From sequences to enzymes: heterologous expression of genes from marine microbes

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Abstract

Heterologous expression is an easy and broadly applicable experimental approach widely used to investigate protein functions without the need to genetically manipulate the original host. The approach is used to obtain large quantities of the desired protein, which can be further analyzed from a biochemical, structural and functional perspective. The expression system consists of three main components: i) a foreign DNA sequence coding for the protein of interest; ii) a suitable expression vector; iii) a suitable host (bacterial, yeast or mammalian cells) which does not encode or express the protein of interest. Here we show how to apply an *Escherichia coli*-based expression system to overexpress protein encoding genes from marine microbes.

Keywords: *E. coli*, expression vector, heterologous expression, recombinant protein, uncultured microorganisms, metagenomes

1 Introduction

Heterologous expression refers to the expression of a gene into a host organism that does not naturally express the gene (*I*). This process allows the production and isolation of a protein of

interest from difficult to grow or uncultured organisms to investigate the protein function and its biological implications (2). Conventional genetic platforms established for heterologous expression consist of bacterial, yeast, algal or mammalian cell systems. Bacterial heterologous hosts are the most desirable in terms of low-cost production and availability of genetic tools for gene cloning (3). Heterologous expression systems are widely used for large-scale production of proteins with significant biotechnological applications. One of the most famous examples is *Taq* DNA Polymerase, a well-known and widely used enzyme, initially isolated from its native host, *Thermus aquaticus*, in 1976 (4), and then cloned and overexpressed in *Escherichia coli* (5). Heterologous expression offers additional advantages besides the potential for high yields, including heterologous expression of genes obtained from uncultured microorganisms (6), metagenomes or Metagenome Assembled Genomes (MAGs) (7) obtained from diverse samples (e.g. marine water, sediments, fluids), as well as expression of ancestral reconstructed genes obtained from comparative genomic analysis (as reported in Chapter [From sequences to enzymes: comparative genomics to study evolutionary conserved protein functions in marine microbes]). This has the potential to unlock information from the large fraction of genes encoding hypothetical proteins (8). In marine microbiology, heterologous expression approaches can be coupled to comparative genomics methods to study the functions and the evolutionary history of genes of interest, providing insight into their biochemical, structural and functional properties.

Heterologous expression methods are well established in molecular biology, and it is achieved by cloning the DNA fragment, previously isolated from the organism or metagenome of interest, into a suitable expression vector and introducing the recombinant plasmid into a host that will provide the biochemical apparatus to generate and express the 'foreign' protein. *E. coli* is the most popular host used to express recombinant proteins for several reasons: i) it is easy to handle; ii) it grows rapidly; iii) it is easy to transform; iv) it does not have specific nutritional needs, and v) a large number of expression vectors are available for *E. coli* (3). These features make *E. coli*-based heterologous expression a feasible and versatile tool to probe marine functional diversity.

Here we illustrate how to apply a heterologous expression method to express a protein coding gene from marine microbes. Briefly, the target DNA is cloned downstream and in frame with a sequence that encodes an N-terminal polyhistidine tag (His-tag), under the control of a strong bacteriophage T7 promoter. The His-tag acts as a metal-binding domain essential for the subsequent protein purification step. *E. coli* BL21 (DE3) (Promega) is an ideal host for recombinant protein expression of T7 regulated genes because it contains the DE3 bacteriophage lambda lysogen (λ DE3) that carries the T7 RNA polymerase gene under the *lacUV5* control. Using Isopropyl- β -D-1-thiogalactopyranoside (IPTG), an allolactose analogue, it is possible to induce the expression of T7 RNA polymerase to express the gene cloned under the T7 promoter's control (3). The method requires several individual steps: (i) cloning and propagation of the recombinant expression vector, (ii) expression and extraction of the recombinant protein, (iii) detection of the recombinant protein by SDS-PAGE and western blotting analysis.

