Purification of rho1D4-tagged Membrane Proteins Using PureCube Rho1D4 Agarose and PureCube 1-step Batch Midi Plus Columns

Overview

Tagging a membrane protein with the rho1D4 epitope to purify on an immunoaffinity matrix loaded with the rho1D4 antiobdy has proven to be an effective purification method for membrane proteins. Once the process is optimized, pure protein fractions (>85% purity) can generally be obtained. With a binding capacity of 3-4 mg protein per mL resin, PureCube Rho1D4 Agarose is a high-quality affinity matrix designed for the efficient purification of rho1D4-tagged membrane proteins.

The protocol uses the revolutionary 1-step batch Midi Plus Columns that feature the SelfSeal™ membrane technology, saving time and pipetting steps. They replace disposable gravity flow columns used in standard protocols. Volumes up to 20 mL can be applied to a Midi Plus Column. For small scale experiments, 1-step batch Mini Columns are available.

This protocol is optimized for tagged proteins expressed in *E. coli* and a bed volume of 0.1-1 mL. It is possible to scale up the protocol for higher volumes. The rho1D4-tagged target protein is purified from the cleared lysate under native conditions in a bind-wash-elute procedure. Binding is performed in batch mode (as opposed to on-column binding). This method is most efficient, especially when the target protein is present at low concentrations. Batch binding can be done directly in the 1-step batch Midi Plus column to simplify the procedure.

This procedure should be preceeded with screens for an optimal expression system and solubilization detergent. Cube Biotech provides general detergent screen protocols (www.cube-biotech.com/protocols). Also note that if the expressed protein is found mainly in inclusion bodies, it may be preferable to purify the protein on PureCube His Affinity matrices under denaturing conditions.

Please contact us if you have questions or need assistance optimizing a protocol for your application (contact@cube-biotech.com). Other protocols, e.g. for Western Blots using rho1D4 antibodies can also be found at www.cube-biotech.com/protocols.

Equipment

- ☐ PureCube 1-step batch Midi Plus Columns (Cube Biotech #63203)
- ☐ Ultrasonic homogenizer
- ☐ Ice bath
- ☐ Refrigerated centrifuge for 50 mL tubes (min 10,000 x g) and 2 mL tubes
- ☐ Refrigerated superspeed or ultracentrifuge capable of 100.000 x g
- ☐ End-over-end rotator
- ☐ 2 mL microcentrifuge tubes
- ☐ 15 mL polypropylene tube (e.g. Falcon)
- ☐ 50 mL polypropylene tube (e.g. Falcon)
- ☐ 50 mL polycarbonate high speed centrifuge tube
- ☐ Micropipettor
- ☐ Micropipetting tips
- ☐ pH meter
- ☐ UV/VIS spectrophotometer
- ☐ SDS-PAGE equipment
- ☐ Optional: Western Blot equipment

Materials

- ☐ Cell pellet from a 400-500 mL *E.coli* culture (approximately 1 g)
- ☐ PureCube Rho1D4 Agarose (1 mL; Cube Biotech #33101, 5 mL; #33102)
- ☐ Rho1D4 peptide (5 mg; Cube Biotech #16201)
- ☐ Sodium phosphate monobasic (NaH_2PO_4)
- ☐ Sodium chloride (NaCl)
- ☐ Detergent (e.g. OG, DDM, see Cube Detergents)
- ☐ Glycerol
- ☐ Lysozyme
- ☐ Benzonase® nuclease (e.g. Merck Milipore, #707464)
- ☐ Protease inhibitor cocktail (e.g. Roche cOmplete, #04693116001)
- ☐ Sodium dodecyl sulfate (SDS)
- ☐ Bromophenol blue
- ☐ Tris base
- ☐ Dithiothreitol (DTT)
- ☐ Triton X-100
- ☐ Bromophenol blue
- ☐ Sodium hydroxide (NaOH)
- ☐ Hydrochloric acid (HCl)
- ☐ Optional: Rho1D4 antibody (Cube Biotech #40020)

Solutions and buffers

Rho Buffer, 200 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
NaH_2PO_4*	10 mM	119.98	0.5 M	29.99 g/ 500 mL	4 mL
NaCl*	150 mM	58.44	5 M	146.1 g/ 500 mL	6 mL
Glycerol	10 % (v/v)	-	100 %	-	20 mL
Protease inhibitor	1x		follow supplier`s instructions		
Instructions: Mix in 160 mL water. Adjust the pH to 7.0 using NaOH and then add water to a total volume of 200 mL. Add the protease inhibitor directly before use. ***Note:** Depending on the protein purified, PBS at pH 7.4 may yield better results.					

Lysis Buffer, 20 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Rho Buffer	1x	-	1x	-	20 mL
Lysozyme	1 mg/mL				20 mg
Instructions: Always prepare fresh.					

Equilibration and Wash (EW) Buffer, 50 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Rho Buffer	1x	-	1x	-	50 mL
Detergent	based on screen**	-	-	-	based on screen**
Instructions: Always prepare fresh. ** Typically 1.5-2x critical micellar concentration (CMC) of detergent. Use the detergent that yielded the best solubilization reults in the detergent screen; see Cube protocol „Screening Detergents for Optimal Solubiization and Purification of Membrane Proteins"					

Elution Buffer, 10 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Rho Buffer	1x	-	1x	-	10 mL
Detergent	based on screen**				based on screen**
Rho1D4 peptide§	200 µM	947	10 mM	5 mg / 530 µL ddH_2O	200 µL
Instructions: Always prepare fresh. ** Typically 1.5-2x critical micellar concentration (CMC) of detergent. Use the detergent that yielded the best solubilization reults in the detergent screen; see Cube protocol „Screening Detergents for Optimal Solubiization and Purification of Membrane Proteins" § The recommended concentration of rho1D4 peptide in the elution buffer is 200 µM-1 mM. See the rho1D4 peptide Datasheet for further instructions to recontitute the lyophilized peptide.					

