

## High-level Production of Recombinant Membrane Proteins Using the Engineered *Escherichia coli* Strains SuptoxD and SuptoxR

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**[Abstract]** We have previously described the development of two specialized *Escherichia coli* strains for high-level recombinant membrane protein (MP) production. These engineered strains, termed SuptoxD and SuptoxR, are capable of suppressing the cytotoxicity caused by MP overexpression and of producing greatly enhanced MP yields. Here, we present a Bio-protocol that describes gene overexpression and culturing conditions that maximize the accumulation of membrane-integrated and well-folded recombinant MPs in these strains.

**Keywords:** Recombinant protein production, Membrane protein, Toxicity, *Escherichia coli*, SuptoxD, SuptoxR

**[Background]** MPs perform a variety of critical functions in the cells of all living organisms (Wagner *et al.*, 2006; Schlegel *et al.*, 2010) and constitute major targets for current and future pharmaceuticals (Yildirim *et al.*, 2007). Acquiring sufficient amounts of isolated protein is a prerequisite for their biochemical and structural studies, which in turn can lead to a deeper understanding of their functions and the discovery of new MP-targeting drugs.

Because MPs are typically encountered in their native environments at very low abundances, heterologous hosts have been routinely used for their recombinant overexpression and subsequent purification. Many different systems have been utilized as overexpression hosts for a large variety of MPs of both prokaryotic and eukaryotic origin (Wagner *et al.*, 2006). Among these, *Escherichia coli* has been one of the most popular ones, owing to its very low cost and ease of use (Makino *et al.*, 2011). Indeed, this bacterium has been successfully utilized for the production of approximately 20% of all recombinantly produced MP structures that are deposited in the Protein Data Bank (Dilworth *et al.*, 2018). Despite these advantages and successes, the use of *E. coli* as a heterologous host for MP production is often accompanied by severe toxicity, low levels of final biomass and minute final yields (Miroux and Walker, 1996; Wagner *et al.*, 2007; Link *et al.*, 2008; Gubellini *et al.*, 2011).

In order to address these challenges, we have recently developed two specialized *E. coli* strains, named SuptoxD and SuptoxR, which enable high-level production of recombinant MPs (Gialama *et al.*, 2017a and 2017b). When used as expression hosts, these strains exert a dual positive effect compared to wild-type bacteria:

(1) They suppress the toxicity that frequently accompanies the MP overexpression process, thus enabling enhanced levels of final bacterial biomass, and

(2) They markedly increase the cellular accumulation of membrane-incorporated and properly folded protein.

Combined, these two positive effects result in dramatically enhanced volumetric yields for various recombinant MPs (Gialama *et al.*, 2017a and 2017b). Importantly, these strains have been optimized to enable the production of high-quality recombinant MPs at quantities sufficient for functional and structural studies (Michou *et al.*, 2019). Up to now, we have tested a broad panel of recombinant MPs of both prokaryotic and eukaryotic origin and with different characteristics, all of which are described in Gialama *et al.* (2017a and 2017b) and in Michou *et al.* (2019).

The toxicity-suppressing and cellular production-promoting capabilities of SuptoxD and SuptoxR are based on the overexpression of either one of the effector genes *djlA* or *rraA*, respectively (Gialama *et al.*, 2017a). *DjlA* (*DnaJ*-like protein *A*) is a single-pass integral MP that functions mainly as a co-chaperone for the central bacterial molecular chaperone DnaK (Clarke *et al.*, 1996). On the other hand, RraA (Regulator of ribonuclease activity A) is known to act as a regulator of the mRNA-degrading activity of RNase E, and *rraA* overexpression has been found to affect the levels of more than 2,000 different mRNAs in *E. coli* (Lee *et al.*, 2003). We have previously found that (i) *DjlA* and *RraA* act independently, *i.e.*, the beneficial effects of each protein on recombinant MP production occur through a mechanism that does not involve the other, and in a non-additive manner; (ii) full-length and membrane-bound *DjlA* is required for exerting its beneficial effects on recombinant MP production in *E. coli* SuptoxD; (iii) the MP production-promoting properties of *DjlA* in SuptoxD are mediated through the action of the molecular chaperone DnaK; (iv) the observed *RraA*-mediated effects in *E. coli* SuptoxR involve the ribonucleolytic activity of RNase E; and (v) *DjlA* and *RraA* are unique among similar *E. coli* proteins in their ability to promote bacterial recombinant MP production (Gialama *et al.*, 2017b). The exact molecular mechanism with which *DjlA* and *RraA* suppress MP-induced toxicity and enhance recombinant MP production in *E. coli* SuptoxD and SuptoxR, respectively, is still under investigation.