2 Materials

2.1 Making *Escherichia coli* DH5 α and BL21 (DE3) chemically competent cells

1. Sterile loops, 2 mL sterile tubes and 100 mL flasks.
2. A basket filled with ice.
3. Cuvette UV/VIS.
4. Tryptone-Yeast (TY) liquid medium (1 L final volume): 10 g Tryptone, 5 g Yeast extract, 8 g NaCl. Add into a cylinder containing at least 800 mL of deionized water and a small stirring magnet at the bottom. Mix until the components are completely dissolved and add water up to 1 litre. Autoclave before use.
5. Tryptone-Yeast (TY) agar plates: add 1.8 % agar (w/v) to TY liquid medium and autoclave before use (see **Note 1**).
6. A fresh stock of 50 mM CaCl₂ solution (500 mL final volume): add 2.77 g of CaCl₂ into a becher containing 400 mL of water. Let it dissolve completely and add water up to 500 mL. Autoclave before use (See **Note 2**).
7. 100 % Glycerol (see **Note 3**).
8. *E. coli* DH5 α glycerol stock.
9. *E. coli* BL21 (λ DE3) glycerol stock.

2.2 Molecular cloning step

1. 0.2 mL and 0.5 mL sterile tubes.
2. Template genomic or metagenomic DNA to be used to amplify the target protein encoding gene.
3. Taq DNA polymerase with proofreading activity with the appropriate buffer.
4. Forward and reverse primers targeting the 5' and 3' of the gene of interest (**Note 4**).
5. 10 mM dNTP.
6. Sterile deionized H₂O (ddH₂O).
7. Thermocycler.
8. pRSET-A expression vector (Invitrogen).

9. Restriction endonucleases with appropriate buffers.
10. T4 ligase enzyme with appropriate buffer (Promega).
11. A tube rack.
12. Gel extraction kit (Qiagen).
13. PCR purification kit (Qiagen).

2.3 Bacterial transformation

1. 90 mm TY agar plates supplemented with ampicillin (100 µg/mL) (see **Note 5**).
2. TY liquid medium (see **Note 3**).
3. *E. coli* DH5α or BL21 λDE3 competent cells.
4. Plasmid DNA or ligation mix (product of 2.2 Molecular cloning step).
5. 1.5 mL sterile tubes.
6. A basket filled with ice.
7. A tube rack.

2.4 Agarose Gel Electrophoresis

1. Comb, gel tray and electrophoresis apparatus.
2. 1% Agarose gel, made in 1× TAE buffer and casted to size according to the gel tray used.
3. Ethidium bromide solution, molecular grade (10 mg/mL).
4. TAE 50×: 2 M Tris Base, 1 M Glacial Acetic Acid, 0.5 M EDTA (pH 8.6) (see **Note 7**).
5. Loading buffer 6× (10 mL final volume): 0.25 % bromophenol blue, 0.25 % xylene cyanol, 30 % glycerol, water.
6. UV light transilluminator.

2.5 DNA plasmid extraction

1. TY liquid medium (see **Note 3**).
2. A basket filled with ice.
3. Ampicillin stock solution (100 mg/mL) (see **Note 6**).
4. Solution 1: 50 mM glucose, 10 mM EDTA, 25 mM Tris Base-HCl, pH 8.0 (see **Note 3, 7**).
5. Solution 2: 0.2 M NaOH, 1 % SDS (see **Note 3, 9**).
6. 3 M Sodium acetate pH 5.2 (100 mL final volume). Add 24.6 g of sodium acetate to 80 mL of water. Adjust pH if needed and add water up to 100 mL (see **Note 3**).
7. Phenol:Chloroform:Isoamyl alcohol (25:24:1, v:v).
8. EtOH 100 %, EtOH 70 % (see **Note 10,15**).
9. RNase A stock solution (10 mg/mL) (see **Note 11**).
10. Sterile 100 % Glycerol
11. 1.5 mL, 2 mL and 15 mL sterile tubes.

2.6 Protein expression and extraction

1. *E. coli* BL21 (DE3) competent cells.
2. Recombinant plasmid (product of DNA plasmid extraction, section 2.5).
3. TY agar plate supplemented with Ampicillin (see **Note 5**).
4. TY liquid medium.
5. Ampicillin stock solution (100 mg/mL) (see **Note 6**).
6. Sterile loops.
7. 2 mL, 1.5 mL, 50 mL sterile tubes, and flasks.
8. Cuvette for UV/VIS.
9. IPTG stock solution 100 mM (see **Note 12**).
10. PBS-Triton X-100 1 % solution (1 L final volume): 0.1 M NaCl, 15 mM Na₂HPO₄, 4.5 mM NaH₂PO₄, 1 % Triton X-100, pH 7.3.
11. Dry ice-ethanol bath or liquid nitrogen.

