; CBD-EGFP Expression, Purification & Reactive Fluorophore Labeling

Introduction

This procedure describes the expression and purification of CBD-EGFP (MeroCBD) and its subsequent covalent labeling with reactive fluorophore to generate a biosensor for studying Cdc42 activation (Nalbant et al., 2004).

CBD-EGFP is expressed in the form of C-terminal 6xHis fusion from the prokaryotic expression vector pET23. This vector has a strong T7 promoter, and is designed to work with BL21(DE3) strains of *E.coli* (available from Stratagene). The highest levels of expression are observed when a plain T7 promoter (not T7*lac*) is used in combination with a BL21(DE3) strain, not the more stringent BL21(DE3)pLysS, which allows for leaky expression. The protein is expressed at lowered temperature (30°C), to increase the portion of the correctly folded, soluble CBD-EGFP. The CBD Cdc42 sensor is then created by covalently labeling the protein with I-SO a dye designed specifically to report protein conformational changes and protein interactions in living cells (Touchkine et al., 2003).

A Methods in Enzymology article describes the photobleach corrections necessary for this Cdc42 biosensor (Hodgson et al., 2006) and the Matlab routines cited in this publication can be found at the Hahn lab web page, <http://www.hahnlab.com>.

Materials

Reagents:

BL21(DE3) cells, pET23-PBD, LBamp plate, LB media, carbenicillin, IPTG, T4 lysozyme and DNase, imidazole, Aquacide powder, NaP buffer (pH 7.3 & 7.5), ISO-IAA dye, aluminum foil, DMSO, Talon resin, a dialysis "bag" (Slide-A-Lyzer, from PIERCE)

Hahn laboratory plasmids are available at addgene, a non-profit constructs repository <http://www.addgene.org/pgvec1?f=c&cmd=showcol&colid=177>

Lysis buffer

50 mM NaH₂PO₄, pH 7.6,

300 mM NaCl,

10% glycerol,

5 mM MgCl₂,

2 mM β-ME,

1 mM PMSF

Equipment:

Cell culture equipment, centrifuge, incubator, ultrafree –15 centrifugal filter device, spectrophotometer, eppendorf benchtop microcentrifuge

Time Taken

Four to five days

Procedure

CBD-EGFP Expression & Purification

Day 1

- 1) Competent BL21(DE3) cells are transformed with pET23-CBD-EGFP according to standard protocols (Maniatis), and plated on LBamp plates. We usually split the transformation volume over 3 plates.
- 2) Incubate the plate at 37°C overnight.

Day 2

- 3) The next morning, the plates should have colonies colored GFP green.
- 4) Inoculate 500ml (up to 700ml) of LB-*carb* (100µg/ml carbenicillin) with colonies from the plates. For that, 5ml of media are added on each plate and cells are resuspended with that media. The cell suspension is transferred into the 500 ml LB-*carb*. and grown in a shaker at 37°C to OD600 = 0.8-0.9. The culture is briefly chilled on ice to 30°C, then put back in the shaking incubator turned down to 30°C. Cells do not degrade carbenicillin as quickly as ampicillin. Therefore a higher percentage of cells retain the vector at the culture density appropriate for induction.
- 5) Add IPTG (1M stock in water, kept at -20°C) to a final concentration of 0.2mM, and allow the cultures to grow for another 5 hours at 30°C (shaker). In the case of CBD-EGFP one can use different concentrations of IPTG ranging from 0.2-0.5mM.
- 6) Collect the cells by centrifugation (**Beckman J-6M rotor, 20 min, 4000 rpm**), and stored as a pellet at -20°C until use. Approximately 2.5-3 g of cells is usually obtained from each liter of culture.

