Copolymer Purification Protocol for Screening Approach

Based on the Masterclass by MIT's Greg Dodge (PhD): SMALPs as a platform to study bacterial membrane proteins in a native-like environment

Overview

Efficient copolymer solubilization and stabilized membrane protein purification are essential for ensuring quality and suitability in various applications. Streamlining the screening process is key to maximizing the output in minimal time. This collaborative protocol by Dr. Greg Dodge (Barbara Imperiali Group: MIT) and Cube Biotech offers a systematic approach to copolymer purification, prioritizing the efficiency to expedite research and development.

It is fast, reliable and can be performed in all laboratories.

For optimal results you will need access to an ultracentrifuge for membrane preparation. For a rapid readout and stability screening over a wide range of temperatures the use of Nanotemper's Prometheus Panta is recommended.

Attention: The kit can also be used without an ultracentrifugation step and the Prometheus Panta device! Other DLS instruments can be used in addition.

Please contact us if you have questions or need assistance optimizing the protocol for your application: contact@cube-biotech.com

What you need:

1. Cube Biotech Synthetic Nanodisc Screening Kit MAXI
2. Buffers (optimized for protein of interest)
3. pH-Meter
4. Protease inhibitors
5. Magnetic stirrer and stirrer magnets
6. Cell Disruptor e.g. Sonicator/ French Press (Optional for mammalian cells)
7. Protein purification resin or magnetic beads
8. Optional but advised:
   - (Ultra-) Centrifuge with rotor for small quantities e.g. TLA-100
   - Nanotemper's Prometheus Panta or other DLS device
9. Spectrophotometer / BCA - / Bradford assay
10. Gel electrophoresis apparatus and SDS-gel
11. Personal protective equipment (lab coat, gloves, goggles)
12. Pipettes and pipette tips (various sizes)
13. Glassware (flasks, beakers, test tubes)
14. Laboratory consumables (e.g., tubes, vials, sealing films)

What you can extract:

Membrane Proteins from all organisms and all membranes. All mammalian cells can be lysed directly by the copolymers found in the kit without the necessity of membrane preparation or cell lysis. For maximum purity it is advised to prepare membrane pellets.

Working with bacterial (exception: outer membrane localization) or plant cells requires a cell lysis pre polymer step.

Protocol for Membrane Protein Purification from *E. coli* Membrane Pellets using Agarose: e.g. Strep-Tactin®XT Resin

Buffers:

Buffer composition highly depends on target protein properties, used polymers and chromatography resins. Please research the optimal buffer conditions for your target upon starting. The following buffers are a suggestion:

A: 50 mM Hepes pH 8.0, 150 mM NaCl

B: 50 mM Hepes pH 8.0, 150 mM NaCl, 50 mM Biotin

1. Lyse the cell pellet in buffer A, include protease inhibitors (adjust pH).
2. Perform a 9000 x g centrifugation for 30 min to spin down cell debris, use the supernatant and take an SDS sample.
3. Perform a 100.000 x g centrifugation for 60 min to isolate the membrane fraction / cell envelope fraction (CEF) and take an SDS sample of the supernatant.
4. Dissolve the membrane pellet / CEF in a small amount of buffer without protease inhibitors or any additives absorbing at 280 nm or nearby.
5. Measure the absorption of the mix in a Nanodrop or UV-VIS at 280 nm. 50 mg/mL is an adequate amount for further solubilization studies (1 AU = ~1 mg/mL protein). If the concentration is too high, dilute with buffer A.

> Note: The total protein amount determination via UV absorption does not give you any insight into the amount of target membrane protein. Make sure your cells have a sufficient amount of overexpressed target protein before starting the screening. It is strongly recommended to check the screening success via Western Blot and SDS-PAGE after elution.

6. Open the tubes from the Cube Synthetic Nanodisc Screening Kit MAXI and set to a stock concentration of 10 % for each polymer - one 50 mg tube equals 500 µl 10 % solution.
7. This protocol uses a 1 % final polymer concentration and a 150 µl final volume per sample, which equals 15 µL of polymer stock per sample. This is a starting point. If a turbid solution is still observed after solubilization, a higher polymer concentration, e.g., 2.5 % is strongly advised to boost the final target protein yield. Directly starting with 2.5 % polymer concentration right away is also an option.
8. Label the required number of clean tubes for each screening approach (23 tubes for whole screening kit).
9. Dispense 60 µL buffer A into each tube (1 % final polymer concentration) / 37.5 µL buffer A (2.5 % final polymer concentration).
10. Dispense 75 µL CEF @ 50 mg/mL into each tube.
11. Pipette 15 µL of each polymer for a 1 % final concentration / 37.5 µl for a 2.5 % final concentration into corresponding tubes, pipette up and down to mix.