Here, we present a protocol that describes gene overexpression and culturing conditions that maximize the accumulation of membrane-integrated and well-folded recombinant MPs when using SuptoxD and SuptoxR. As structural biology of MPs has entered a new era, we believe that these specialized strains will be broadly utilized to address some of the important challenges of MP production and will facilitate the acquirement of sufficient quantities of high-quality recombinant MPs.

## **Materials and Reagents**

1. Nylon-membrane Syringe filter 0.2  $\mu\text{m}$  pore size, 25 mm diameter, sterile (Corning, catalog number: CLS431224)
2. HiLoad Superdex 200 16/600 column (GE Healthcare, catalog number: GE28-9893-35)
3. Sterile pipette tips, 10-200  $\mu\text{l}$  (Greiner Bio, catalog number: 739290)
4. Sterile pipette tips, 200-1,000  $\mu\text{l}$  (Greiner Bio, catalog number: 740290)
5. Sterile culture tubes (Sigma-Aldrich, catalog number: C1048-72EA)
6. Sterile closures for culture tubes (Sigma-Aldrich, catalog number: C1298-100EA)

7. Sterile centrifugation tubes 1.5 ml (Eppendorf, catalog number: 616201)
8. Sterile Falcon tubes 15 ml (Greiner Bio, catalog number: 188271)
9. Sterile Falcon tubes 50 ml (Greiner Bio, catalog number: 227261)
10. Semi-micro-cuvettes (Greiner, catalog number: 613101)
11. Polypropylene centrifuge bottles, 500 ml (Celltreat, catalog number: 229468)
12. Polycarbonate ultracentrifuge bottles, 3 ml (Beckman, catalog number: 355618)
13. Polypropylene chromatography columns, 5 ml (Pierce, catalog number: 29922)
14. Amicon® Ultra-15 Centrifugal Filter Unit (Merck, catalog number: UFC901024)
15. 96-well black plates for fluorescence measurements (Greiner, F-bottom, catalog number: 655076)
16. Polyvinylidene fluoride (PVDF) membrane (Merck, catalog number: IPVH00010)
17. *E. coli* SuptoxD cells  
Genotype: F-  $\lambda^-$   $\Delta(ara-leu)7697$  [araD139]<sub>B/r</sub>  $\Delta(codB-lacI)3$  galK16 galE15 e14- mcrA0 relA1 rpsL150(StrR) spoT1 mcrB1 hsdR2(r-m+) pSuptoxD  
Note: *E. coli* SuptoxD carry either the pSuptoxD or the pSuptoxD[untagged] plasmid (see below).
18. *E. coli* SuptoxR cells  
Genotype: F-  $\lambda^-$   $\Delta(ara-leu)7697$  [araD139]<sub>B/r</sub>  $\Delta(codB-lacI)3$  galK16 galE15 e14- mcrA0 relA1 rpsL150(StrR) spoT1 mcrB1 hsdR2(r-m+) pSuptoxR  
Note: *E. coli* SuptoxR carry either the pSuptoxR or the pSuptoxR[untagged] plasmid (see below).
19. *E. coli* MC1061 cells (Coli Genetic Stock Center, catalog number: 6649)  
Genotype: F-  $\lambda^-$   $\Delta(ara-leu)7697$  [araD139]<sub>B/r</sub>  $\Delta(codB-lacI)3$  galK16 galE15 e14- mcrA0 relA1 rpsL150(StrR) spoT1 mcrB1 hsdR2(r-m+)  
Note: We have used *E. coli* MC1061 as a background host for the co-expression the *djlA* and *rraA* effector genes and for the evaluation of the performance of SuptoxD and SuptoxR extensively and successfully. However, we have found that the beneficial effects of *DjlA* and *RrraA* on MP productivity are independent of the use of MC1061 and that other *E. coli* K-12 and *B* strains can also be utilized as background hosts (Gialama *et al.*, 2017a).
20. Plasmid pASK-MP  
Notes:
  - a. This plasmid can be generated by cloning a gene encoding a prokaryotic or eukaryotic MP into the pASK75 vector backbone (Biometra, Göttingen) (Skerra, 1994). We recommend flanking the MP's sequence between the XbaI and anyone of the remaining restriction sites of the plasmid's multiple cloning site (MCS), in order to remove the OmpA signal sequence.
  - b. In order to facilitate MP purification via immobilized metal affinity chromatography (IMAC), we recommend inserting a poly-Histidine tag in-frame of the expressed MP. However, other tags and means of MP purification can be used according to the user's particular preferences and needs.
  - c. In order to easily monitor MP production, we recommend fusing the GFP reporter protein downstream of the MP of interest (Drew *et al.*, 2001 and 2008).

