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## Production and purification of a GFP-tagged ABC transporter *CaCdr1p*

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**Abstract:** The production and purification are the first steps needed in any functional or structural study of a protein of interest. In the case of membrane proteins, these tasks can be difficult due to low expression levels and the necessity to extract them from their membrane environment. This chapter describes a convenient method based on GFP tagged to the membrane protein to facilitate these steps. Production is carried out in the yeast *S. cerevisiae* and purification steps are carried out and monitored taking advantage of an anti-GFP nanobody. We show how GFP can be a very helpful tool for controlling the correct addressing of the protein and for probing and optimizing purification. These methods are described here for producing and purifying *CaCdr1p*, an ABC exporter conferring multi-antifungal resistance to *C. albicans*. This purification method can be amenable to any other GFP-tagged protein.

### Introduction

Since its discovery in 1962, the jellyfish *Aequorea victoria* green fluorescent protein (*avGFP*) has proven to be a very powerful tool in biology and biochemistry. It is often used as a reporter for DNA and protein localization (1),(2), as a folding reporter (3). In the case of membrane proteins, GFP as a tag has been shown to provide valuable information, as if it is not folded properly when overexpressed, it does not fluoresce (4). Furthermore, GFP has been used in preliminary structural studies to assess protein mono-dispersity and stability of protein-detergent complexes (5).

All these examples show the power of such a versatile tag. In our protocol, we decided to use this tag not only as a localization reporter, but also during an affinity purification using an anti-GFP camelid single chain VHH antibody domain (nanobody) (6),(7). Such nanobodies are characterized by a small size (about 15 kDa), stability (8) and the possibility to produce them in bacteria, yeast or mammalian cells (9). Recently, synthetic single domain antibodies for the conformational trapping of membrane proteins were developed using an *in vitro* selection platform (10), highlighting the potential of these nanobodies in the study of membrane proteins, in this case of ATP-binding cassette (ABC) transporters.

ABC transporters are a large class of membrane proteins found across all kingdoms. They are capable of importing nutrients inside the bacterial cells or efflux a plethora of different compounds: antibiotics, anticancer drugs, lipids etc. (11), (12), (13). Proteins of this class share a common topology consisting of two transmembrane domains (TMD) involved in substrate binding and translocation and two soluble nucleotide-binding domains (NBD), involved in ATP binding and hydrolysis.

These transporters are involved in different physiological processes (14). In the case of *CaCdr1p*, its overexpression leads to a drug resistance phenomenon (15), (16). Indeed, this protein is capable of exporting azoles, the major antifungal drug class, conferring a resistance to *Candida albicans* species. Until very recently (17), no structure of a yeast PDR subfamily ABC transporter was solved, so the exact mechanism of substrate promiscuity and transport remains not well understood. The need to overexpress these proteins in a functional form, then solubilize them makes them extremely difficult to study. Here we present a protocol for the production and purification of GFP-tagged *CaCdr1p* using anti-GFP nanobodies.

## **Materials**

### **2.1: Lab equipment**

- Incubator (ThermoScientific Heraeus New Brunswick Inova™ 44), temperature regulated.
- Centrifuge for 15/50 mL Falcon-type tubes (ThermoScientific Heraeus X1R multifuge) and a centrifuge (Beckmann Coulter Avanti® J-E) for large volumes
- Cell disrupter (CellD, Constant Systems Ltd.)
- Typhoon FLA9500 fluorescence imager
- Mini Protean Tetra Cell (BioRad) and related devices: 1-mm spacer plates, 10-well combs and a power generator
- 96-well black plates (Greiner) and a spectrophotometer-fluorimeter (Safas Xenius)

### **2.2 Yeast cell culture and overexpression**

- Yeast strain *Saccharomyces cerevisiae* AD1 8u- (*MAT $\alpha$*  *PDR1–3* *ura3* *his1*  $\Delta$ *yor1::hisG*  $\Delta$ *snq2::hisG*  $\Delta$ *pdr10::hisG*  $\Delta$ *pdr11::hisG*  $\Delta$ *ycf1::hisG*  $\Delta$ *pdr3::hisG*  $\Delta$ *pdr5::hisG*  $\Delta$ *pdr15::hisG*) containing the plasmid *pSK-PDR5 PPUS* (Nakamura 2001) encoding for *CaCdr1p* (Uniprot [Q5ANA3](#))-GFP was a kind gift from R.D. Cannon. A Precision protease cleavage site was inserted between the sequence of *CaCdr1p* and *GFP*.

