

# Rho1D4 Agarose

## Purification of rho1D4-tagged Membrane Proteins

Product	Cat#	Package size
Rho1D4 Agarose 1mL (2mL 50% suspension)	S5384.0001	2mL
Rho1D4 Agarose 5mL (10mL 50% suspension)	S5384.0005	10mL

All volume specification relating to the sedimented agarose resin.

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### 1. Overview

Proteins tagged with the epitope sequence (TETSQVAPA) of the rho1D4 antibody can be purified with this affinity matrix. Based on [BioWorks Workbeads](#), consisting of 7.5% cross-linked agarose, Genaxxon offers the first commercially available immunoaffinity resin for the Rho1D4 purification system.

- demonstrably suitable for membrane protein research
- binding capacity of 3-4 mg protein per mL resin
- purifies protein with high specificity
- gentle protein elution based on competitive binding
- can be regenerated for reuse

Rho1D4 refers to the last 9 amino acids of the intracellular C-terminus of bovine rhodopsin. The name comes from the monoclonal antibody that specifically binds to the sequence. Combined with the rho1D4 antibody, this epitope can serve as a highly specific purification tag suitable for membrane proteins. A membrane protein of interest can be genetically modified to incorporate the rho1D4 tag at the C-terminus. Once outfitted with this sequence, the target protein can be captured on an affinity matrix loaded with rho1D4 antibody and subsequently eluted by adding an excess of rho1D4 peptide to competitively bind with the matrix antibody. This provides for gentler elution conditions than, for example, changing pH.

Genaxxon Rho1D4 Agarose can be used for batch purification, low pressure column purification, and is compatible with all prokaryotic and eukaryotic expression systems.

Rho1D4 agarose is delivered as a 50% suspension. Therefore, 2mL suspension will yield a 1mL bed volume. The suspension contains 20% ethanol to prevent microbial growth.

### 1.1 Specifications

particle size	40µm
pH stability	6-8
recommended flow rate	up to 6mL/min. (optimal 0.25 - 1mL/min)
formulation	50% suspension in 20% ethanol
binding*/loading capacity Genaxxon Rho1D4 agarose	3-4mg protein/mL agarose
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 Agarose

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, the result is generally large and pure fractions (>85% purity) of eluted protein. With a binding capacity of 3-4 mg protein per mL resin, Genaxxon Rho1D4 Agarose is a highquality affinity resin designed for the efficient purification of rho1D4-tagged membrane proteins. This protocol is optimized for tagged proteins expressed in *E. coli* and a bed volume of 0.1-1mL. It is possible to scale up the protocol for higher through-put volumes.

This procedure should be proceeded 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 Ni-IDA matrices under denaturing conditions.

### 2.1

Equipment	Materials
<input type="checkbox"/> Ultrasonic homogenizer <input type="checkbox"/> Refrigerated tabletop centrifuge <input type="checkbox"/> Refrigerated superspeed or ultracentrifuge capable of 100,000×g (e.g., Sorvall® LYNX 6000 Superspeed centrifuge) <input type="checkbox"/> 2mL microcentrifuge tubes <input type="checkbox"/> 15mL polypropylene centrifuge tubes (e.g. Falcon) <input type="checkbox"/> 50mL polypropylene tubes, conical (e.g. Falcon) <input type="checkbox"/> pHmeter <input type="checkbox"/> 50mL polycarbonate high speed centrifuge tube <input type="checkbox"/> End-over-end rotator <input type="checkbox"/> UV/VIS Spectrophotometer <input type="checkbox"/> SDS-PAGE and Western blotting equipment <input type="checkbox"/> Disposable gravity flow columns (1mL bed volume)	<input type="checkbox"/> Cell pellet from <i>E. coli</i> culture (typically 1g) <input type="checkbox"/> Genaxxon Rho1D4 Agarose (1 ml; Genaxxon #S5384) <input type="checkbox"/> Rho1D4 peptide (5mg; Genaxxon #S5385) <input type="checkbox"/> Detergent (e.g., DM, OG, LDAO) <input type="checkbox"/> Sodium dihydrogen phosphate (NaH <sub>2</sub> PO <sub>4</sub> ) <input type="checkbox"/> Sodium chloride (NaCl) <input type="checkbox"/> Glycerol <input type="checkbox"/> Protease inhibitors <input type="checkbox"/> Benzonase® nuclease (Novagen®) <input type="checkbox"/> Lysozyme <input type="checkbox"/> Hydrochloric acid (HCl) <input type="checkbox"/> Sodium hydroxide (NaOH) <input type="checkbox"/> EDTA <input type="checkbox"/> Dithiothreitol (DTT) <input type="checkbox"/> Trishydrochloride (Tris-HCl, powder) <input type="checkbox"/> Triton X-100 <input type="checkbox"/> Glycerol <input type="checkbox"/> Sodium dodecyl sulfate (SDS) <input type="checkbox"/> 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 <sub>2</sub> PO <sub>4</sub> *	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<sub>2</sub>PO<sub>4</sub> 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 <sub>2</sub> PO <sub>4</sub> *	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 10mL. Always prepare fresh.

