Purification of rho1D4-tagged Membrane Proteins Using PureCube Rho1D4 Agarose and PureCube 1-step Batch Mini 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 Mini Columns that feature the SelfSeal™ membrane technology, saving time and pipetting steps. They replace disposable gravity flow columns used in standard protocols. Volumes of up to 600 µL can be applied to a Mini Column. For larger scale experiments, 1-step batch Midi Plus Columns are available.

This protocol is optimized for tagged proteins expressed in *E. coli* and a bed volume of 0.1-0.5 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 Mini Columns (Cube Biotech #63103)
- ☐ 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 100 mL *E.coli* culture (ca. 100 mg)
- ☐ PureCube Rho1D4 Agarose (1 mL; Cube Biotech #33101)
- ☐ 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, 100 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	2 mL
NaCl*	150 mM	58.44	5 M	146.1 g/ 500 mL	3 mL
Glycerol	10 % (v/v)	-	100 %	-	10 mL
Protease inhibitor	1x		follow supplier`s instructions		
Instructions: Mix in 80 mL water. Adjust the pH to 7.0 using NaOH and then add water to a total volume of 100 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, 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
Lysozyme	1 mg/mL				10 mg
Instructions: Always prepare fresh.					

Equilibration and Wash (EW) Buffer, 30 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Rho Buffer	1x	-	1x	-	30 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 1 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 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 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 200 µL of the 50% suspension (corresponding to 100 µL bed volume) into the batch incubation chamber of the spin column barrel. Close the chamber and spin the resin at 10.000-14.000 x g for 20 sec.
2. Add 600 µL of EW Buffer and centrifuge again at 10.000-14.000 x g for 20 sec.
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 2 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. The maximum loading volume is 600 µL. Invert 2-3 times to mix sample and resin. Incubate at 4°C overnight on an end-over-end shaker.
7. Centrifuge at at 10.000-14.000 x g for 20 sec, or until the lysate has completely passed through, and collect the flow-through.

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

8. Wash twice with 600 µL each of Wash Buffer.

These are the **wash fractions.**

9. Replace the 2 mL microcentrifuge tube with a fresh one, and elute the rho1D4-tagged protein by adding 50-600 µL Elution Buffer, incubating at 4°C for 1 h, and centrifuging for 20 sec at 10.000-14.000 x g.
10. Repeat step 9 four times, for a total of five elutions. Collect each elution fraction separately.

These are the **elution fractions.**

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:

1. Oprian, D.D., et al. 1987. Expression of a synthetic bovine rhodopsin gene in monkey kidney cells. Proc Natl Acad Sci USA 84: 8874-8878.
2. Takayama, H. et al. 2008. High-level expression, single-step immunoaffinity purification and characterization of human tetraspanin membrane protein CD81. PLoS ONE 3: e2314 (DOI: 10.1371/journal.pone.0002314).
3. Zhong, M. and Molday, R.S. 2010. Biding of retinoids to ABCA4, the photoreceptor ABC transporter associated with Stargardt Macular Degeneration. Methods Mol Biol 652: 163-176.
4. Leck, K.-J., et al. 2010. Study of bioengineered zebra fish olfactory receptor 131-2: receptor purification and secondary structure analysis. PLoS ONE 5: e15027(DOI:10.1371/journal.pone.0015027).
5. Bonar, P. and Casey, J.R. Purification of functional human Cl-/HCO3- exchanger, AE1, overexpressed in Saccharomyces cerevisiae. Protein Express Purif 74: 106-115.
6. Wang, X. et al. 2011. Study of two G-protein coupled receptor variants of human trace amine-associated receptor 5. Sci Rep 1: 102 (DOI:10.1038/srep00102).
7. Wang, X. and Zhang, S. 2011. Production of a bioengineered G-protein coupled receptor of human formyl peptide receptor 3. PLoS ONE 6: e23076 (DOI: 10.1371/journal.pone.0023076).
8. Corin, K. et al. 2011. Structure and function analyses of the purified GPCR human vomeronasal type 1 receptor 1. Sci Rep 1: 172 (DOI: 10.1038/srep00172).