- Four baffled flasks of 2 L containing 500 mL of autoclaved YEPD media: 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose in distilled water + one 250 mL-flask containing 40 mL of the same YEPD media + one 500 mL-bottle containing 250 mL of YP 5X media: yeast extract 10 g/L, peptone 20 g/L.

### **2.3 Bacterial culture and overexpression**

- *E. coli* BL21 DE3 Gold *pLysS* cells containing the plasmid *pOPINE GFP nanobody*, a gift from Brett Collins (Addgene plasmid # 49172; <http://n2t.net/addgene:49172>; RRID: Addgene\_49172) (Kubala 2010).

- Four 5 L- flasks containing 1 L of autoclaved LB media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl).

- 50 mg/mL ampicillin (fresh, dissolved in distilled water) and 25 mg/mL chloramphenicol (fresh, dissolved in ethanol).

- 0,7 M IPTG.

## 2.4 Nanobody purification

- 200 mL Cell lysis buffer: 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0,1 g/L of chymostatin, leupeptin, antipain, pepstatin and 0,4 g/L of aprotinin (CLAPA), 1 mM EDTA pH 8.8.
- 200 mL buffer A : 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 40 mM imidazole, CLAPA
- 200 mL buffer B : 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 60 mM imidazole
- 200 mL elution buffer: 50 mM HEPES-HCl pH 8.0, 100 mM NaCl, 250 mM imidazole.
- A 5 mL-HisTrap Ni-NTA column (Generon) and an AKTApure FLPC system.
- 2 L dialysis buffer: 50 mM HEPES-HCl pH 8.0, 100 mM NaCl and ≈10 cm of dialysis membrane (MWCO 3,5 kDa).

## 2.5 Yeast lysis and membrane isolation

- 500 mL Suspension buffer: 50 mM Tris-HCl pH 8.0, 20% glycerol, CLAPA + 5 µL of Benzonase (after lysis)
- 15-mL glass homogenizer with pestle

## 2.6 Detergent screening and protein purification

- 1 mL of different detergent solutions (10% w/v) in suspension buffer.
- 200 mL buffer A2 : 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, CLAPA, 20 mM imidazole, 4-trans-0.005% (4-trans-Propylcyclohexyl) -cyclohexyl α-maltoside (t-PCCαM)
- 200 mL buffer B2 : 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, CLAPA, 40 mM imidazole, 0.005% t-PCCαM.
- 200 mL elution buffer : 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, CLAPA, 250 mM imidazole, 0.005% t-PCCαM.
- A 5 mL-HisTrap Ni-NTA column (Generon) and an AKTApure FLPC purification system.
- A 50-mL Amicon® centrifugal filter (MWCO 100k), a Superdex 200 10/300 GL Increase gel filtration column and an AKTApure FPLC system.

## Methods

### 3.1: Yeast cell culture and overexpression

1. In the morning, inoculate a 40-mL YEPD preculture with a fresh colony of *S. cerevisiae* AD1-8u- *pSK-PDR5 PPUS Cdr1p-GFP* and incubate at 30°C, 200 rpm for 6-8 hours. (Note 1)
2. Pour 10 mL of the preculture in each of the baffled flasks containing 500 mL of YEPD, and incubate them at 30°C, 200 rpm until reaching 1.5 OD<sub>600 nm</sub>. Add 50 mL YP 5x and incubate again until reaching 3.5 OD<sub>600 nm</sub>. (Note 2)
3. Harvest yeast by centrifugation for 5 min. at 6000 xg, 4°C (JLA 9.1000 rotor, Beckmann Coulter, 2 1-L bottles) , discard the supernatant, then resuspend the pellet in 500 mL/bottle of ice-cold distilled water and centrifuge again at for 5 minutes at 6000 xg, 4°C.
4. Suspend the yeast pellet in 175 mL of ice-cold ddH<sub>2</sub>O and centrifuge again during 5 minutes at 6000 xg, 4°C.
5. Suspend the yeast pellet in 85 mL suspension buffer and proceed to lysis, otherwise freeze them in liquid nitrogen and store -80°C until use.