Day 3

- 7) Purification of CBD-EGFP-6xHis is performed essentially as described in the Clontech manual for Talon affinity resin.
- 8) Thaw the cells (3-5g) in 20-30 ml of the Lysis buffer and homogenize by pipetting up and down, then sonicate (4 pulses, 20 sec each).
- 9) Centrifuge the cells at **13000 rpm for 20-30 min**, and carefully transfer the supernatant containing CBD-EGFP into a 50ml Falcon tube.
- 10) While the cells are spinning, transfer 2ml of Talon resin into a 50ml Falcon tube and centrifuge at 700xg. We use 1ml resin per 2.5g cell pellet. Depending on the expression level of your protein you can increase or decrease the amount of resin. **To pellet the resin we use a swinging bucket centrifuge at a setting of "3", 2-3 minutes.**
- 11) Wash the resin twice with 10 volumes of the lysis buffer (no β-ME and PMSF) in a 50ml Falcon tube. Again, pellet the resin by centrifuging at 700g
- 12) Add the cell lysate to the 2ml of washed Talon resin in the 50ml falcon tube and inverted gently using an orbit shaker at room temperature for 40 min (Wrap it with foil to avoid unnecessary bleaching of the EGFP). Then separate the resin by centrifugation (700g) in a swinging bucket centrifuge.
- 13) Remove and save the supernatant (unbound fraction).
- 14) Wash the resin twice (5 min each, room temperature, orbit shaker) with 20ml of the lysis buffer, without PMSF and β-ME.
- 15) Perform a third (final) wash with 10ml lysis buffer + 5mM imidazole (add 1 M stock in water, kept at room temperature).

- 16) Perform the elution by adding 10ml Lysis buffer + 100mM imidazole to the resin and rotation (orbit shaker) at room temperature for 5 min. Pellet the resin again by centrifugation.
- 17) Remove the supernatant and saved (elution).
- 18) Add another 2-3ml of elution buffer to the resin and separate by centrifugation to obtain residual eluted protein.
- 19) Concentrate the resulting 10-12ml eluate with the “ultrafree –15 centrifugal filter device” from Millipore by centrifugation at 4 °C (read manual). This step can take a long time (2-3 hrs) and needs to be monitored. Check every 20 min to be sure the concentration process does not go too far. Also check the concentration. Stop at around 120µM.
- 20) Take a part of the concentrated protein for labeling the next day, and dialyze overnight against 2 L of 50mM NaH₂PO₄ (monobasic sodium phosphate) buffer, pH 7.5. Use a dialysis “bag” (Slide-A-Lyzer, from PIERCE) with a molecular weight cut-off of 3,500 kDa.
- 21) Measure the concentration of CBD-EGFP by taking a small aliquot (5-10µL) and diluting into 50mM Tris HCl (pH 7.5-8.0). Measure the absorbtion at 280 and use 28260 (cm⁻¹/M⁻¹) as extinction coefficient for the protein. On average, 10-15mg of CBD-EGFP is obtained per liter culture.

$$[\text{CBD-EGFP}] = (\text{OD}_{280} * \text{dilution factor}) / 28260 \text{ (in mol/L)}$$

- 22) For storage, the rest of the protein is dialyzed over night against 2 L of storage buffer (50mM Tris-HCl, pH 7.5, 50mM NaCl, 5mM MgCl₂, 10% Glycerol) and is shock frozen on dry ice or liquid nitrogen.

Covalently labeling with I-SO IAA dye

- 1) To attach dyes to the cysteine, prepare CBD-EGFP in 50mM NaH₂PO₄ buffer, pH 7.5 at a protein concentration of around 100µM.
- 2) Prepare a fresh solution of the dye in pure DMSO, by adding a small amount of dye to 30-40µL DMSO. Determine the exact concentration of the dye spectrophotometrically by diluting the DMSO solution 1:5000 in methanol. The ISO-IAA dye extinction coefficient at maximum (approx. 600 nm) = 125,000. Usually we obtain concentrations around 20-25mM for the original DMSO stock.

$$[\text{ISO-IAA}] = (\text{OD}_{600} * \text{dilution factor}) / 125000 \text{ (in mol/L)}$$

- 3) Transfer a 300µL aliquot of fresh CBD-EGFP protein (see CBD-EGFP expression & purification protocol) into a 2ml eppendorf tube wrapped in foil (to protect from light).
- 4) Add the dye in 2-3 aliquots to the CBD-EGFP solution to make the final dye:protein ratio in the reaction about 5:1 or 6:1 (e.g. if the protein concentration is 100µM, the dye concentration would be 600µM). When adding dye, vortex gently, i.e. using a setting of 3 on the vortexer.
- 5) Vortex the protein-dye mix once briefly at higher setting to mix dye and protein completely.
- 6) Completely wrap the tube in foil and put on a wheel at room temperature for 1 hour.
- 7) Add 1µL β-ME to the reaction mix and incubate the tube for another 5-10 min while repeatedly inverted on a rotating wheel. This assures that there are no reactive species left during the following separation. This may also solubilize and remove non-covalently bound dye molecules from CBD-EGFP.
- 8) Centrifuge the tube at room temperature for 2 min at 13,000 rpm (e.g.: Eppendorf benchtop microcentrifuge) to sediment insoluble material. Load the supernatant onto a small G25 gel filtration column (0.5 cm x 6-8 cm) to separate the conjugate from the free dye.
- 9) Equilibrate the column and eluted with 50 mM NaH₂PO₄ buffer, pH 7.5. The first colored band to elute contains CBD-EGFP (MeroCBD) conjugated to dye. Collect fractions of approximately 200µL (approximately 4 drips).