d. We have used the vector pASK-MP extensively for production of various recombinant MPs in *E. coli* SuptoxD and SuptoxR. However, we have found that the beneficial effects of these strains on MP productivity are independent of the use of the tet promoter of pASK75 (Gialama *et al.*, 2017a). Thus, recombinant MPs can be overexpressed in these strains using other types of promoters and plasmids as well.

21. pSuptoxD[untagged]

Note: This plasmid overexpresses *djlA*, the gene encoding the *E. coli* membrane-bound DnaK co-chaperone *DjlA*, upon induction with L(+)-arabinose.

22. pSuptoxD

Notes:

- a. This plasmid overexpresses *djlA*, the gene encoding the *E. coli* membrane-bound DnaK co-chaperone *DjlA*, with a poly-histidine tag at its C-terminus, upon induction with L(+)-arabinose.
- b. Overexpression of the SuptoxD system can be monitored by western blotting using an anti-His antibody, according to standard protocols. The molecular weight of the expressed protein, *i.e.*, *DjlA-His<sub>6</sub>*, is ~32 kDa.
- c. As we have found that the presence of the poly-histidine tag does not affect the activity of *DjlA*, we recommend using an untagged version of pSuptoxD, *i.e.*, pSuptoxD[untagged], for MP purification via IMAC.

23. pSuptoxR[untagged]

Note: This plasmid overexpresses *rraA*, the gene encoding the inhibitor of the *E. coli* RNase E *RraA*, upon induction with L(+)-arabinose.

24. pSuptoxR

Notes:

- a. This plasmid overexpresses *rraA*, the gene encoding the inhibitor of the *E. coli* RNase E *RraA*, with a poly-histidine tag at its C-terminus upon induction with L(+)-arabinose.
- b. Overexpression of the SuptoxR system can be monitored by western blotting using an anti-His antibody, according to standard protocols. The molecular weight of the expressed protein, *i.e.*, *RraA-His<sub>6</sub>*, is ~20 kDa.
- c. As we have found that the presence of the poly-histidine tag does not affect the activity of *RraA*, we recommend using an untagged version of pSuptoxR, *i.e.*, pSuptoxR[untagged], for MP purification via IMAC.