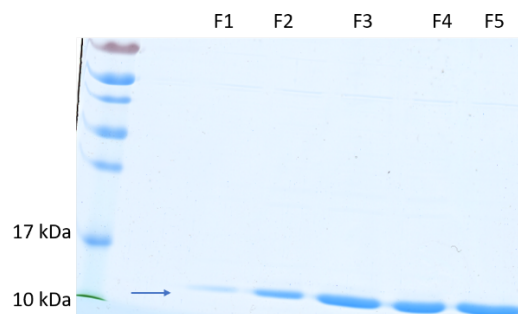
### 3.2 Bacterial culture and overexpression

1. Inoculate 40 mL of LB media supplemented with 50 µg/mL of ampicillin and kanamycin with a colony of *E. coli* BL21 DE3 Gold *pLysS* cells containing the plasmid *pOPINE GFP nanobody*. Incubate at 37°C overnight, 200 rpm.
2. The next day, dispatch 10 mL of this culture into each of the flasks containing LB media and 50 µg/mL of ampicillin and kanamycin. Incubate at 37°C under agitation and check the OD<sub>600</sub> periodically.
3. Once the OD<sub>600</sub> = 0.5, cool down the flasks to 20°C (~1h), add 0.7 mM IPTG to induce nanobody production. Incubate again at 20°C, 200 rpm for 16-20 h.
4. Harvest bacteria by centrifugation for 5 min. at 6000 xg, 4°C (JLA 9.1000 rotor, Beckmann Coulter, 4 1-L bottles) . Wash the pellets once with ddH<sub>2</sub>O.
5. Weigh the wet bacteria pellet, suspend them in cell lysis buffer to a 3:1 ratio (v/w), divide the total volume in four falcon tubes (1/L of biomass) and proceed to lysis, otherwise freeze them in liquid nitrogen and store -80°C until use.

### 3.3 Nanobody purification

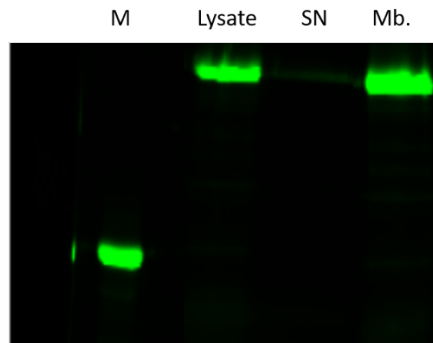
1. If frozen, thaw the bacteria suspension on ice for 1-2 h. Usually, we work with 1-2 aliquots at a time.
2. Lyse cells with a high-pressure cell disrupter such as a French press, Emulsiflex (Avestin), or Microfluidizer (Microfluidics), or here a Cell Disrupter (Constant Systems Ltd.). Follow the manufacturer's guidelines for operation of the cell disrupter but normally 2-3 passages through the chosen disrupter are more than sufficient for disrupt 100 % of the cells. Here, the Cell Disrupter (Constant Systems Ltd.) is operating at a pressure of 1.5 kbar and we make 1 passage only.
3. Centrifuge at 5000 xg for 5 min. and discard the pellet, then centrifuge again at 10,000 xg and keep the supernatant.

