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Rho1D4 MagBeads

Purification of rho1D4-tagged Proteins

Product	Cat#	Package size
Rho1D4 MagBeads 5% suspension	**S5394.0001**	**1mL**
Rho1D4 MagBeads 5% suspension	**S5394.0005**	**5mL**

1. Overview

Genaxxon Rho1D4 MagBeads were developed for the affinity purification of proteins with the rho1D4-tag (protein sequence TETSQVAPA), and are compatible with all prokaryotic and eukaryotic expression systems. The affinity matrix is based on spherical magnetic agarose beads, consisting of 6% cross-linked agarose. The material is highly porous to allow optimal protein interaction. Cross-linked agarose is also physically very stable, making it suitable for purification processes without deformation or destruction. Our magnetic beads are very homogeneous in size with a medium particle diameter of 30µm, yielding a high degree of reproducibility between individual purification runs. The Rho1D4 antibody is coupled to the agarose resin in such a way as to obtain a matrix with highest binding capacity towards rho-tagged proteins and enhanced storage stability. Genaxxon Rho1D4 MagBeads are delivered on ice as a 5% suspension. Therefore, 1mL suspension will yield a 50µL bed volume. The suspension contains 20% ethanol to prevent microbial growth.

1.1 Specifications

particle size	30µm
pH stability	6.0-8.0
formulation	5% suspension in 20% ethanol
binding capacity*	up to 3mg/mL settled beads
antimicrobial agent	20% ethanol
stability	2 years
storage	2°C - 8°C, do not freeze!

*as determined by purification of a 35 kDA rho1D4-tagged membrane protein, and quantified via spectrophotometry.

2. Purification of rho1D4-tagged Membrane Proteins expressed in *E.coli* using Genaxxon Rho1D4 MagBeads

Tagging a membrane protein with the rho1D4 epitope to purify on an immunoaffinity matrix loaded with the rho1D4 antibody has proven to be an effective purification method for membrane proteins. Once the process is optimized, pure fractions (>85% purity) of eluted protein can be obtained[1-8].
Genaxxon Rho1D4 MagBeads are well-suited to purify rho1D4-tagged membrane proteins from dilute solutions, such as cell culture or medium supernatants. This protocol is optimized for tagged proteins expressed in ca. 20-60ml *E. coli* culture volume and a Magbead volume of 200µl. It is possible to scale up the protocol for higher throughput volumes, or to scale it down for smaller culture volumes and expression levels.
This procedure should be preceeded with screens for an optimal expression system and solubilization detergent. Also note that if the expressed protein is found mainly in inclusion bodies, it may be preferable to purify the protein on Genaxxon NTA or IDA matrices under denaturing conditions.

2.1

Equipment	Materials
☐ Ultrasonic homogenizer ☐ Refrigerated tabletop centrifuge ☐ Refrigerated superspeed or ultracentrifuge capable of 100,000×g (e.g., Sorvall® LYNX 6000 Superspeed centrifuge) ☐ 15mL polypropylene tubes, conical (e.g. Falcon) ☐ 50mL polypropylene tubes, conical (e.g. Falcon) ☐ 50mL polycarbonate high speed centrifuge tube ☐ End-over-end rotator ☐ UV/VIS Spectrophotometer ☐ SDS-PAGE and Western blotting equipment ☐ Magnetic holder for Microcentrifuge tubes, for separation of magnetic beads ☐ pH-meter	☐ Cell pellet from *E.coli* culture (typically 1 g) ☐ Genaxxon Rho1D4 MagBeads (1 ml; Genaxxon #S5394) ☐ Rho1D4 peptide (5 mg; Genaxxon #S5385) ☐ Detergent (e.g., DM, OG, LDAO) ☐ Sodium dihydrogen phosphate (NaH_2PO_4) ☐ Sodium chloride (NaCl) ☐ Glycerol ☐ Protease inhibitors ☐ Benzonase® nuclease (Novagen®) ☐ Lysozyme ☐ Hydrochloric acid (HCl) ☐ Sodium hydroxide (NaOH) ☐ EDTA ☐ Dithiothreitol (DTT) ☐ Tris base ☐ Triton X-100 ☐ Glycerol ☐ Sodium dodecyl sulfate (SDS) ☐ Bromophenol blue

needed but not supplied

2.2 Solutions and Buffers

Lysis buffer, 100mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for final buffer
NaH_2PO_4*	10mM	119.98	1M	59.99g/500mL	1mL
NaCl	150mM	58.44	5M	146.1g/500mL	3mL
Glycerol	10%	-	100%		10mL
Protease inhibitor+	1x	-	-		follow supplier's instructions
Instructions: Mix in 80mL water. Adjust pH to 7.0 using NaOH, then add water to a total of 100mL. Always prepare fresh.					