25. Sodium chloride for analysis, ACS, ISO (Applichem, catalog number: 131659)

26. Tryptone BioChemica BC (Applichem, catalog number: A1553)

27. Yeast extract BioChemica BC (Applichem, catalog number: A1552)

28. Agar bacteriology grade BC (Applichem, catalog number: A0949)

29. Ampicillin sodium salt (Sigma-Aldrich, catalog number: A9518)

30. Chloramphenicol (Sigma-Aldrich, catalog number: C0378)

31. L(+)-Arabinose (Applichem, catalog number: A9728)

32. Anhydrotetracycline hydrochloride (Sigma-Aldrich, catalog number: 37919)
33. Sodium hydroxide (NaOH)
34. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9333)
35. Sodium phosphate dibasic (Na<sub>2</sub>HPO<sub>4</sub>) (Sigma-Aldrich, catalog number: S7907)
36. Sodium dihydrogen phosphate, anhydrous (Chemlab, catalog number: CL00.1496)
37. Potassium phosphate monobasic (KH<sub>2</sub>PO<sub>4</sub>) (Sigma-Aldrich, catalog number: P5655)
38. Tris Base (Fisher, catalog number: BP154-1)
39. Tween 20 (Fisher, catalog number: BP337)
40. Mini-PROTEAN TGX Precast 4-20% resolving gels (Bio-Rad, catalog number: 456-1094)
41. Non-fat dry milk (Sigma-Aldrich, catalog number: M7409)
42. Monoclonal anti-polyhistidine-peroxidase antibody produced in mouse (Sigma-Aldrich, catalog number: A7058)
43. Pierce ECL western blotting substrate kit (Thermo Fisher Scientific, catalog number: 32106)
44. n-dodecyl β-D-maltoside (DDM) (Glycon Biochemicals, catalog number: D97002)
45. Glycerol (Fisher, catalog number: G/0650/21)
46. Imidazole (Applichem, catalog number: A1073)
47. β-mercaptoethanol (Sigma, catalog number: M6250)
48. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626)
49. Ni-NTA Agarose (Qiagen, catalog number: 30230)
50. Ethanol absolute (ACROS Organics, catalog number: 448450025)
51. Luria-Bertani broth (LB) (see Recipes)
52. Antibiotics (see Recipes)
53. Inducers of protein production (see Recipes)
54. Lysis buffer (see Recipes)
55. Wash buffer (see Recipes)
56. Elution buffer (see Recipes)
57. Sodium dodecyl sulphate (SDS) sample buffer (6x) (see Recipes)
58. Tris-buffered saline with Tween-20 (TBST) (see Recipes)
59. Phosphate-buffered saline (PBS) (see Recipes)
60. DDM solubilization buffer (see Recipes)
61. Size exclusion buffer (see Recipes)

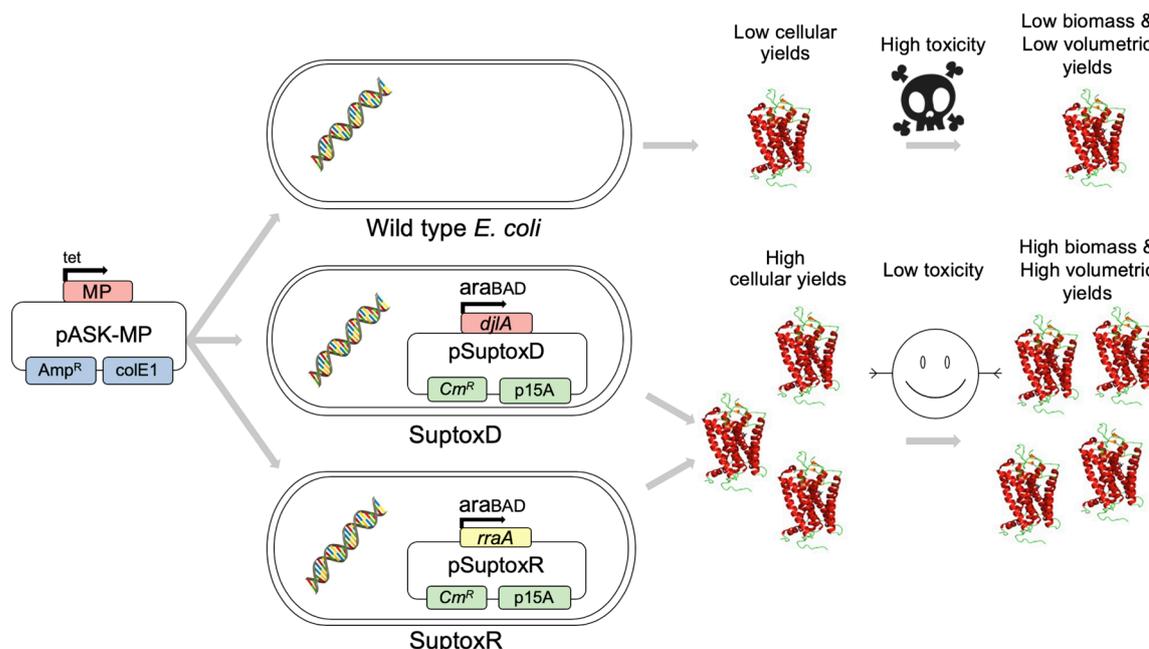
## **Equipment**

1. Centrifuge MiniSpin (Eppendorf, Mini Spin, catalog number: 5452000018)
2. High-speed refrigerated centrifuge (Kubota, catalog number: 7780)
3. Ultracentrifuge (Beckman Coulter, model: Optima LE-80K)
4. Shaking incubator (Eppendorf, model: New Brunswick™ Innova® 44, catalog number: M1282-0006)

