Chapter 2

Development of *Mycoplasma hyopneumoniae* Recombinant Vaccines

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1 Introduction

Respiratory diseases are among the most important health problems associated with swine production. *Mycoplasma hyopneumoniae* is the principal etiological agent responsible for enzootic pneumonia (EP), a chronic respiratory disease in pigs. This infection is highly prevalent (ranging between 38 and 100 %), in almost all areas of pig production worldwide, and *M. hyopneumoniae* infections cause significant economic losses [1]. The control of EP should focus on the optimization of management practices and housing conditions [2], the use of antimicrobial medication [3], and vaccination. Several commercial vaccines consisting of the inactivated adjuvanted whole cell lysates of *M. hyopneumoniae* are available and used worldwide. Though these vaccines have been proven to be effective in reducing the clinical signs, they provide only partial protection against the development of lesions [4].

Recombinant DNA technology could be employed to overcome problems encountered with conventional vaccines. The small genome of this pathogen, as well as the limited number of secreted or surface proteins, favors the use of reverse vaccinology approach [5]. However, *Mycoplasma* sp. uses an unusual genetic code. The amino acid tryptophan is not encoded by TGG, as in most organisms, but by TGA, which is a stop codon [6]. This difference has hampered the expression of genes of *M. hyopneumoniae* containing TGA codons in *Escherichia coli*, the most attractive system used for production of recombinant proteins [7]. However, mutations that can replace TGA codons with TGG have been used to solve this problem [8].

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Constant effort is being directed toward the investigation of new vaccines that may offer a better protection against *M. hyopneumoniae* infections. Several studies have evaluated recombinant proteins of *M. hyopneumoniae*, in different forms of administration and formulations, seeking to develop more effective vaccines against EP. Some of them were evaluated individually [9, 10], and others were associated with attenuated bacterial or viral vectors [11–18], fused to mucosal adjuvants [19, 20], and also evaluated as a cocktail of antigens [21, 22]. Only a few of these recombinant proteins were used in challenge experiments in pigs; most of them were evaluated only in mice. Differences in the immunity induced by these antigens were observed, which can be influenced by differences in the vaccine construction, the route of immunization, the correct folding, and/or other posttranslational modification that may contribute to the ability to generate antibodies by the antigens [23]. However, these evaluations suggest that these new vaccine approaches may represent promising new strategies and may be economically feasible to control EP.

2 Materials

2.1 Strains and Plasmids

1. *M. hyopneumoniae* strain for genomic DNA extraction.
2. *E. coli* TOP10 (Invitrogen, USA) as a host strain.
3. *E. coli* BL21 (DE3) RIL (Invitrogen, USA) as expression strain.
4. Vector Champion pET200D/TOPO (Invitrogen, USA) for cloning and expression.

2.2 Bioinformatics Software

1. Bioinformatics softwares: Pfam, SignalP, PROSITE, and NNPREDICT for in silico selection of coding sequences.
2. Vector NTI® 11 (Invitrogen, USA) for primer design.

2.3 Cloning of M. hyopneumoniae Coding Sequences

1. Genomic DNA extracted of *M. hyopneumoniae* strains.
2. GFX genomic blood DNA and gel band purification kit (GE Healthcare, USA).
3. PCR reagents: 50 ng of *M. hyopneumoniae* genomic DNA, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 10 pmol of each primer, 2.5 units of Platinum *Pfx* DNA polymerase, 1× reaction buffer, and 1× enhancer buffer (Invitrogen, USA).
4. Champion pET200D/TOPO (Invitrogen, USA) expression vector.
5. Restriction enzymes and 10× buffers (Invitrogen, USA).
6. T4 DNA ligase and 10× buffers (Invitrogen, USA).
7. Agarose, loading dye, and nucleic acid stain suitable for gel electrophoresis (Invitrogen, USA).