*PBS or equivalent can replace NaH_2PO_4 as buffer component.
+Optional.

Equilibration and Wash buffer, 10mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for final buffer
NaH_2PO_4*	10mM	119.98	1M	59.99g/500mL	100µL
NaCl	150mM	58.44	5M	146.1g/500mL	300µmL
Glycerol	10%	-	100%	-	1mL
Protease inhibitor+	1x	-	-	-	follow supplier's instructions
Detergent	based on screen*	-	-	-	-
Instructions: Mix in 8mL water. Adjust pH to 7.0 using NaOH, then add water to a total of 100mL. Always prepare fresh.					

*PBS or equivalent can replace NaH_2PO_4 as buffer component.
+Optional.
*Typically 1.5-2X critical micellar concentration (CMC) of detergent. Use the detergent that yielded the best solubilization in the detergent screen.

Elution buffer, 10mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for final buffer
NaH_2PO_4*	10mM	119.98	1M	59.99g/500mL	100µL
NaCl	150mM	58.44	5M	146.1g/500mL	300µmL
Glycerol	10%	-	100%	-	1mL
Protease inhibitor$^+$	1x	-	-	-	follow supplier's instructions
Detergent	based on screen*	-	-	-	-
Rho1D4 peptide	200µM$^▲$	947	10 mM	-	200µL
Instructions: Mix in 8mL water. Adjust pH to 7.0 using NaOH, then add water to a total of 10mL. Always prepare fresh.					

*PBS can replace NaH_2PO_4 as buffer component.
$^+$Optional
*Typically 1.5–2X critical micellar concentration (CMC) of detergent. Use the detergent that yielded the best solubilization in the detergent screen.
$^▲$The recommended concentration of rho1D4 peptide in the elution buffer is 200µM – 1mM. See the rho1D4 peptide Datasheet for instructions to reconstitute the lyophilized peptide.

5X SDS-PAGE buffer, 10mL

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

2.3 Procedure

A. Solubilization of the membrane protein

1. Complete the Lysis buffer by adding 1mg lysozyme per mL buffer used.

Optional: If the lysate is very viscous, add Benzonase® Nuclease to the lysis buffer (30U/mL).

2. Thaw the cell pellet on ice for 15 min and resuspend in the lysozyme-Lysis buffer. Use 10mL Lysis buffer per g cell pellet.
3. Pour the resuspended pellet into a 50mL conical centrifuge tube and incubate on an end-over-end rotator at 4°C for 60 min.
4. Divide the lysate into two equal volumes and disrupt the cells using a sonicator. Sonicate each volume twice in short bursts for 3 min each time. Pool the lysates in one tube.

Note: Keep the lysates on ice to prevent warming.

5. Centrifuge the lysate for 15 min at 900xg and 4°C to remove cell debris.
6. Carefully transfer the supernatant to a fresh tube. Centrifuge for 30 min at 7,000xg and 4°C to precipitate inclusion bodies.

Recommendation: Analyze the resulting pellet to assess if target protein is present in inclusion bodies. To capture these proteins, we recommend purification via His-tag under denaturing conditions, using Genaxxon Ni-IDA matrices.

7. Carefully transfer the supernatant to a polycarbonate high-speed centrifuge tube and centrifuge at 100,000×g for 1h at 4°C.
8. Discard the supernatant and resuspend the pellet in 5mL Lysis buffer. Determine protein concentration and adjust the volume with Lysis buffer to a concentration of 5mg/mL. Note the adjusted volume.
9. Based on the results from detergent screen, calculate the amount of detergent needed to solubilize the protein in the adjusted volume. Add the detergent.

10. Transfer the suspension to a clean 15mL polypropylene centrifuge tube. Incubate on an end-over-end rotator using the incubation conditions determined in the detergent screen.
11. Remove a 20µL aliquot. Store the aliquot in 5µl 5X SDS-PAGE buffer at −20°C for analysis by SDS-PAGE.

This is the total protein fraction.

12. Transfer the supernatant to a polycarbonate high-speed centrifuge tube and centrifuge at 100,000×g for 1 h at 4°C.
13. Transfer the supernatant to a fresh tube. This is the soluble membrane fraction from which the protein is purified in part B.
14. Remove a 20µL aliquot. Store the aliquot in 5µl of 5X SDS-PAGE buffer at −20°C for analysis by SDS-PAGE.

This is the soluble membrane fraction.