12. Incubate mix while rotating at room temperature for 1-2 hour (e.g. put tubes into a 50 mL tube and rotate/ use mini magnet stirrers and place tubes on a stirrer plate)

13. Label a set of tubes for the ultracentrifugation rotor (e.g. TLA-100 rotor)

14. After 1-2 h, transfer each solubilization sample to labeled TLA-100 tubes and take an SDS sample. Spin down at 100.000 x g for 30 - 60 min at 4 °C and take an SDS sample of the supernatant afterwards. Go on working with the supernatant.

15. While the centrifuge is running, pipette 50 % Strep-Tactin®XT slurry into a clean tube. Spin for 30 seconds at 700 g to pellet the resin. Carefully aspirate the supernatant and add resin volume of buffer A (e.g. 500 µL for 1 ml 50 % Strep-Tactin®XT slurry). Pipette up and down to mix the resin. Repeat this step 3 times to equilibrate the beads.

16. Resuspend fully equilibrated beads and distribute 40 µL into fresh tubes, one for each polymer.

17. After the centrifuge finishes, carefully transfer supernatant from each centrifuge tube to a fresh tube containing the Strep-Tactin®XT resin. Pipette up and down to mix.

18. Batch bind to resin, rotating the mixture at RT for at least 30 min.

19. After incubation, pellet the resin in a tabletop centrifuge for 30 seconds at 700 x g.

20. Carefully remove supernatant and discard.

21. Add 500 µL buffer A to each tube, to the pelleted resin and pipette up and down for proper washing

22. Pellet resin in a tabletop centrifuge for 30 seconds at 700 x g.

23. Repeat wash and pellet steps 3 times to fully remove unbound material and excess polymer.

24. At the end of the last wash, be sure to remove as much of the buffer as possible so your elutions are highly concentrated.

25. Add 30 µL buffer B to each tube, pipetting up and down to agitate resin. Incubate at RT for 10 minutes, spin for 30 seconds at 700 x g and transfer the elution into a fresh clean tube. Take an SDS sample.

26. Perform this step multiple times to elute all bound target protein.

27. Eluted samples will be very stable and can be stored at 4 °C for several days/weeks.

28. Load all taken SDS samples on a Gel and run an SDS-PAGE as well as a Western-Blot to check protein quality. The pre-binding samples give you valuable insight into your workflow.

29. The eluted target protein concentration can be measured via spectroscopy, Bradford assays or other protein determination assays.

For further quality control Nanotemper's Prometheus Panta is advised

Protocol for a Membrane Protein Purification from a Mammalian Cells Membrane Pellet using Magnetic Beads: e.g. Rho1D4

Mammalian cell lines often have lower overexpression levels than bacterial cells. This protocol uses double the CEF and 2.5 % polymer to increase the target protein elution quantity. In case of poor elution yield it is advised to increase CEF and buffer volumes / polymer concentration.

Buffers:

Buffer composition highly depends on target protein properties, used polymers and chromatography resins. Please research the optimal buffer conditions for your target upon starting. The following buffers are a suggestion:

A: 20 mM HEPES pH 7.5, 150 mM NaCl

B: 20 mM HEPES pH 7.5, 150 mM NaCl, 0.2-0.5 mg/ml Rho1D4 Peptide (recheck pH)

1. Lyse the cell pellet in buffer A, include protease inhibitors (if you want to work with whole cells, please refer to protocol addition below).
2. Perform a 9000 x g centrifugation for 30 min to spin down cell debris, use the supernatant and take an SDS sample.
3. Perform a 100.000 x g centrifugation for 60 min to isolate the membrane fraction/ cell envelope fraction (CEF) and take an SDS sample of the supernatant.
4. Dissolve the membrane pellet / CEF in a small amount of buffer without protease inhibitors or any additives absorbing at 280 nm or nearby.
5. Measure the absorption of the mix in a Nanodrop or UV-VIS at 280 nm. 50 mg/mL is an adequate amount for further solubilization studies (1 AU = ~1 mg/mL protein). If the concentration is too high, dilute with buffer A.

> Note: The total protein amount determination via UV absorption does not give you any insight into the amount of target membrane protein. Make sure your cells have a sufficient amount of overexpressed target protein before starting the screening. It is strongly recommended to check screening success via Western Blot analysis and SDS-PAGE after elution.