\*PBS or equivalent can replace NaH<sub>2</sub>PO<sub>4</sub> 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 <sub>2</sub> PO <sub>4</sub> *	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<sub>2</sub>PO<sub>4</sub> 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 TrisHCl 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 (0.5mL 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.
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.
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.
7. Carefully transfer the supernatant to a polycarbonate high-speed centrifuge tube and centrifuge at 100,000xg 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.

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

**Note:** Keep the lysates on ice to prevent warming.

**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.

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.
12. Transfer the supernatant to a polycarbonate highspeed 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.

#### B. Purification of the membrane protein

1. Resuspend Genaxxon Rho1D4 Agarose by inverting the bottle until the slurry is homogeneous. Transfer 0.2mL of the suspension into a 15mL conical centrifuge tube. Allow the resin to settle by gravity and remove the supernatant.
2. Add 1mL (10bv) of Equilibration and Wash buffer to the Genaxxon Rho1D4 Agarose and gently resuspend the slurry to equilibrate the resin. Allow the resin to settle by gravity and remove the supernatant. Repeat once.
3. Pipet the soluble membrane fraction onto the equilibrated Genaxxon Rho1D4 Agarose and incubate overnight at 4°C on an end-over-end rotator.
4. Pour the binding suspension into a disposable gravity flow column with a capped bottom outlet.
5. Remove the bottom cap of the column and collect the flow-through. Remove a 20µL aliquot. Store the aliquot in 5µl 5X SDS-PAGE buffer at –20°C for analysis by SDS-PAGE.
6. Wash four times with 0.5ml of Equilibration and Wash buffer (5bv). From each wash, remove a 20µL aliquot. Store the aliquot in 5µl 5X SDS-PAGE buffer at –20°C for analysis by SDS-PAGE.
7. Elute the rho1D4-tagged protein. Replace the bottom cap of the column and add 0.2ml Elution buffer (2bv). Cap and rotate the column for 1h at 4 °C. Remove the top and bottom cap of the column and collect the eluate.
8. Repeat the elution (step 7) 9 additional times, but with a shorter incubation time of 30 min.
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.

This is the total protein fraction.

**Note:** Genaxxon Rho1D4 Agarose is supplied as a 50% slurry corresponding to a 100µL bed volume (bv). If you expect a higher protein concentration in your sample, scale up the bed volume.

**Note:** The binding suspension is the Genaxxon Rho1D4 Agarose plus the soluble protein fraction.

This is the flow-through fraction.

This is the wash fraction.

This is elution fraction 1.

These are elution fractions 2-10.