12. 37 °C water bath.

2.7 Bradford assay

1. 2 mL tubes.
2. Cuvette for UV/VIS.
3. Pasteur pipettes.
4. Bovine Serum Albumin (BSA) stock solution (0.1 mg/mL).
5. Bradford reagent (1 L final volume): 100 mg Coomassie Brilliant Blue G250, 50 mL 95 % ethanol, 100 mL 85 % (w/v) phosphoric acid, add ddH₂O up to 1 L.
6. Deionized H₂O (ddH₂O).
7. Spectrophotometer UV/VIS.

2.8 SDS-Polyacrylamide electrophoresis (SDS-PAGE)

1. Glass plates, comb, spacer, casting gel apparatus.
2. Resolving gel (12.5 %) (10 mL final volume): 4 mL 30 % Acrylamide, 2.6 mL 1 M Tris Base-HCl pH 8.8, 100 µl 10 % SDS, 100 µl 10 % APS, ddH₂O up to 10 mL, 5 µl Tetramethylethylenediamine (TEMED) (see **Note 13**).
3. Stacking gel (5 %) (4 mL final volume): 670 µl 30 % Acrylamide, 500 µl 1 M Tris Base-HCl pH 6.8, 40 µl 10 % SDS, 40 µl 10 % APS, ddH₂O up to 4 mL, 4 µl TEMED (see **Note 13**).
4. 2-propanol 100 %.
5. Deionized H₂O (ddH₂O).
6. Clean absorbent paper.
7. Electrophoresis apparatus.
8. TGS 10× running buffer: 0.25 M Tris Base, 1.92 M Glycine, 1 % SDS, pH 8.3 (see **Note 14**).
9. *5× Loading sample buffer* (10 mL final volume): 1 mL 1 M Tris Base-HCl pH 6.8, 1.4 mL 10 % SDS, 2 mL of 20 % glycerol, 2.5 mL of 25 % β-mercaptoethanol and 1 % Bromophenol blue. Add water up to 10 mL and aliquot into clean 2 mL tubes (see **Note 15**).

10. Coomassie staining solution: 0.2 % Coomassie Brilliant-blue R, 25 % 2-propanol and 10 % acetic acid (see **Note 16**).
11. Destaining solution: 10 % acetic acid, 30 % denatured ethanol. Prepare 500 mL of destain solution adding 50 mL of acetic acid and 150 mL denatured ethanol to 300 mL of ddH₂O (see **Note 17**).

2.9 Immunoblotting and detection

1. A spatula and clamps.
2. Nitrocellulose membrane 6 cm x 9 cm.
3. 6 absorbent filter paper 6 cm x 9 cm for a single gel.
4. Plastic containers.
5. 10× Tris Base-glycine (see **Note 18**): 0.25 M Tris Base pH 8.3, 1.92 M glycine.
6. 100 % methanol.
7. Ponceau S.
8. 10× Phosphate buffered saline (PBS): 80 mM NaHPO₄; 20 mM NaH₂PO₄ × 2 H₂O; 100 mM NaCl, pH 7.2.
9. 5% milk-blocking solution: add 2.5 g skimmed milk to 50 mL of 1× PBS (see **Note 19**).
10. Anti-His tag antibody.
11. Chemiluminescent substrate.
12. Deionized H₂O.

3 Methods

Carry out all the procedures under sterile conditions unless otherwise specified.

3.1 Cloning and propagation of the recombinant expression vector in *Escherichia coli*

Prepare *E. coli* DH5α and BL21 (DE3) competent cells following the standard calcium chloride method (**9**) prior to cloning procedures:

1. Streak *E. coli* from a glycerol stock or a liquid culture on a TY agar plate with a sterile loop and incubate the plate overnight at 37 °C.
2. Inoculate a single colony of *E. coli* in 5 mL of TY liquid medium and incubate overnight at 37 °C with vigorous shaking.
3. Add 1 mL of the overnight *E. coli* culture to 99 mL of fresh TY liquid medium and incubate at 37 °C with shaking until OD_{600nm} reaches 0.5.
4. Place the culture on ice for 30 minutes then centrifuge at 4 °C at 4000 g for 10 minutes and discard the supernatant.
5. Gently resuspend the pellet with 100 mL ice-cold 50 mM CaCl₂, then centrifuge at 4 °C at 4000 g for 10 minutes and discard the supernatant.
6. Resuspend the pellet with 50 mL of ice-cold 50 mM CaCl₂ and incubate for 15 minutes on ice, then centrifuge at 4 °C at 4000 g for 10 minutes and pour off the supernatant.
7. Lastly, resuspend the pellet with 8.5 mL of ice-cold 50 mM CaCl₂ and 1.5 mL of Glycerol (15 % final concentration) and place on ice for 1 hour.
8. Aliquot 400 µl of the competent cells into sterile 2 mL tubes. The competent cells are ready to be used or can be stored at - 80 °C (see **Note 20**).