B. Purification of the membrane protein

1. Resuspend Genaxxon Rho1D4 MagBeads by vortexing. Transfer 0.2mL of the suspension into a conical microcentrifuge tube.

Note: Genaxxon Rho1D4 MagBeads are supplied as a 5% suspension. If you expect a higher protein concentration in your sample, increase the suspension volume.

2. Add 0.5mL of Equilibration and Wash buffer to the Genaxxon Rho1D4 MagBeads and mix gently. Place the tube on a magnetic microtube stand until the beads are separated and remove the supernatant. Repeat once. Allow the resin to settle by gravity and remove the supernatant. Repeat once.
3. Pipet the soluble membrane fraction onto the equilibrated Genaxxon Rho1D4 MagBeads and incubate overnight at 4°C on an end-over-end rotator.
4. Place the tube on the magnetic microtube stand until the beads separate and remove the supernatant. Remove a 20 µL aliquot. Store the aliquot in 5µl 5X SDS-PAGE Buffer at −20°C for analysis by SDS-PAGE.

This is the flow-through fraction.

5. Remove the tube from the magnet. Add 0.5mL of Equilibration and Wash buffer and mix gently. Place the tube again on the magnetic microtube stand and allow the beads to separate.
6. Repeat twice. From each wash, remove a 20 µL aliquot. Store the aliquot in 5µl 5X SDS-PAGE Buffer at −20°C for analysis by SDS-PAGE.

This is the wash fraction.

7. Elute the Rho1D4-tagged protein by adding 100µL elution buffer. Mix gently and rotate the microcentrifuge tube for 1 h at 4°C. Place the tube on the magnetic microtube stand and allow the beads to separate. Collect the supernatant.

This is elution fraction 1.

8. Repeat the elution 4 additional times, but with a shorter incubation time of 30 min.

These are elution fractions 2-5.

9. Determine the protein concentration of each elution fraction by Bradford assay or spectrophotometry (OD 280).
10. Analyze all collected fractions (total protein, soluble membrane, flow-through, wash, elution) by SDS-PAGE.

Tip: To avoid membrane protein aggregation, do not boil the fraction aliquots; instead, incubate at 45°C.

3. Regeneration of Rho1D4 MagBeads

Used beads can be regenerated for re-use by washing them with more than 10 times alternating high pH (0.1 M Tris-HCl, *0.5* M NaCl, pH 8.5), low pH (0.1 M sodium acetate, *0.5* M NaCl, pH 4.5) buffers, and re-equilibrated in DPBS buffer (pH7.4). The re-generation protocols needs to be performed in empty column (30 ml). The peptides can be detected by Nanodrop at OD_{220nm}. Washing until the OD_{220nm} was below 0.1.

4. technical and performance parameters

pH stability	6-8
chem. stability	very stable and can resist low concentrations of detergents
detergents	based on screen
additives	Ethanol: 20%

5. References

1. Oprian, D.D., et al. 1987. Expression of a synthetic bovine rhodopsin gene in monkey kidney cells. Proc Natl Acad Sci USA 84:88748878.
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5. Bonar, P. and Casey, J.R. Purification of functional human Cl/HCO3exchanger, AE1, overexpressed in *Saccharomyces cerevisiae*. Protein Express Purif 74: 106115.
6. Wang, X. et al. 2011. Study of two Gprotein coupled receptor variants of human trace amineassociated receptor 5. Sci Rep 1:102 (DOI:10.1038/srep00102).
7. Wang, X. and Zhang, S. 2011. Production of a bioengineered Gprotein coupled receptor of human formyl peptide receptor 3. PLoS ONE 6: e23076 (DOI: 10.1371/journal.pone.0023076).
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6. Important Information

Genaxxon Rho1D4 MagBeads are developed, designed and sold for research purposes only. It is not to be used for human, diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this manual.

7. Warranty

Genaxxon guarantees only for the described properties of the Rho1D4 MagBeads over a period of 2 years (for Certificate of Analysis Date) if this product is used according to the information given in this publication. However, if you are not satisfied with this product, please contact Genaxxon Bioscience GmbH using given contact form or one of its authorized distributors.

8. Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online as pdf-file or on request (info@genaxxon.com).