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

### 3. Washing and Regenerating Rho1D4 Affinity Resins

Used Rho1D4 Agarose can be regenerated for re-use by washing 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 PBS buffer (pH7.4). The re-generation protocol needs to be performed in empty columns (30 ml, Biorad). The peptides can be detected by Nanodrop at OD<sub>220nm</sub>. Washing until the OD<sub>220nm</sub> 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

- Oprian, D.D., et al. 1987. Expression of a synthetic bovine rhodopsin gene in monkey kidney cells. Proc Natl Acad Sci USA 84:88748878.
- Takayama, H. et al. 2008. Highlevel expression, single-step immunoaffinity purification and characterization of human tetraspanin membrane protein CD81. PLoS ONE 3: e2314 (DOI: 10.1371/journal.pone.0002314).
- 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: 163176.
- Leck, K.J., et al. 2010. Study of bioengineered zebra fish olfactory receptor 1312: receptor purification and secondary structure analysis. PLoS ONE 5: e15027(DOI:10.1371/journal.pone.0015027).
- Bonar, P. and Casey, J.R. Purification of functional human Cl/HCO<sub>3</sub>exchanger, AE1, over-expressed in *Saccharomyces cerevisiae*. Protein Express Purif 74: 106115.
- Wang, X. et al. 2011. Study of two Gprotein coupled receptor variants of human trace amine-associated receptor 5. Sci Rep 1:102 (DOI:10.1038/srep00102).
- 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).
- 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).

### 6. Important Information

Genaxxon Rho1D4 agarose is 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 agarose 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

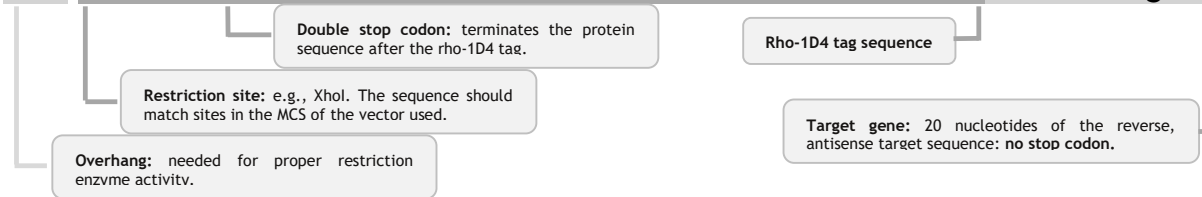
When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online as pdf-file or on request ([info@genaxxon.com](mailto:info@genaxxon.com)).

### 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)

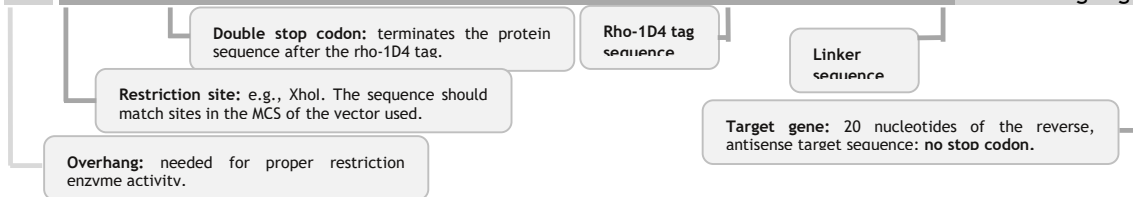
5'-GGG CTC GAG TCA TCA AGC TGG CGC CAC CTG GGA AGT CTC GGT + 20 nt target gene-3'



Copy-paste sequence: GGGCTCGAGTCATCAAGCTGGCGCCACCTGGGAAGTCTCGGT

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

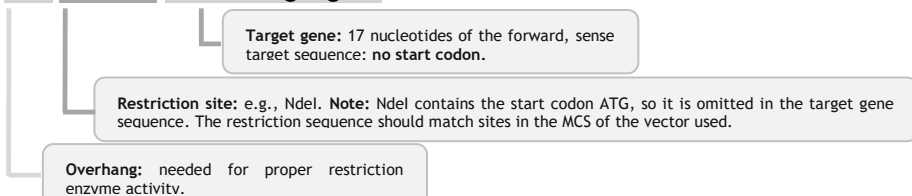
5'-GGG CTC GAG TCA TCA AGC TGG CGC CAC CTG GGA AGT CTC GGT GCC GGA GGA GCC + 20 nt target gene-3'