4. Perform the purification using a 5-mL Ni-NTA column (Generon) equilibrated with buffer A. Pass the sample through it at a flow rate of 2 mL/min.
5. Wash thoroughly the column with buffer B until the  $Abs_{280nm}$  falls back to 0 after at least 3 resin volumes.
6. Elute the anti-GFP nanobodies with buffer C at a flow rate of 1.0 mL/min and collect fractions of 1 mL.
7. Pool the fractions and dialyze them against 1 L of the dialysis buffer using a 3.5 kDa MWCO membrane during the day, then change the buffer and let the dialysis overnight at 4°C.
8. After dialysis, the nanobodies can be stored at 4°C or at -80°C until use. Estimate the quantity of pure protein using a Nanodrop or by performing a BCA assay. In our case, for a purification, we obtain 3-4 mg of anti-GFP nanobody per purification run.



### 3.4 Yeast lysis and membrane isolation

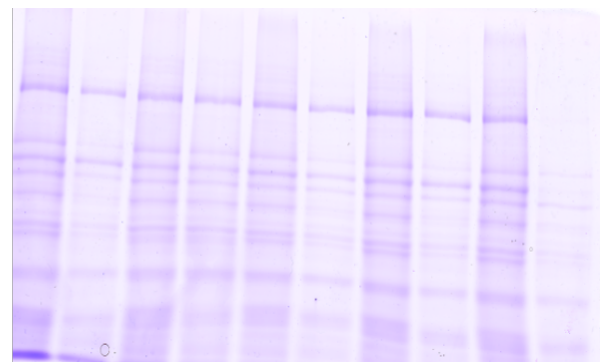
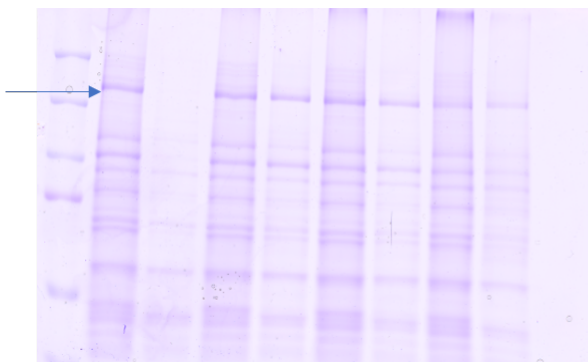
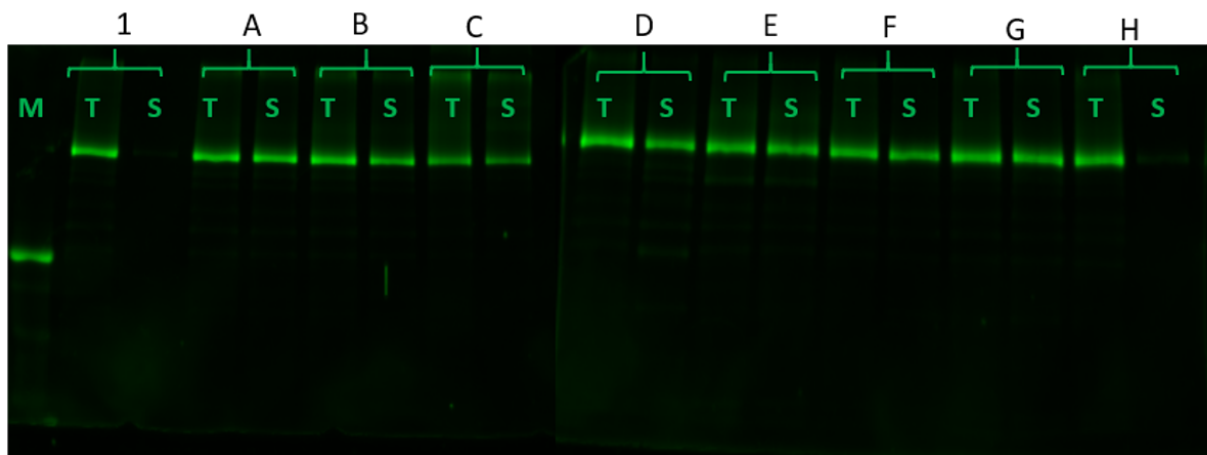
1. If frozen, let the yeast suspension defreeze on ice for 1-2 hours. It is important that the solution is homogeneous and without clumps.
2. Lyse cells with a high-pressure cell disrupter Constant Systems (Ltd.). Follow the manufacturer's guidelines for operation of the cell disrupter. We have observed that, with an increased number of passages, the membranes must be pelleted at higher centrifugation speeds. Here, we describe a protocol where we do just one passage.
3. Centrifuge 3 min. at 3000 xg then 5 min. at 6000 xg to get rid of unbroken cells and debris.
4. Dilute the supernatant with suspension buffer (7 mL lysate + 21 mL buffer per tube, here we are using a JA 20 rotor (Beckmann Coulter)) and pellet the membranes at 20,000 xg, 1 h, 4 °C. Take a small aliquot of the supernatant for further analysis.
5. Resuspend the membranes in ~10 mL of suspension buffer, adjust to the desired protein concentration and freeze them in liquid nitrogen.
6. To check the quality of the membrane preparation, perform a SDS-PAGE (8% in our case). The samples (5 µL each) were deposited per well and migration was carried on at 180 V. The in-gel fluorescence was visualized with an imager (Typhoon 9500 FLA, GE Healthcare, excitation at 475 nm, emission >510 nm). Alternatively, fluorescence can be measured directly in solution with a fluorimeter.