6. Open the tubes from the Cube Synthetic Nanodisc Screening Kit MAXI and set to a stock concentration of 10 % for each polymer - One 50 mg tube equals 500 µl 10 % solution.
7. This protocol uses 2.5 % final polymer concentration and a 300 µl final volume per sample, which equals 30 µL of polymer stock per sample. This is a starting point. If a turbid solution is still observed after solubilization, a higher polymer concentration or lower CEF is strongly advised to boost final target protein yield.
8. Label the required number of clean tubes for each screening approach (23 tubes for whole screening kit).
9. Dispense 75 µL buffer A into each tube (2.5 % final polymer concentration).

10. Dispense 150 µL CEF @ 50 mg/mL to each tube.

11. Pipette 75 µL each polymer for a 2.5 % final concentration into corresponding tubes, pipette up and down to mix.

12. Incubate mix while rotating at room temperature for 1-2 hours (e.g. put tubes into a 50 mL tube and rotate/ use mini magnet stirrers and place tubes on a stirrer plate).

13. Label a set of tubes for the ultracentrifugation rotor (e.g. TLA-100 rotor).

14. After 1-2 h, transfer each solubilization sample to labeled TLA-100 tubes and take an SDS sample. Note: You can adapt the sample quantity to the size of your preferred rotor.

15. Spin at 100.000 x g for 30 - 60 min at 4 °C and take an SDS sample of the supernatant afterwards. Go on working with the supernatant.

16. While the centrifuge is running, pipette 25 % Cube Biotech HighSpec Rho1D4 MagBeads (MBs) solution into a clean tube (use 25 µL of pure MBs per sample). Use a magnet to pull down the MBs. Carefully aspirate the supernatant and add buffer A. Pipette up and down to mix the resin. Repeat this step 3 times to equilibrate the beads.

17. Resuspend fully equilibrated beads and dispense 50 µL of freshly equilibrated 50 % MBs into fresh tubes, one for each polymer.

18. After the centrifuge finishes, carefully transfer the supernatant from each centrifuge tube to a fresh tube containing Cube Biotech Rho1D4 MBs.

19. Batch bind to resin by rotating at RT for at least 60 min.

20. After incubation, use a magnet to separate the MBs from the solution.

21. Carefully remove the supernatant and discard.

22. Add 500 µL buffer A to each tube, making sure to resuspend the pelleted MBs to wash.

23. Use a magnet to separate the MBs from the solution.

24. Repeat the wash and separation steps 5 times to fully remove all unbound material and excess polymer.

25. At the end of the last wash, be sure to remove as much of the buffer as possible so your elutions are highly concentrated.

26. Add 30 µL of buffer B to each tube, pipetting up and down to agitate the resin. Incubate at RT for 30 minutes and transfer the elution into clean tubes. Take an SDS sample.

27. Perform this step multiple times to elute all bound target protein.

28. Eluted samples are very stable and can be stored at 4 °C for several days/weeks.

29. Load all taken SDS samples on a Gel and Western-Blot to check protein quality. The pre-binding samples give you valuable insight into your workflow.

30. Eluted target protein concentration can be measured via spectroscopy, Bradford assays or other protein determination assays.

For further quality control Nanotemper's Prometheus Panta is advised

Protocol for a Membrane Protein Purification from a Mammalian Cells Membrane Pellet using Magnetic Beads: e.g. Rho - Without manual cell lysis and ultracentrifugation

When working with mammalian cells it is possible to solubilize and stabilize membrane proteins with the Cube Copolymer Platform directly from whole cells. It is important to add DNAse / Benzonase to the Mix to avoid an increase in viscosity. Adding Protease Inhibitors is advised. Be aware that protease inhibitors do interfere with UV-Vis measurement and some affinity purification materials.

Buffers:

Buffer composition highly depends on target protein properties, used polymers and chromatography resins. Please research the optimal buffer conditions for your target upon starting. The following buffers are a suggestion:

A: 20 mM HEPES pH 7.5, 150 mM NaCl

B: 20 mM HEPES pH 7.5, 150 mM NaCl, 0.2-0.5 mg/ml Rho1D4 Peptide (recheck pH)

1. Resuspend the cell pellet in buffer A in a 1:1 ratio, include protease inhibitors and DNAse/ Benzonase (e.g. 5 mL cell pellet volume in 5 mL buffer A)

2. Open the tubes from the Cube Synthetic Nanodisc Screening Kit MAXI and set to a stock concentration of 10 % for each polymer - One 50 mg tube equals 500 µl 10 % solution.