Copy-paste sequence: GGGCTCGAGTCATCAAGCTGGCGCCACCTGGGAAGTCTCGGTGCCGAGGAGCC

#### 5'-primer

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



Copy-paste sequence: GGGCATATG

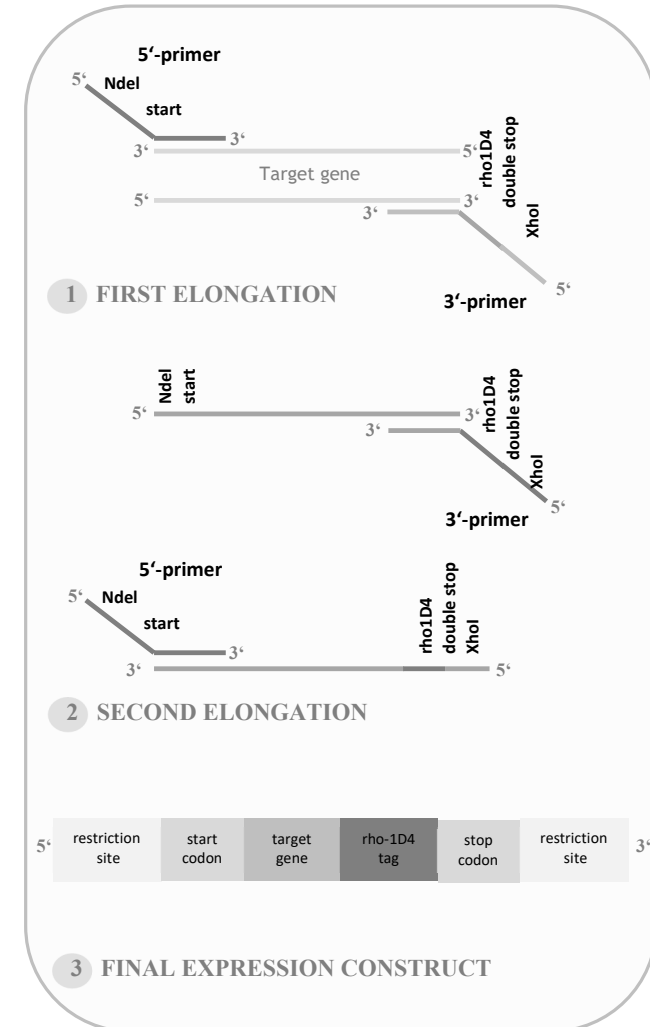


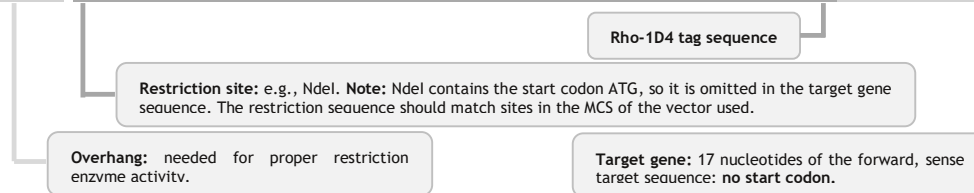
Fig. 1: The primers are designed to generate an expression construct with the rho-1D4 sequence at the 3'-end of the gene of interest. As a result, the rho-1D4 tag appends to the C-terminus of the protein.