8. Agarose gel electrophoresis (for 150 ml of 1.0 % agarose gel, use 1.5 g of ultrapure agarose with 150 ml of 1× TBE. Prepare 1 l of 1× TBE solution in ultrapure water with 10.8 g of Tris base, 5.5 g of boric acid, and 4 ml of EDTA 0.5 M, and adjust to pH 8.0).
9. Gel documentation system (Loccus, Brazil) or equivalent.
10. QIAquick Gel Extraction Kit (Qiagen, Germany).
11. *E. coli* TOP10 electro-competent cells (Invitrogen, USA).
12. Electroporator: Bio-Rad Gene Pulser®II, BTX® ECM® 630 (Bio-Rad, USA) or equivalent.
13. Electroporation cuvette of 0.1 cm (Bio-Rad, USA) or equivalent.
14. Luria–Bertani (LB) medium (to 900 ml of distilled H_2O, add 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 10 g NaCl. Adjust to 1 l with distilled H_2O. Sterilize by autoclaving and store up to 3 months at room temperature).
15. Kanamycin (100 μg/ml) (Sigma-Aldrich, USA).
16. Phenol–chloroform (Sigma-Aldrich, USA) and lysis buffer (sample buffer 6× [24]: 3 ml glycerol (30 %); 25 mg bromophenol blue (0.25 %); dH_2O to 10 ml. Lysis buffer: 100 μl of sample buffer + 900 μl of dH_2O + 1 μl RNAse).
17. DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, USA).
18. MegaBACE 500 (GE Healthcare, USA) or equivalent.
19. Shaking and non-shaking incubator at 37 °C (DeLeo, Brazil) or equivalent.
20. Illustra plasmidPrep Mini Spin Kit (GE Healthcare, USA).

2.4 Expression of Recombinant Proteins and Solubility Testing

1. *E. coli* BL21 (DE3) RIL (Invitrogen, USA) expression competent cells.
2. Recombinant plasmid DNA.
3. Solid and liquid LB medium (to 900 ml of distilled H_2O, add 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 10 g NaCl. Adjust to 1 l with distilled H_2O. Sterilize by autoclaving and store up to 3 months at room temperature. For solid medium, add 1.5 % of agar).
4. Kanamycin (100 μg/ml) (Sigma-Aldrich, USA).
5. Isopropylthio-b-d-galactosidase (IPTG) (Invitrogen, USA) with a final concentration of 0.3 mM.
6. Sterile inoculation loops.
7. Sterile round-bottom snap-cap tubes of 15 ml (Sigma-Aldrich, USA).
8. UV spectrophotometer (Biochrom, USA) or equivalent.

9. Shaking and non-shaking incubator (DeLeo/Brazil).
10. Phosphate-buffered saline 0.1 M (PBS, pH 7.4).
11. Loading buffer: (5×) (62.5 mM Tris–HCl pH 6.8, 10 % glycerol, 5 % 2β-mercaptoethanol, 2 % SDS).
12. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) 12 % running gel: 10.2 ml of H_2O; 7.5 ml of 1.5 M Tris–HCl pH 8.8; 0.15 ml of 20 % (w/v) SDS; 12.0 ml of acrylamide/bis-acrylamide (30 %/0.8 % w/v); 0.15 ml of 10 % (w/v) ammonium persulfate (APS); 0.02 ml of TEMED. Cast gel within a 7.25 cm × 10 cm × 1.5 mm gel cassette. Allow space for stacking the gel and gently overlay with water. Prepare the stacking gel by mixing 3 ml of H_2O; 1.25 ml of 0.5 M Tris–HCl pH 6.8; 0.1 ml of 20 % (w/v) SDS; 0.67 ml of acrylamide/bis-acrylamide (30 %/0.8 % w/v); 0.04 ml of 10 % (w/v) ammonium persulfate (APS); 0.005 ml of TEMED. Insert a ten-well gel comb immediately without introducing air bubbles.
13. Coomassie Blue (Sigma-Aldrich, USA).
14. Lysozyme 1 mg/ml (Sigma-Aldrich, USA).
15. Phenylmethanesulfonyl fluoride (PMSF) 1 mM (Sigma-Aldrich, USA).
16. Microcentrifuges (Thermo Scientific, USA).
17. Ultrasonic probe sonicator (Qsonica LLC, USA).