Once *E. coli* competent cells are ready, subject your target DNA to PCR amplification using the following method:

1. In a 0.2 mL sterile tube combine 10-50 ng of DNA target, 0.4 µM of appropriate primers forward and reverse, 0.3 mM dNTP, 1× buffer (supplied by the manufacturer of the DNA polymerase), 1 U of Taq DNA polymerase with proofreading activities. Prepare a negative control mix by adding the same reagents except for the DNA target in another 0.2 mL sterile tube.
2. Add the right amount of sterile water to reach 50 µl final volume per reaction and gently pipette up and down.
3. Place the PCR samples into the thermocycler and start the PCR amplification program as follows: a) initial denaturation step at 94 °C for 5 minutes; b) denature 15 seconds at 94 °C; c) anneals primers at 56 °C for 30 seconds; d) extend the DNA at 68 °C for 90 seconds; e) repeat steps b-d for 30 times; f) final extension at 68 °C for 5 minutes.
4. In the meantime prepare 1 % agarose gel by mixing 1 g agarose with 100 mL of TAE 1× buffer previously prepared (see **Note 22**).

5. Melt agarose by heating in a microwave oven until the solution boils and then leave the gel solution to cool at room temperature. Chill until the gel reaches approximately 50 °C.
6. Gently pour the gel into a gel tray, add 0.5 µg/mL of ethidium bromide and evenly mix into the agarose gel with the help of a pipette tip.
7. Add the comb to one side of the gel and remove any bubbles in the solution.
8. Let the gel harden for approximately 20 minutes until it becomes milky and opaque. Remove the comb and soak the gel into an electrophoresis tank with 1× TAE running buffer.
9. Prepare the samples by adding 6× loading buffer to the DNA samples and the DNA ladder.
10. Load 5 µl of the DNA target into the wells and close the lid of the electrophoresis chamber. Set the run at 120 volts (usually 30 minutes to 1 hour).
11. Visualize the gel under UV light to reveal the DNA bands.
12. Purify the PCR products with a PCR purification kit and proceed with the cloning step.

Prior to proceeding with the cloning step, digest both the DNA insert and the expression plasmid with the selected restriction enzymes applying the following digestion procedure:

1. add the following reagents in a sterile 1.5 mL tube: 1 µg of DNA target, 10 units of the selected restriction endonuclease, 1× restriction enzyme Buffer (supplied by the manufacturer of the restriction endonuclease), sterile water to reach the 50 µl final volume. Gently mix by pipetting up and down.
2. Incubate the tube at 37 °C for at least 1 hour.
3. Load 5 µl of the digested DNA on 1 % agarose gel, together with the undigested DNA (100 ng) and run the gel by starting the electrophoresis process for 20 minutes at 200 volts (see **Note 22**).
4. Purify the digested DNA with a gel extraction kit.
5. Set up the ligation mixture reaction in a sterile tube using a 1:5 molar ratio of vector:insert DNA following the equation (see **Note 21**):

$$(ng\ of\ vector \times kb\ insert \div kb\ vector) \times (insert : vector\ molar\ ratio)$$

6. Add the following reagents in a 20 µl final volume: water, 1× ligation buffer (supplied by the manufacturer of the T4 ligase), DNA vector, DNA insert, T4 DNA ligase (1 unit). Mix gently (T tube). Carry out a negative control mix containing all the reagents except the DNA insert (C tube).