5. Roller mixer (Kisker, catalog number: L005-SLN)
6. Microplate reader (Tecan, model: Safire II)
7. Electrophoresis power supply (Consort, catalog number: EV231)
8. Imaging system for DNA and protein analysis (ChemiDoc-It2 Imaging System (UVP))
9. Sonicator equipped with a 3 mm diameter probe (Qsonica, catalog number: Q125-110)
10. Spectrophotometer UV-VIS (Hitachi, model: U2000)
11. Mini PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad, catalog number: 1658005)
12. Type 70Ti fixed-angle titanium rotor (Beckman, catalog number: 337922)
13. Homogenizer (Heidolph, model: RZR1)

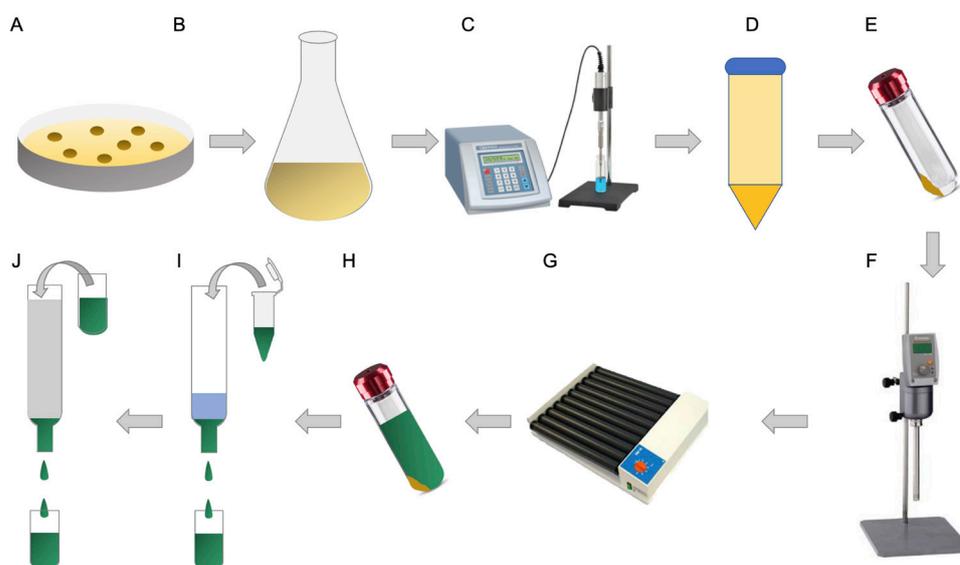
## Procedure

The use of the engineered *E. coli* strains SuptoxD and SuptoxR for the high-level production of recombinant MPs is outlined in Figure 1.



**Figure 1. Overview of the *E. coli* SuptoxD and SuptoxR systems.** The toxicity-suppressing and cellular production-promoting capabilities of *E. coli* SuptoxD and SuptoxR that result in dramatically enhanced volumetric yields for various recombinant MPs, are based on the overexpression of the *E. coli* genes *djlA* or *rraA*, respectively. The effector genes are overexpressed from the vectors pSuptoxD and pSuptoxR under the control of the araBAD promoter and its inducer L(+)-arabinose. For the production of recombinant MPs in these strains, we typically use pASK75-based plasmids under the control of a *tet* promoter and its inducer aTc (pASK-MP vector).

The procedure of the recombinant MP production and purification is outlined in Figure 2.



**Figure 2. Overview of the recombinant MP production and purification procedure using the *E. coli* SuptoxD and SuptoxR strains.** A. Transform either one of the SuptoxD or SuptoxR strains with the plasmid encoding the MP of interest. B. Overexpress the MP of interest together with the pSuptoxD or pSuptoxR system, at 25 °C for 16 h and using the appropriate inducers. C. Lyse cells by sonication. D. Centrifuge the total cell lysates and transfer the soluble supernatant into an ultracentrifugation tube. E. Ultracentrifuge the soluble cell lysate and collect the pelleted membranes. F. Mechanically resuspend the membranes in DDM-solubilization buffer using a homogenizer. G. Rotate for at least 1 h at 4 °C. H. Ultracentrifuge and collect the supernatant containing the solubilized membranes. I-J. Purify the solubilized membranes using IMAC and SEC.