### 3.5 Detergent screening and protein purification

Due to the high sensitivity of the method, to screen for detergents which will best solubilize the protein of interest, small quantities of membranes can be used.

1. Thaw the frozen membranes on ice for 1-2h. Adjust them to the needed protein concentration (here 2 g/L).
2. Due to the smallest volume we can centrifuge, we use 190  $\mu\text{L}$  of membranes for each condition: in this case add 10  $\mu\text{L}$  of a 10% detergent solution and incubate under gentle agitation for 1h at 4°C.
3. Remove the non-solubilized material by centrifugation at 100,000  $\times g$  for 1 h, 4 °C. (Rotor MLA 120, Beckmann Coulter).
4. Run a SDS-PAGE 8%, with 10  $\mu\text{L}$  of sample per well and visualize the in-gel fluorescence (as described in §3.4). Here, we have analyzed the total content of samples prior to centrifugation and the supernatants obtained after this step.

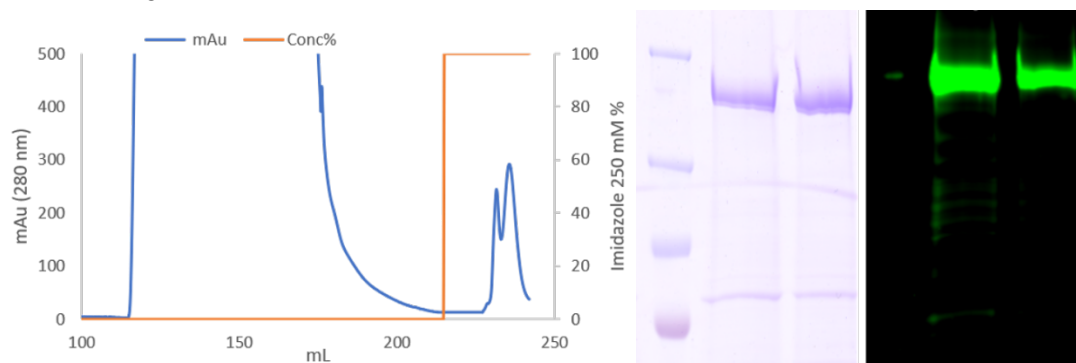


Most of the detergents work well in extraction, with the exception of 9b-CHS, which is unable to extract Cdr1p-GFP. In the case of OG, a fluorescence decrease is detected in both T and S fractions, probably hinting at an aggregation of a part of Cdr1p.

This sensitivity of detection can allow to carry on several purifications of small quantities of proteins, allowing to perform ATPase and binding assays on the purified protein.