7. Incubate both ligation mixtures for 12 hours at 16 °C.
8. Add 200 µl of *E. coli* competent cells into the T tube gently pipette up and down. Do the same with the C tube.
9. Incubate on ice for 30-minute.
10. Heat-shock both tubes at 42 °C for 2 minutes and then transfer at room temperature for 5 minutes.
11. Add 800 µl of prewarmed TY liquid medium and incubate at 37 °C for 1 hour.
12. Spread 1:10 and 1:100 dilutions from the T and C tubes on TY agar plates containing 100 µg/mL ampicillin and incubate at 37 °C for 12-15 hours.
13. Inoculate 10 colonies obtained from the transformation of *E. coli* cells with T tube and 1 colony obtained from the transformation of *E. coli* cells with C tube (control colony), in 5 mL TY liquid medium supplemented with 100 µg/mL ampicillin and incubate at 37 °C for 12 hours with energetic shaking for the subsequent plasmid DNA extraction.
14. Centrifuge bacterial cultures at 10,000 g for 5 minutes and discard the supernatant.
15. Resuspend the pellet in 100 µl of Solution 1 and incubate for 5 minutes at room temperature.
16. Add 200 µl of Solution 2 and quickly place it on ice.
17. Add 150 µl of 3 M Sodium acetate and place on ice for 5 minutes.
18. Centrifuge at 10,000 g for 5 minutes and collect 400 µl of supernatant into 2 mL sterile tubes.
19. Add 1 volume of phenol:chloroform:Isoamyl alcohol solution (25:24:1, v/v) and centrifuge at 10,000 g for 5 minutes.
20. Transfer the aqueous phases in a new 2 mL tube and add 3 volumes of Ethanol (EtOH) 100 % (v/v) and 1/10 volume of 3M Sodium acetate.
21. Centrifuge at 10,000 g for 5 minutes and wash two more times using EtOH at 70 % final concentration.
22. Discard the supernatant and let samples dry at room temperature.
23. Resuspend the pellet in 50 µl of sterile water containing 20 µg of RNase (10 mg/mL) and incubate at 37 °C for 30 minutes.
24. Load 5 µl of the isolated plasmid DNA on 1 % agarose gel, together with 5 µl of plasmid DNA extracted from the control colony and run the gel by starting the electrophoresis process for 20 minutes at 200 volts (see **Note 22**).

25. Visualize the gel under UV light to reveal the DNA bands.
26. Digest the selected plasmid DNA with one or two one cutter restriction endonucleases based on the sequence of the recombinant plasmid, to verify the success of the cloning procedure and load the digested DNA on 1 % agarose gel for 20 minutes at 100 Volts.
27. Prepare a glycerol stock of the strain containing the recombinant plasmid, by adding glycerol at 30 % final concentration to the bacterial culture collected in step 13. Store it at -80 °C (Figure 1).
28. Use 10-50 ng of the recombinant plasmid to transform *E. coli* BL21 (DE3) competent cells and to select the recombinant strain on TY agar plate supplemented with 100 µg/mL ampicillin, as reported above.

3.2 Expression and extraction of recombinant protein

1. Inoculate a single BL21 (DE3) recombinant colony in 5 mL TY liquid medium containing ampicillin 100 µg/mL using a sterile loop and incubate the culture at 37 °C for 12 hours.
2. Add 1 mL of the culture to 49 mL of fresh TY liquid medium and incubate at 37 °C with shaking until OD_{600nm} reaches 0.4.
3. Split the cultures into 2 sterile flasks containing 25 mL of culture each one.
4. Add IPTG to a final concentration of 1 mM only to one flask (labelled induced flask) and incubate both flasks at 37 °C with vigorous shaking.
5. After 6 hour induction, collect the cultures from both flasks and centrifuge at 10,000 g for 10 minutes. Discard the supernatant.
6. Resuspend the pellets in 4 volumes of fresh and cold PBS-Triton X-100 1 % and aliquote the samples into clean 2 mL tubes.
7. Freeze in a dry ice-ethanol bath for 10 minutes. Immediately, thaw at 37 °C water bath for 10 minutes.
8. Repeat step 7 for 5 times.
9. Centrifuge at 10,000 g for 15 minutes at 4 °C.
10. Collect the supernatants into clean 2 mL tubes and resuspend the pellets with the same PBS-Triton X-100 1% volumes used in step 6.
11. Quantify the protein concentration of the soluble (supernatant) and insoluble (pellet) fractions using the Bradford assay. Warm up the spectrophotometer before use.
12. Prepare a 0.1 mg/mL BSA stock solution to use as standard.