A. MP overexpression in shake flasks (Under sterile conditions)

1. Transform (chemically or electro-) competent SuptoxD or SuptoxR cells with the pASK-MP vector and plate on LB agar containing 100 µg/ml ampicillin and 40 µg/ml chloramphenicol.

*Notes:*

- a. When performing recombinant production of a specific MP of interest for the first time, we recommend using both the SuptoxD and SuptoxR strains, in order to determine the particular strain that yields higher levels of MP production.
- b. For the expression of highly toxic or otherwise difficult-to-express MPs that accumulate at very low yields, we highly recommend to use the pSuptoxD or pSuptoxR plasmid in isolated form and perform double transformation of pSuptoxD or pSuptoxR with the pASK-MP plasmid in fresh competent *E. coli* MC1061 cells. Other *E. coli* strains can also be used as hosts for the pSuptox vectors.
- c. We have used the pASK75 vector backbone for the expression of the MP of interest extensively. However, other expression vectors can also be used, provided that they are

*compatible with the pSuptoxD/R system (chloramphenicol resistance, p15A origin of replication, araBAD promoter) and preferably contain an inducible promoter with tight regulation.*

2. Pick a single transformed bacterial colony and inoculate liquid LB cultures supplemented with the selection antibiotics 100 µg/ml ampicillin and 40 µg/ml chloramphenicol for pASK-MP and SuptoxD/R system maintenance. Incubate at 37 °C for 16 h with constant shaking at 200-220 rpm.

*Notes:*

- a. *We highly recommend using freshly transformed E. coli cells for all MP production experiments in order to obtain maximum protein yields.*
- b. *The volume of the LB cultures should be enough for next-day's inoculation.*
3. The following day prepare a 2% sub-culture in fresh LB supplemented with the appropriate antibiotics, as well as 0.01% or 0.2% (w/v) L(+)-arabinose for the induction of the SuptoxD and SuptoxR system respectively. Incubate at 30 °C with shaking until an optical density at 600 nm (OD<sub>600</sub>) of 0.5.

*Notes:*

- a. *The recommended L(+)-arabinose concentrations have been determined following expression optimization in SuptoxD/R using certain model recombinant MPs (Michou *et al.*, 2019). Other MPs, however, may require other concentrations for their optimal accumulation.*
- b. *Keep working stock solution of arabinose (20% w/v) in aliquots to avoid contamination.*
4. Initiate overexpression of the target MP by adding 0.2 µg/ml anhydrotetracycline (aTc) and incubate overnight at 25 °C with shaking.

*Notes:*

- a. *We highly recommend using freshly prepared and light-protected aTc.*
- b. *We highly recommend initiating MP overexpression at an OD<sub>600</sub> of precisely 0.5 as we have found that deviations result in lower protein yields.*
- c. *We recommend to not exceed 16 h of overexpression in order to minimize the accumulation of genomic/plasmid mutations that suppress MP production.*
- d. *The recommended aTc concentration has been determined following expression optimization in SuptoxD/R using certain model recombinant MPs (Michou *et al.*, 2019). Other MPs, however, may require other concentrations for their optimal accumulation.*
5. Harvest cells by centrifugation at 4,000 x g for 10 min at 4 °C.
6. Check for MP production by western blotting or activity assay according to standard protocols.

*Notes:*

- a. *Due to the MP-induced toxicity, it is not unusual for genomic/plasmid mutations to accumulate and bypass the overproduction of MP. This will result in a high increase of the culture's OD<sub>600</sub> and low yield of MP production. For this reason, MP production should always be checked before continuing with MP purification.*
- b. *When overexpressing an MP-GFP fusion, the bacterial fluorescence can be used as an*

*indicator of protein production. In this case, cells corresponding to an  $OD_{600} = 1$  should be resuspended in 100  $\mu$ l PBS and fluorescence should be measured:*

- i. Using a 96-well plate reader at 510 nm after excitation at 488.*
- ii. Using a flow cytometer equipped with a 488 nm solid state laser for excitation and a 530/30 nm band pass filter for detection. In this case, events should be first gated in a forward scatter (FSC) vs. side scatter (SSC) plot in order to eliminate non-cellular events and then plotted in a histogram of fluorescence intensity detected by the FITC (fluorescein isothiocyanate) channel photomultiplier tube (PMT) vs. number of events, in order to estimate the mean fluorescence of the population.*