9.1 Cloning Strategy and Primer Design for C-terminal Rho-1D4 Fusion Proteins

The following diagrams lay out primer design to generate a construct for the addition of rho-1D4 to the C-terminus of a protein of interest (Fig. 1), that can be inserted into expression vectors used with *E.coli* expression systems. Adding a C-terminal rho-1D4 tag to a protein of interest is preferable when the protein has an intracellular C-terminus. Generally, the tag position should be chosen to minimize interference with binding sites of the native protein. **Note: Primers should be purified by HPLC**

3' rho-1D4 primer without linker (TETSQVAPA)

5'-**GGG CTC GAG TCA TCA AGC TGG CGC CAC CTG GGA AGT CTC GGT** + 20 nt target gene-3'

- **Double stop codon:** terminates the protein sequence after the rho-1D4 tag.
- **Rho-1D4 tag sequence**
- **Restriction site:** e.g., XhoI. The sequence should match sites in the MCS of the vector used.
- **Target gene:** 20 nucleotides of the reverse, antisense target sequence: **no stop codon.**
- **Overhang:** needed for proper restriction enzyme activity.

Copy-paste sequence: GGGCTCGAGTCATCAAGCTGGCGCCACCTGGGAAGTCTCGGT

3' rho-1D4 primer with linker (TETSQVAPAGSSG)

5'-**GGG CTC GAG TCA TCA AGC TGG CGC CAC CTG GGA AGT CTC GGT GCC GGA GGA GCC** + 20 nt target gene-3'

- **Double stop codon:** terminates the protein sequence after the rho-1D4 tag.
- **Rho-1D4 tag sequence**
- **Linker sequence**
- **Restriction site:** e.g., XhoI. The sequence should match sites in the MCS of the vector used.
- **Target gene:** 20 nucleotides of the reverse, antisense target sequence: **no stop codon.**
- **Overhang:** needed for proper restriction enzyme activity.

Copy-paste sequence: GGGCTCGAGTCATCAAGCTGGCGCCACCTGGGAAGTCTCGGTGCCGGAGGAGCC

5'-primer

5'-**GGG CAT ATG** + 17 nt target gene-3'

- **Target gene:** 17 nucleotides of the forward, sense target sequence: **no start codon.**
- **Restriction site:** e.g., NdeI. **Note:** NdeI contains the start codon ATG, so it is omitted in the target gene sequence. The restriction sequence should match sites in the MCS of the vector used.
- **Overhang:** needed for proper restriction enzyme activity.

Copy-paste sequence: GGGCATATG

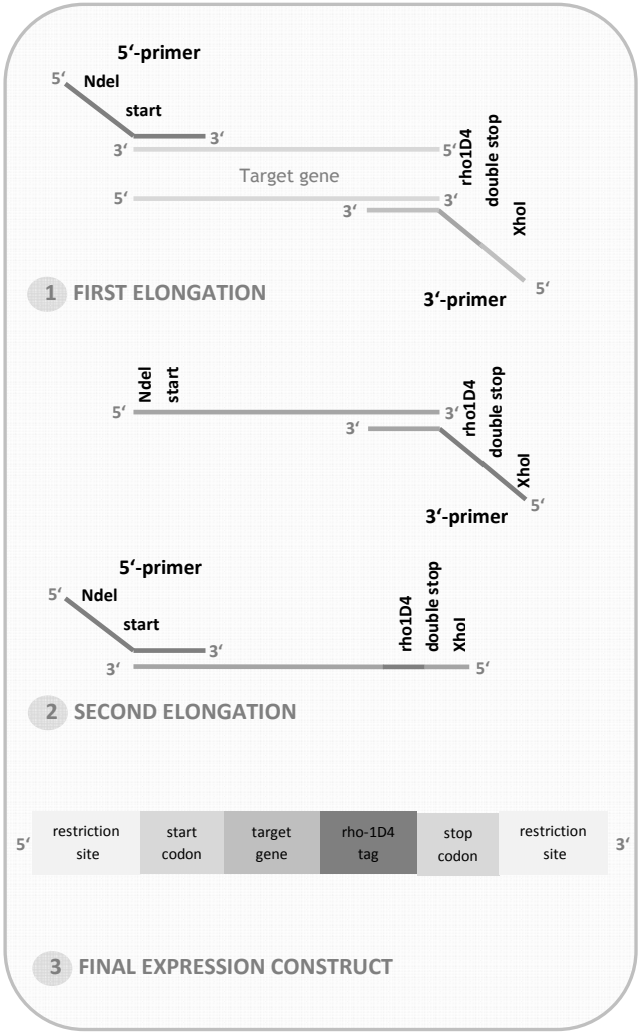

Fig. 1: The primers are designed to generate an expression construct with the rho-1D4 sequence at the 3'-end of the gene of interest. As a result, the rho-1D4 tag appends to the C-terminus of the protein.