13. Dilute the Bradford reagent 5-fold in deionized H₂O to minimize analysis bias.
14. Create the blank sample adding 1 mL Bradford reagent into a clean 2 mL tube.
15. Prepare the standard samples into 2 mL clean tubes using increasing concentrations of BSA to elaborate the standard curve (i.e. 2, 3, 6, 8 µg). When pipetting, be careful to release the samples at the bottom of the tube (see **Note 23**).
16. Add 1 mL Bradford reagent to all the BSA samples, mix well and incubate for 20 minutes at room temperature.
17. Dilute the unknown samples if necessary in deionized H₂O (generally 10-100 fold) to obtain approximately 5-50 µg of protein.
18. Set up a triplicate of each unknown sample using increasing volumes (i.e. 20, 30, 40 µl) to get approximately increasing protein concentration.
19. Add 1 mL Bradford reagent to all the unknown samples, mix well and incubate for 20 minutes.
20. With the help of Pasteur pipettes, transfer each sample into clean UV/VIS cuvettes to read the absorbance.
21. Use the blank sample as the zero to calibrate the spectrophotometer. Measure the absorbance of each sample (BSA standards and unknown samples) at 595 nm.
22. Calculate the concentration of the unknown samples based on the linear equation of the standard curve.
23. Load 15 µg of each soluble fraction or 15 µl of each pellet fraction on SDS-PAGE gel and run the electrophoresis process as follows.

3.3 Recombinant protein detection by SDS-PAGE

The first step of SDS-PAGE consists of casting the polyacrylamide gel in two distinct layers: the stacking gel (5 %) and the resolving gel (12.5 %). The polyacrylamide gel is composed of

30	%	acrylamide/bis	acrylamide	29:1.
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1. Assemble the glass plates and the spacer in the gel casting apparatus. Accurately mix the resolving gel mixture before pouring it into the glass plates. Make sure to reach a level below approximately 2 cm from the shorter plates.
2. Add a layer of 2-propanol 100 % with the help of a pipette to avoid bubbles formation. This step also prevents the gel from drying out.
3. Let the resolving gel polymerize for 20 minutes at room temperature.

4. Drain the 2-propanol 100 %, and add ddH₂O to wash out any 2-propanol or gel residual. Wipe any residual water trapped into the glass plates with the help of clean absorbent paper.
5. Mix the stacking gel components and carefully pour the gel above the resolving gel to fill the glass plates. Insert the comb at the top of the gel.
6. Stand at room temperature for at least 20 minutes. Once the gel has polymerized, remove the comb and wash the wells with ddH₂O.
7. Place the casted gel into the electrophoresis apparatus and fill it with TGS 1× running buffer.
8. Accurately prepare the samples resuspending in 1× loading sample buffer.
9. Incubate the samples at 100 °C for 5 minutes. Add a protein marker into the first well and then load 15 µg or 15 µl of the samples into the wells.
10. Run the gel at 120 V until the samples reach the bottom of the gel. Stop the run and pick up the gel. Carefully remove the glass plate and place the gel into a plastic container. Wash the gel with ddH₂O.
11. Drain the water and add approximately 20 mL of Coomassie staining solution. Incubate 1 hour at room temperature with shaking.
12. Remove the staining solution and add 5 mL of destaining solution for 1 minute. Discard the destain solution and add 20 mL of destaining solution. Place at room temperature with shaking until completely destained. Wash with ddH₂O and visualize (Figure 2).

3.4 Recombinant protein detection by Western blotting

To detect the recombinant fusion protein by western blot analysis, we use an anti-His antibody against the N-terminal His-tag. Separate the proteins by SDS-PAGE as reported above and then proceed with the electro-transfer. Be careful to avoid the staining step with Coomassie staining solution when performing Western blotting analysis.

1. Once the electrophoresis is completed, collect the gel with the help of a spatula and remove the stacking gel.
2. Place the gel and the nitrocellulose membrane in separate plastic containers with the 1× Tris Base-glycine, 20 % methanol transfer buffer (see **Note 24**). Incubate for 30 minutes at 4 °C.