As an example, here we show a purification procedure using trans-PCC  $\alpha$ -M :

1. Thaw the needed quantity of membranes on ice (here we use 10 mL at a concentration of 2 g/L).
2. Thaw the needed quantity of nanobodies (We put always an excess of nanobodies ex. We use 1 mg per purification, after that, the nanobodies can be re-used several times)
3. Extract the GFP-protein for 1 h under gentle agitation at 4 °C and centrifuge at 100,000 xg, 1 h to remove unsolubilized material.
4. Perform the purification using a 5-mL Ni-NTA column (Generon) equilibrated with buffer A2. Pass the nanobody solution at a flow rate of 2 mL/min. At this step, the nanobodies are bound to the Ni<sup>2+</sup> resin, wash with 3 CV of buffer A2 and pass the sample containing Cdr1p-GFP at a flow rate of 0.5 mL/min.
5. Wash thoroughly the column with buffer B2 until the Abs<sub>280nm</sub> falls back to 0.
6. Elute the whole complex using elution buffer at a flow rate of 1 mL/min, collect fractions of 1 mL.



This protocol allows to keep the whole complex together and the GFP can be used as a marker to choose the right fractions to analyze and use for biochemical assays, estimate the final quantity of proteins etc.

This purification highlights the efficiency of this strategy, aiming to harness the full potential of the GFP, as an antigen during the affinity purification step, and as a reporter. Alternatively, other purification methods can be considered, where the GFP can be cleaved using to elute only CaCdr1p. After this first purification step, the protein is ready for further usage.

## Figure captions

*Figure 1 : 14 % SDS-PAGE of purified anti-GFP nanobody. 10  $\mu$ L of different IMAC fractions (1-5) were visualized with Coomassie Brilliant Blue, with the nanobody band appearing at 13 kDa.*

*Figure 2 : In-gel fluorescence of CaCdr1p-GFP after SDS-PAGE migration. Lane 1: marker (75 kDa), lane 2: total lysate, lane 3: supernatant, lane 4: membranes containing CaCdr1p-GFP*

*Figure 3A: In-gel fluorescence of 10- $\mu$ L aliquots of total proteins (T) after 1 h of detergent extraction and of the supernatants (S) after centrifugation. Lane 1 is a negative control, while the detergents used for screening are: DDM (A); UDM (B); OG (C); LMNG (D); FC12 (E); GDN (F); trans-PCC  $\alpha$ -M (G); 9b-CHS (H).*

*Figure 3B: SDS-PAGE of total proteins after extraction and of the supernatants after centrifugation, as described in Fig.3A, with the band of Cdr1p-GFP shown by the blue arrow.*

*Figure 4A: An example of an IMAC chromatogram, with the apparition of two peaks during elution. A fluorescence measure on each fraction allowed to determine that the Cdr1p-GFP-anti GFP nanobody elutes in the first peak, while the excess nanobodies are in the second peak.*

*Figure 4B, C: An SDS-PAGE and in-gel fluorescence analysis of the main fractions of the peak containing Cdr1p-GFP, showing a predominant band at approximately 200 kDa, corresponding to the CaCdr1-GFP.*

## NOTES

1. If inoculated in the morning of Day 1, the 40-mL preculture will have a small OD<sub>600nm</sub> at the end of the day (0.1-0.2). Thus, the 500-mL culture will have a very low starting OD<sub>600nm</sub> and will be reaching the desired OD (3.5) by the very end of the next day. To avoid this, a 10-mL preculture can be inoculated overnight at 30°C before Day 1 and this can be used to inoculate the 40-mL preculture at a higher starting OD<sub>600nm</sub> (ex.0.2).
2. It is best if OD<sub>600nm</sub> is reached early in the morning, so you can add the YP 5X medium and wait for a final OD<sub>600nm</sub> of 3.5 (it will take 4-6 hours). To do so, prepare the precultures as described at Note 1, then inoculate the 500-mL culture with the desired initial OD<sub>600nm</sub> after calculating the number of generation cycles needed to reach OD<sub>600nm</sub> = 1.5 in the morning (estimated generation time = 120 min.)

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