5X SDS-PAGE Buffer, 10 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Tris-HCl, pH 6.8–7.0	300 mM	121.14	1 M	121.14 g/ 1 L	3 mL
Glycerol	50% (v/v)	–	100% (v/v)	–	5 mL
SDS	5% (w/v)	–	–	–	0.5 g
Bromophenol blue	0.05% (w/v)	–	4%	–	125 µL
DTT	250 mM	154.25	1 M	1.54 g/ 10 mL	125 µL/aliquot
Instructions: Make sure to prepare a 1 M Tris-HCl stock by dissolving Tris base in 500 mL deionized water, adding HCl to a pH of 6.8–7.0, and adding water to a final volume of 1 L. For the SDS-PAGE Buffer, mix all components listed **except DTT** and add water to a total of 10 mL. Freeze 20 aliquots (375 µL each) at –20°C. Before use, add DTT to the needed single aliquots.					

Procedure

A. Solubilization of the membrane protein

1. Thaw the *E. coli* cell pellet on ice for 15 min.

   Optional: Freezing the cell pellet at –20°C for 30 min prior to incubation at room temperature improves lysis by lysozyme.

2. Resuspend the cell pellet in Lysis Buffer. Use 10 mL Lysis Buffer per g cell pellet. Pour it into a 50 mL conical centrifuge tube.
3. If the solution is very viscous, add 3 units Benzonase® per mL *E.coli* culture volume to the lysis buffer. Alternatively or additionally, sonicate the lysate to improve cell disruption.

   Note: Keep the lysates on ice to prevent warming.

4. Incubate on an end-over-end shaker at 4°C for 1 h.
5. Centrifuge the lysate for 15 min at 900 x g and 4°C to remove cell debris.

   Note: The supernatant contains the **cleared lysate fraction.**
   We recommend to take aliquots of all fractions for SDS-PAGE analysis.

6. Carefully transfer the supernatant to a fresh tube. Centrifuge for 30 min at 7,000 x g and 4°C to precipitate inclusion bodies.

   Tip: Analyze the resulting pellet by SDS-PAGE to assess if target protein is present in inclusion bodies. To capture these proteins, we recommend purification via His-tag under denaturing consitions, using PureCube His Affinity matrices. Alternatively, optimize expression conditions to bring the target protein into the membrane fraction.

7. Carefully transfer the supernatant to a polycarbonate high-speed centrifuge tube and centrifuge at 100,000xg for 1 h at 4°C.
8. Discard the supernatant and resuspend the pellet in 5 mL EW Buffer. Determine protein concentration and adjust the volume with EW Buffer to a concentration of 5 mg/mL. Note the adjusted volume.

   Note: The solution contains the **total membrane protein fraction.**

9. Based on the results from the detergent screen, calculate the amount of detergent needed to solubilize the protein in the adjusted volume. Add the detergent.

   See: Cube Protocol: "Screening Detergents for Optimal Solubilization and Purification of Membrane Proteins"

10. Transfer the suspension to a clean 15 mL polypropylene centrifuge tube. Incubate on an end-over-end rotator using the incubation conditions determined in the detergent screen.
11. Transfer the suspension to a polycarbonate high-speed centrifuge tube and centrifuge at 100,000 x g for 1 h at 4°C.
12. Transfer the supernatant to a fresh 15 mL tube and use it in part B of the protocol.

   Note: The solution contains the **solubilized membrane protein fraction.**

B. Purification of the membrane protein

1. Resuspend the PureCube Rho1D4 Agarose by inverting the bottle until the suspension is homogeneous. Transfer 400 µL of the 50% suspension (corresponding to 200 µL bed volume) into the batch incubation chamber of the spin column barrel. Use the ***clear*** spin push cap to close the chamber and spin the resin at 400 x g for 5 min.
2. Add 10 mL of EW Buffer and centrifuge again for 5 min at 400 x g.
3. Repeat the step to completely remove any residual ethanol that might interfere with protein binding to the affinity resin.
4. Immediately before loading, filter the cleared lysate prepared in step A.12 through a 0.2 µm filter (e.g. syringe filter) to remove any solid material that might clog the column.

Note: It is critical to perform this filter step immediately before loading the column.

5. Empty the 50 mL centrifuge tube and place the spin column barrel containing the equilibrated purification resin back into it.
6. Load the lysate filtered in step 4. Tightly screw the ***yellow*** batch incubation cap and invert 2-3 times to mix sample and resin. Incubate at 4°C overnight on an end-over-end shaker.
7. After batch incubation, replace the ***yellow*** cap with the ***clear*** spin push cap. Centrifuge at 400 x g for 5 min, or until the lysate has completely passed through, and collect the flow-through.

This is the **flow-through fraction.**

8. Wash at least 4 times with 5 mL each of EW Buffer.

These are the **wash fractions.**

9. Replace the 50 mL conical centrifuge tube, and elute the rho1D4-tagged protein by adding 0.5 mL Elution Buffer, and incubating at 4°C for 1 h.

These are the **elution fractions.**

10. Repeat step 9 four times, for a total of five elutions. Collect each elution fraction separately.
11. Determine the protein concentration of the elution fractions with Bradford assay, using BSA as protein standard.
12. Analyze all fractions by SDS-PAGE.

Note: Do not boil membrane proteins. Instead, incubate samples at 46°C for 30 min in preparation for SDS-PAGE analysis.

13. Optional: Perform a Western Blot assay using Rho1D4 antibody.

References:

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