3. Assemble the transfer sandwich with the help of clean clamps and using absorbent filter papers. Rinse 3 filter paper into the transfer buffer and stack them on top of each other. Make sure the nitrocellulose membrane is on the cathode and the gel on the anode. Stack the other 3 filter paper rinsed into the transfer buffer onto the sandwich.
4. Eliminate any potential air bubbles trapped into the sandwich with the help of a roller.
5. Run the electro-transfer at 15 V for 30 minutes.
6. Shut the transfer machinery and pick up the membrane with the help of clean clamps. Place it into a plastic container.
7. To reveal the transferred bands, stain the membrane with Ponceau S dye for a couple of minutes.
8. Once the protein bands display, de-stain thoroughly with 1× PBS for further immuno-revelation steps.
9. Incubate the membrane with 1× PBS-milk 5 % blocking solution for 30 minutes on a shaker.
10. Add 1:1,000 HRP conjugate anti-His tag antibody to the blocking 5 % solution and incubate for 1 hour.
11. Rinse the blot 3-4 times with 1× PBS for 5 minutes.
12. Reveal using the chemiluminescent substrate according to the manufacturer instructions.
13. Visualize the chemiluminescent signal using a CCD camera-based imager.

Notes

1. To prepare 400 mL of TY agar plate, weigh 7.4 g of agar and transfer it into a clean bottle. Adjust the volume to 400 mL using fresh TY liquid medium and autoclave. Let it cool at room temperature before pouring onto Petri dishes in sterile conditions.
2. We find that preparing this fresh each time will implement the results.
3. We find best results when using it sterile.
4. Primers are designed by using the web-based program *Primer3*. The forward primer includes 15-18 nucleotides complementary to the sequence of interest starting from its start codon and a selected restriction site at the 5' end. The reverse primer includes 15-18 nucleotides complementary to the sequence of interest starting from the stop

codon and a selected restriction site at the 5' end of this primer. The restriction sites are chosen amongst that present in the Multi-Cloning-Site (MCS) of the vector and should guarantee that the DNA fragment is inserted *in frame* with the start codon of the expression vector

5. Before pouring onto Petri dishes, add 400 μ L ampicillin into a 400 mL TY agar plate. We maintain a ratio of 1:1,000 when adding ampicillin to medium or suspension. This means that for 1 mL of medium, 1 μ L of ampicillin (100 μ g/mL) is added.
6. To prepare ampicillin 100 mg/mL stock, weigh 1 g of ampicillin (keep the powder stock stored at 4 °C). Transfer into a sterile 15 mL falcon having care not to miss any granule of the ampicillin powder (help yourself with a washing flask). Add water up to 10 mL. Vortex to help to dissolve ampicillin. Filter the solution with a 0.22 μ M filter and aliquot into sterile 2 mL tubes. Store at -20 °C.
7. To prepare 500 mL 50 $\times$ TAE, weigh 121 g of Tris Base and dissolve in approximately 300 mL of deionized water. Add 28.6 mL of 100 % Glacial Acetic Acid and 50 mL of 0.5 M EDTA. Tris Base will take a while to dissolve completely, thus we find it convenient to use it as the first ingredient. Store stock solution at room temperature.
8. Prepare Tris Base-HCl 1 M solution and adjust the pH to 8. Weigh 0.90 g of glucose and add to a becher containing 50 mL of water. Add 2.5 mL of Tris Base-HCl 1 M, 2 mL of 0.5 M EDTA. Add water up to 100 mL and mix until dissolved. Adjust the pH to 8 if needed.
9. Mix 10 mL of 10 % SDS and 5 mL of 4 M NaOH into 8.5 mL of distilled water. Add deionized water up to 100 mL.
10. Always use cold freeze EtOH. When preparing or handling EtOH wear gloves and a mask to prevent from inspiring any fumes. To prepare 100 mL of EtOH 70 %, add 30 mL of EtOH to 70 mL of deionized water. Store at - 20°C.
11. To prepare RNase A 10 mg/mL stock, weigh 10 mg of RNase A (keep the powder stock stored at 4 °C). Transfer into a 2 mL sterile tube having care not to miss any granule of the RNase powder (help yourself with a washing flask and a rack to stabilize the sterile tube). Add water up to 1 mL. Vortex to help to dissolve the powder. Store at -20°C.
12. To prepare a stock of IPTG 100 mM, weigh 0.24 g of IPTG (keep the powder stock stored at -20°C). Transfer into a sterile 15 mL falcon tube having care not to miss any granule of the powder (help yourself with a washing flask). Add deionized water up to 10 mL. Vortex to help to dissolve the powder. Filter the solution with a 0.22 μ M filter and aliquot into sterile 2 mL tubes. Store at -20 °C.
13. In a 15 mL conical tube add the acrylamide, Tris Base-HCl and SDS. Carefully mix and then add APS. Reach the final volume by adding ddH₂O and mix well. Add

TEMED at last, evenly mix and pour it into the glass plates directly from the tube. This will lessen bubbles forming at the top of the gel. Always add TEMED as the last ingredient because this will initiate the polymerization reaction.