## B. Bacterial membrane isolation and MP extraction

1. Resuspend a cell pellet from a 1 L culture in 10 ml ice-cold lysis buffer.
2. Lyse cells by sonication for 4 x 25 s on ice, with a 1 min interval between each sonication.
3. Centrifuge the total cell lysates (10,000 x g, 15 min, 4 °C) and transfer carefully the soluble supernatant into an ultracentrifugation tube.
4. Ultracentrifuge the soluble fraction at 130,000 x g for 1 h at 4 °C using a 70-Ti rotor, in order to pellet the bacterial membranes.
5. Collect the pelleted membranes using a spatula and mechanically resuspend them in 5 ml ice-cold DDM-solubilisation buffer using a homogenizer. The homogenate should contain no visible particles.

*Note: Care should be taken when collecting the membranes as they form a very sticky and difficult to handle pellet.*

6. Rotate the resuspension at 180 rpm for at least 1 h at 4 °C, in order for MP extraction to take place.

*Note: A range of different DDM concentrations and incubation periods should be tested in order to determine the optimal conditions for MP extraction. When using MP-GFP fusions or chromophore-containing MPs, such as certain bacteriorhodopsins (Michou *et al.*, 2019), this procedure can be easily optimized by regular centrifugations and monitoring of the fluorescence levels or discoloration respectively, of the resulting pellet.*

7. Ultracentrifuge at 130,000 x g for 1 h at 4 °C and collect the supernatant that contains the extracted MP.
8. Check for successful MP extraction by western blotting or activity assay according to standard protocols.

*Note: When overexpressing an MP-GFP fusion, we highly recommend using in-gel fluorescence analysis as a quality control of the extraction of well-folded and active MP (Geertsma *et al.*, 2008). In this case, the following procedure should be followed:*

- a. Mix 100  $\mu$ l of the supernatant containing the extracted MP with 20  $\mu$ l of 6x SDS loading dye.
- b. Load 50  $\mu$ l of sample on a 4-20% precast polyacrylamide gel without prior boiling of the sample and run the gel at a constant voltage of 80 V.

- c. Visualize the MP-GFP fluorescent band on an imaging system, e.g., ChemiDoc-It2 Imaging System, equipped with a CCD camera and a GFP filter, after exposure of about 3 s.
  - d. Western blotting of the analysed proteins according to standard protocols should result in the visualization of the MP's dual band migration, with the lower band corresponding to the fluorescent and well-folded MP-GFP fusion and the upper band corresponding to non-fluorescent and misfolded MP-GFP fusion (Drew *et al.*, 2008; Geertsma *et al.*, 2008).
  - e. Analyzing the same samples after boiling for 10 min with SDS-PAGE and western blotting results in full denaturation of the fusion and single band migration that lacks fluorescence.
9. Store the supernatant on ice until further use or purification.
- C. Small-scale, two-step MP purification for initial studies using immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC) (All steps should be performed at 4 °C)
1. Transfer 1 ml of Ni-NTA agarose resin to a 15 ml tube and remove the supernatant after brief centrifugation at low speed (1,000 x g, 1 min). Resuspend in 2 ml lysis buffer and mix by gently inverting.
  2. Mix the supernatant containing the extracted MP with the equilibrated resin from the above step at 4 °C for 1 h on a roller mixer.
  3. Equilibrate a 5 ml polypropylene chromatography column with 1 column volume (CV) of lysis buffer and load the mixture onto the equilibrated column.
  4. Collect the flow-through, and reload it onto the column. Repeat for a total of 5-6 times.  
*Note: This step should be repeated multiple times in order to ensure complete binding of the MP to the nickel resin. When using MP-GFP fusions or chromophore-containing MPs, this step should be repeated until a non-fluorescent or colourless flow-through is collected.*
  5. Wash the column-bound protein with 40 ml wash buffer.
  6. Elute the MP with 6 ml elution buffer.
  7. Concentrate the elution to a final volume of approximately 500 µl using a centrifugal filter.
  8. Equilibrate a Superdex 200 column with 2 CV of size exclusion buffer at 1 ml/min, ensuring that the pressure does not exceed the maximum limit of 0.3 MPa.
  9. Inject the concentrated sample onto the column and pass 1 CV of size exclusion buffer through the system while collecting the eluted fractions.
  10. Pool together the fractions that correspond to the MP's peak.
  11. Quantify the amount of purified MP by calculating the absorbance at 280 nm of the integrated peak area and using the MP's extinction coefficient ( $\epsilon_{280}$ ).  
*Note: By following this protocol, and using the SuptoxD strain we have obtained 1 mg per L of bacterial culture of purified human bradykinin receptor 2 (BR2), which under normal conditions exhibits very high levels of toxicity and low yields when overexpressed in E. coli (Michou *et al.*, 2019). This amount was 14-fold higher than the corresponding yield from wild-type E. coli. Similar results were achieved by the use of SuptoxR in overexpressing the large bacterial*