## 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)

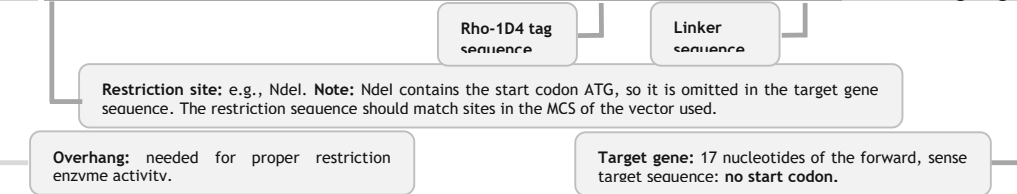
5'-GGG CAT ATG ACC GAG ACT TCC CAG GTG GCG CCA GCT + 17 nt target gene-3'



Copy-paste sequence: GGGCATATGACCGAGACTTCCCAGGTGGCGCCAGCT

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

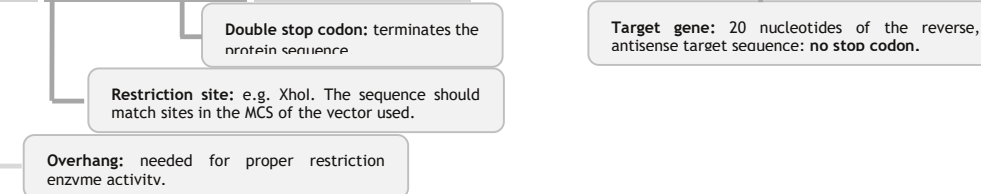
5'-GGG CAT ATG ACC GAG ACT TCC CAG GTG GCG CCA GCT GGA AGC AGC GGA + 17 nt target gene-3'



Copy-paste sequence: GGGCATATGACCGAGACTTCCCAGGTGGCGCCAGCTGGAAGCAGCGGA

### 3'-primer

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



Copy-paste sequence: GGGCTCGAGTCATCA

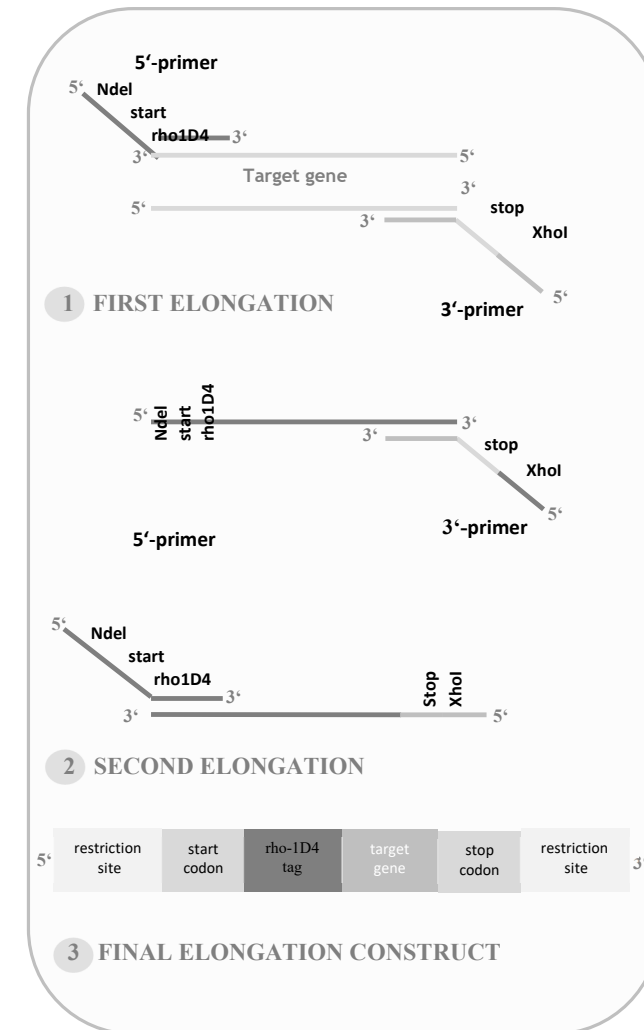


Fig. 2: The primers are designed to generate an expression construct with the rho-1D4 sequence at the 3'-end of the gene of interest. As a result, the rho-1D4 tag appends to the N-terminus of the protein.