14. To prepare 100 mL stock of TGS 10 $\times$, weigh 3 g of Tris Base and 14.4 g of Glycine. Transfer into a bottle and add 10 mL of 10 % SDS. Add deionized water up to 100 mL.

To prepare 1 L of TGS 1 $\times$, add 100 mL of TGS 10 $\times$ to 900 mL of ddH₂O previously spilt into a cylinder. Seal the cylinder with parafilm and carefully mix upside down to evenly blend the ingredients.

15. Always store at -20 °C. When the solution is being used, place it on ice. We find it proper not to let the solutions sit at room temperature.
16. To prepare 400 mL of stain solution, add 0.8 g of Brilliant Blue R to 100 mL of 2-propanol and let the Coomassie powder dissolve completely. Add 40 mL of acetic acid and up to the final volume with ddH₂O.
17. Always wear gloves when handling acetic acid or ethanol and prepare the solution in a fume hood to avoid any fumes inhalation.
18. Always prepare a large stock of 10 $\times$ Tris Base-glycine (as reported in **Note 15** except for SDS) to store at room temperature.
19. Prepare 50 mL of 1 $\times$ PBS-milk 5 % blocking solution, weigh 2.5 g of skimmed milk and transfer into a falcon tube containing 50 mL of 1 $\times$ PBS. Vortex the solution may help dissolve faster.
20. It seems to be common that competent cells may have low-efficiency transformation. We find that this issue can be sorted out by changing the molarity of CaCl₂ or using fresh CaCl₂, or resuspending more gently.
21. Frequently this ligation reaction step does not work as intended causing low or no recombinant colonies downstream the transformation step. Thus, troubleshoot modifying the vector:insert molar ratio for the insert as well as scaling down ng of the vector.
22. Prepare TAE 1 $\times$ adding 20 mL TAE 50 $\times$ to 980 mL ddH₂O evenly mixing.
23. When performing the Bradford assay, put the samples first into the tubes (BSA standard or unknown) paying attention to release the sample completely and then add the Bradford reagent. It is recommended to use the same equipment throughout the assay to minimize biases that frequently can occur when handling with little volumes.
24. Prepare 1 $\times$ Tris Base-glycine-20 % methanol in a becher adding 10 mL 10 $\times$ Tris Base-glycine, 20 mL methanol 100 % and 70 mL ddH₂O. The gel and the nitrocellulose membrane must be thoroughly covered with the transfer buffer solution.

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Figure 1. A simplified scheme of the cloning strategy applied to obtain the recombinant plasmid. Green and red lines indicate forward and reverse primers used for the PCR amplification step. The amplified products (PCR insert) and the expression vector (pRSET-A) are subjected to digestion with the appropriate restriction enzymes, ligated thanks to the action of the T4 ligase enzyme, and cloned into *E. coli* DH5a host cells. The recombinant plasmid is then used to transform *E. coli* BL21 (DE3) competent cells. *Ampicillin*= ampicillin resistance gene; *MCS*= multi cloning site.

Figure 2. An example of SDS-PAGE analysis of soluble and insoluble proteins extracted from induced and uninduced cultures. The image represents an SDS-PAGE analysis of the expression of a novel putative collagenase from a marine microorganism *Sulfurovum riftiae* (10), class *Epsilonproteobacteria* (recently proposed to be reclassified as a new phylum *Campylobacterota*) (11). The arrow indicates a strong expression of the protein in the induced cultures. **M**: Protein marker (Precision Plus Protein Dual Color Standards, BIORAD); **NI_s** indicates soluble proteins extracted from the uninduced BL21 (DE3) strain carrying the recombinant plasmid; **NI_p** indicates insoluble proteins extracted from the uninduced BL21(DE3) strain carrying the recombinant plasmid; **I_s** indicates soluble proteins extracted from the induced BL21 (DE3) strain carrying the recombinant plasmid; **I_p** indicates insoluble proteins extracted from the induced BL21 (DE3) strain carrying the recombinant plasmid.