3. This protocol uses 2.5 % final polymer concentration and a 300 µl final volume per sample which equals 30 µL of polymer stock per sample. This is a starting point. If the elution quantity is unsatisfactory a higher polymer concentration and/ or larger sample volume is strongly advised to boost final target protein yield.

4. Label the required number of clean tubes for each screening approach (23 tubes for whole screening kit).

5. Dispense 270 µL cell suspension into each tube.

6. Pipette 30 µL of each polymer for a 2.5 % final concentration into corresponding tubes, pipette up and down to mix.

7. Incubate mix while rotating at room temperature for 1-2 hours (e.g. put tubes into a 50 mL tube and rotate/ use mini magnet stirrers and place tubes on a stirrer plate). Take an SDS sample.

8. Spin down the solution at 15.000 x g for 2 min to separate solution from cell fragments and take an SDS sample. Go on working with the supernatant.

9. While the centrifuge is running, pipette 25 % Cube Biotech HighSpec Rho1D4 MagBeads (MBs) solution into a clean tube (use 25 µL of pure MBs per sample). Use a magnet to pull down the MBs. Carefully aspirate the supernatant and add buffer A. Pipette up and down to mix the resin. Repeat this step 3 times to equilibrate the beads.

10. Resuspend fully equilibrated beads and dispense 50 µL of freshly equilibrated 50 % MBs into fresh tubes, one for each polymer.

11. After the centrifuge finishes, carefully transfer the supernatant from each centrifuge tube to a fresh tube containing Cube Biotech Rho1D4 MBs.

12. Batch bind to resin by rotating at RT for at least 60 min.
13. After incubation, use a magnet to separate the MBs from the solution.
14. Carefully remove the supernatant and discard.
15. Add 500 µL buffer A to each tube, making sure to resuspend the pelleted resin to wash.
16. Use a magnet to separate the MBs from the solution.
17. Repeat the wash and separation steps at least 5 times to fully remove all unbound material and excess polymer.
18. At the end of the last wash, be sure to remove as much of the buffer as possible so your elutions are highly concentrated.
19. Add 30 µL of buffer B to each tube, pipetting up and down to agitate the resin. Incubate at RT for 30 minutes and transfer the elution into clean tubes. Take an SDS sample.
20. Perform this step multiple times to elute all bound target protein.
21. Eluted samples are very stable and can be stored at 4 °C for several days/weeks.
22. Load all taken SDS samples on a Gel and Western Blot to check protein quality. The pre-binding samples give you valuable insight into your workflow.
23. Eluted target protein concentration can be measured via spectroscopy, Bradford assays or other protein determination assays.

For further quality control Nanotemper's Prometheus Panta is advised

Analysis of Target Protein Native Nanodiscs

The above purification protocol usually yields enough material for both SDS-PAGE analysis and analysis using a Nanotemper's Prometheus Panta nDSF instrument.

SDS PAGE

1. Dispense 2 µL of 6x reducing loading dye into # of PCR tubes corresponding to each polymer screened.
2. Add 10 µL of each elution to corresponding PCR tube.
3. Incubate at 45 °C for 30 min (This is CRITICAL to avoid aggregation of some membrane proteins)
4. Spin down samples, load 8 µL onto a SDS gel (2 µl for Western Blot analysis), run at 200 V for 26 min or as described in producer manual.

nDSF

1. Turn on DSF at the back power switch, open the Panta control software, enter sample names, and select HEPES + NaCl solvent for each sample.
2. Open the sample tray door using the touch screen on the instrument and remove the magnetic clamp. Clean the surface as per the instruction manual of the device.
3. Draw up eluted protein into number of capillaries corresponding to the number of polymers screened, and load onto sample tray of nDSF instrument. Take care to avoid air bubbles, and ensure the capillaries are ~70 % filled.
4. Re-seat magnetic clamp, making sure all 4 corners are fully set before closing the sample tray door.
5. Click start discovery scan in the control software, make sure there's a signal of at least 500 for each of your samples. This is also feasible as a fast insight into protein quantity comparison between the screening samples – the higher the signal, the higher the protein quantity measured by tryptophan fluorescence.
6. Add a measurement for size and run the default isothermal DLS experiment. These runs are quick – usually under 30 mins.
7. Once finished, add a measurement for thermal denaturation, and set the gradient to ~1°C or 1.5 °C per minute from 25 – 90 °C. Each run should take ~45 minutes.
8. Once the run is finished and cooled, dispose of sample capillaries in sharps waste, clean the surface of the sample area with a lens paper, close the same door and shut off the instrument.
9. Save your project file before closing the control software!
10. Data can be analysed and exported (including raw data for processing) using the analysis software module.