*mechano-sensitive ion channel (MscL), in which case we isolated 0.33 mg per L of bacterial shake flask culture, a > 10-fold increased yield compared to wild-type E. coli (Michou et al., 2019). However, other means of MP purification can be used according to the user's particular preferences and needs.*

12. Evaluate MP purity by SDS-PAGE analysis and Coomassie staining.

*Note: For highly concentrated MP fractions, we recommend running the gel at a very low constant current, such as 12-13 mA.*

## **Recipes**

1. Luria-Bertani broth (LB)
  - 10 g/L tryptone
  - 5 g/L yeast extract
  - 10 g/L NaCl
  - Adjust pH to 7.0 with 5 N NaOH
  - Autoclave for sterilization for 20 min at 15 psi
2. Antibiotics
  - a. 40 mg/ml chloramphenicol in ethanol 99.8%, store at -20 °C
  - b. 100 mg/ml ampicillin in MilliQ water, sterilize the solution using a 0.22 µm syringe filter, store at -20 °C
3. Inducers of protein production
  - a. 20% w/v L(+)-arabinose in MilliQ water, sterilize the solution using a 0.22 µm syringe filter, store at room temperature
  - b. 20 mg/L anhydrotetracycline (aTc) in ethanol 99.8%, store at -20 °C
4. Lysis buffer
  - 300 mM NaCl
  - 50 mM NaH<sub>2</sub>PO<sub>4</sub>
  - 15% glycerol
  - 5 mM dithiothreitol (add immediately before use)
  - 0.1 mM PMSF (add immediately before use)
  - Adjust pH to 7.5
  - Store at 4 °C
5. Wash buffer
  - 20 mM imidazole
  - 300 mM NaCl
  - 50 mM NaH<sub>2</sub>PO<sub>4</sub>
  - Adjust pH to 8
  - Add 0.1% w/v DDM (add immediately before use)
  - Store at 4 °C

6. Elution buffer
  - 250 mM imidazole
  - 300 mM NaCl
  - 50 mM NaH<sub>2</sub>PO<sub>4</sub>
  - Adjust pH to 8
  - Add 0.1% w/v DDM (add immediately before use)
  - Store at 4 °C
7. Sodium dodecyl sulphate (SDS) sample buffer (6x)
  - 6% w/v SDS
  - 300 mM Tris
  - 15% v/v glycerol
  - 0.01% w/v bromophenol blue
  - 10% v/v β-mercaptoethanol
  - Adjust pH to 6.8
8. Tris-buffered saline with Tween-20 (TBST)
  - 20 mM Tris base
  - 150 mM NaCl
  - Adjust pH to 7.5
  - 0.1% v/v Tween-20
9. Phosphate-buffered saline (PBS)
  - 137 mM NaCl
  - 2.7 mM KCl
  - 10 mM Na<sub>2</sub>HPO<sub>4</sub>
  - 1.8 mM KH<sub>2</sub>PO<sub>4</sub>
  - Adjust pH to 7.4
  - Autoclave for sterilization for 20 min at 15 psi
10. DDM solubilization buffer
  - Lysis buffer
  - 2.5% w/v DDM (add immediately before use)
11. Size exclusion buffer
  - This buffer should be selected according to the characteristics of the MP of interest. Some examples include:
    - PBS supplemented with 0.1% w/v DDM or 10 mM HEPES, 0.4 M NaCl, 0.05% w/v DDM, pH 7.2

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### **Competing interests**

GS is inventor on a patent application for *E. coli* SuptoxD and SuptoxR (PCT/EP2017/025168). The authors declare no other competing interests.

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