10) Analyze an aliquot (3-5 μ L) of each fraction by 12% SDS-PAGE to confirm the presence of the modified CBD-EGFP and the purity of the sample. Fluorescence visualization of the gel assures labeling with the dye.

Determination of the labeling efficiency (dye : protein ratio)

This can be difficult and will be approximate. For accurate measurements, at least until you obtain consistent readings, it is a good idea to use both methods described here and compare their results.

Protein concentration: Protein concentration can be determined by taking an absorbance spectrum of the conjugate solution.

11) Dilute a small aliquot (5-10 μ L) in 50mM Tris HCl, pH 7.5-8.0 and use OD₂₈₀ for the calculation: [CBD-EGFP] = (OD₂₈₀*dilution factor) / 28260 (in mol/L)

12) Since the protein absorbance at 280 overlaps dye absorption, determine the protein concentration independently by comparing a sample of the labeled material to unlabeled CBD-EGFP using at different sample concentrations. Colorimetric assays, limited to those with readouts that do not overlap dye absorbance, have been more variable in our hands.

Dye concentration: Determine dye concentration by taking an absorbance spectrum of the protein conjugate.

13) Dilute a small aliquot of the conjugate (5-10 μ L) in DMSO. This solvent will overwhelm effects of the protein on the dye absorbance, to produce a consistent dye extinction coefficient. OD at the dye absorbance maximum (approximately 600) is used to calculate the dye concentration.

$$[\text{Dye}] = (\text{OD} \cdot \text{dilution factor}) / 125000 \text{ (in mol/L)}$$

14) Divide labeled CBD-EGFP solution into 15-20 μ L aliquots, flash freeze with dry ice or liquid nitrogen and stored at -80°C. Alternatively, the CBD-EGFP-dye conjugate may be kept without freezing at 4°C for about one week.

Troubleshooting

Using high dye to CBD-EGFP ratios (e. g. 10:1 or 15:1) in the reaction mixture can produce excessive amounts of precipitated material and “over-labeling” (dye:protein in the purified covalent adduct greater than 1). The optimal dye:protein ratio depends on the reactivity of the dye, so it can be different with other dyes.

Critical Steps

- The bacteria strain is critical. We use BL21(DE3). Do not use BL21(DE3)pLysS.
- Use Talon resin (Co²⁺ affinity, Clontech) and not Ni-NTA resin! Don't use too much resin (2ml dry volume of talon resin for 6g of cell pellet).
- Use the suggested buffers.
- Use enough buffer during lysis, e.g. for 6g cell pellet use 50ml total lysis buffer (divide between two 50ml tubes).
- The timing of step 6 in the labeling protocol above is critical, longer time will result in over-labeling.

Anticipated Results

On average, 10-15 mg of CBD-EGFP is obtained per liter of culture. After labeling about 40-60% of the CBD-EGFP is recovered as purified conjugate, since there is some precipitation during

labeling and loss during gel filtration. The final elute concentration is usually 50-60 μ M. Labeling efficiency under these conditions varies between 0.7-0.9 dye: protein.

References

Hodgson L, Nalbant P, Shen F, Hahn K. Imaging and photobleach correction of Mero-CBD, sensor of endogenous Cdc42 activation. *Methods Enzymol.* 2006;406:140-156. [PubMed](#) | [CMC Update article](#).

Nalbant P, Hodgson L, Kraynov V, Touthkine A, Hahn KM. Activation of Endogenous Cdc42 Visualized in Living Cells. *Science* 2004;305:1615-1619. [PubMed](#)

Touthkine A, Kraynov V, Hahn KM. Solvent-Sensitive Dyes to Report Protein Conformational Changes in Living Cells. *J. Amer. Chem. Soc.* 2003;125:4132-4145. [PubMed](#)