9.2 Cloning Strategy and Primer Design for N-terminal Rho-1D4 Fusion Proteins

The following diagrams lay out primer design to generate a construct for the addition of rho-1D4 to the N-terminus of a protein of interest (Fig. 2), that can be inserted into expression vectors used with *E.coli* expression systems. Adding a N-terminal rho-1D4 tag to a protein of interest is preferable when the protein has an intracellular N-terminus. Generally, the tag position should be chosen to minimize interference with binding sites of the native protein. **Note: Primers should be purified by HPLC**

5' rho-1D4 primer without linker (TETSQVAPA)

5'-GGG CAT ATG ACC GAG ACT TCC CAG GTG GCG CCA GCT + 17 nt target gene-3'

- **Rho-1D4 tag sequence**
- **Restriction site:** e.g., NdeI. **Note:** NdeI contains the start codon ATG, so it is omitted in the target gene sequence. The restriction sequence should match sites in the MCS of the vector used.
- **Overhang:** needed for proper restriction enzyme activity.
- **Target gene:** 17 nucleotides of the forward, sense target sequence: **no start codon.**

Copy-paste sequence: GGGCATATGACCGAGACTTCCCAGGTGGCGCCAGCT

5' rho-1D4 primer with linker (TETSQVAPAGSSG)

5'-GGG CAT ATG ACC GAG ACT TCC CAG GTG GCG CCA GCT GGA AGC AGC GGA + 17 nt target gene-3'

- **Rho-1D4 tag sequence**
- **Linker sequence**
- **Restriction site:** e.g., NdeI. **Note:** NdeI contains the start codon ATG, so it is omitted in the target gene sequence. The restriction sequence should match sites in the MCS of the vector used.
- **Overhang:** needed for proper restriction enzyme activity.
- **Target gene:** 17 nucleotides of the forward, sense target sequence: **no start codon.**

Copy-paste sequence: GGGCATATGACCGAGACTTCCCAGGTGGCGCCAGCTGGAAGCAGCGGA

3'-primer

5'-GGG CTC GAG TCA TCA + 20 nt target gene-3'

- **Double stop codon:** terminates the protein sequence.
- **Target gene:** 20 nucleotides of the reverse, antisense target sequence: **no stop codon.**
- **Restriction site:** e.g. XhoI. The sequence should match sites in the MCS of the vector used.
- **Overhang:** needed for proper restriction enzyme activity.

Copy-paste sequence: GGGCTCGAGTCATCA

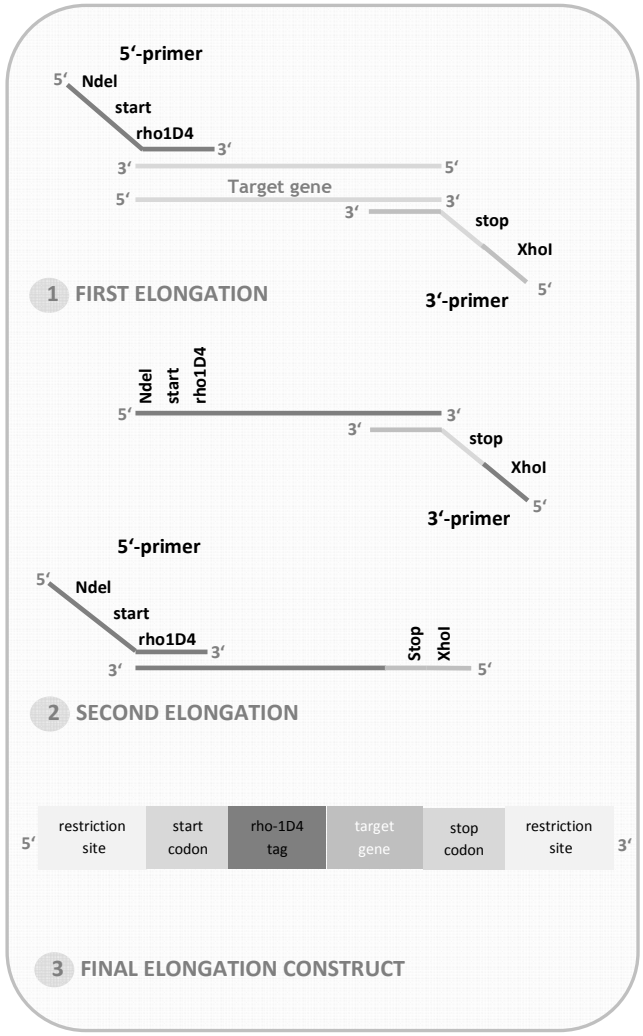

Fig. 2: The primers are designed to generate an expression construct with the rho-1D4 sequence at the 3'-end of the gene of interest. As a result, the rho-1D4 tag appends to the N-terminus of the protein.