2.5 Solubilization and Purification of Recombinant Proteins

1. Buffer A: (200 mM NaH_2PO_4, 500 mM NaCl, 5 mM imidazole, pH 8.0) containing 8 M urea (Sigma-Aldrich, USA).
2. HisTrap™ HP 1 ml columns prepacked with precharged Ni Sepharose™ (GE Healthcare, USA).
3. Buffer B: (200 mM NaH_2PO_4, 500 mM NaCl, 500 mM imidazole) containing 8 M urea (Sigma-Aldrich, USA).
4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) 12 % (*see* Subheading 2.4).
5. PBS (pH 7.4) containing 0.05 % Triton X-100 (Sigma-Aldrich, USA).
6. Refrigerated ultracentrifuge (Thermo Scientific™ Sorvall™ WX Floor, USA).
7. ÄKTAprime™ automated liquid chromatography system (GE Healthcare, USA).
8. Dialysis tubing membrane (Sigma-Aldrich, USA).

2.6 Immunoblotting Components

1. Nitrocellulose membranes (GE Healthcare, USA).
2. Western blot transfer buffer (0.025 M Tris, 0.192 M glycine, and 20 % ethanol) (Sigma-Aldrich, USA).

3. PBS containing 0.05 % Tween-20 (PBST) (Sigma-Aldrich, USA).
4. Blocking solution (5 % powdered milk in PBS). Store at 4 °C.
5. Diluent solution (5 % powdered milk in PBST). Store at 4 °C.
6. Mini-PROTEAN® 3 system glass plates (Bio-Rad, USA) or equivalent.
7. Whatman no. 3 filter paper (GE Healthcare, USA) or similar.
8. Monoclonal antibodies anti-6× His IgG (Sigma-Aldrich, USA).
9. 4-Chloro-1-naphthol.
10. Hydrogen peroxidase.
11. BenchMark™ Prestained Protein Ladder (Invitrogen, USA) or equivalent.

2.7 Vaccine Formulation

1. BCA™ protein assay kit (Pierce, USA).
2. Purified recombinant proteins.
3. Aluminum hydroxide or similar adjuvants.

3 Methods

3.1 Selection of Coding Sequences and Primer Design

1. Coding DNA sequences (CDS) encoding surface-exposed, secreted proteins, related to pathogenesis with up to three tryptophan (TGA) codons should be selected by bioinformatics software.
2. Primers are designed based on GenBank genome sequences by Vector NTI® 11.
3. The mutagenesis procedure required four oligonucleotides: two flanking primers, which are positioned upstream (U) and downstream (D) of the mutation site, and two mutagenic primers, forward (FM) and reverse mutagenic (RM) with at least a 15 bp overlap between adjacent fragments. The mutation site should be located in the middle of the mutagenic primers.
4. Cloning into Champion pET200D/TOPO His-tag expression vector is necessary to add four bases (CACC) in the forward flanking primer to anneal a complementary overhang (GTGG) in the vector.

3.2 The Site-Directed Mutagenesis Method

1. Genomic DNA extraction of *M. hyopneumoniae* strains can be performed with GFX genomic blood DNA purification kit.
2. Site-directed mutagenesis is carried out using a two-step PCR procedure to replace the TGA codons using an overlap extension PCR method [4].
3. The first step, two simultaneous PCR reactions are performed. One reaction is performed with a primer pair that included the

U primer and the RM primer; the other reaction contained the D primer and the FM primer. PCR reactions are carried out with a final volume of 25 μl.

4. PCR reactions are carried out using 50 ng of *M. hyopneumoniae* genomic DNA, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 10 pmol of each primer, 2 units of Platinum® *Pfx* DNA polymerase (extension temperature 68 °C), and 1× reaction buffer in a 25 μl reaction volume. For amplification, a Mastercycler gradient is used with the following settings: 7 min at 95 °C followed by 30 cycles of 60 s at 95 °C, 60 s at 55 °C and 60 s at 68 °C, and then a final extension of 7 min at 68 °C.
5. PCR products are analyzed by 1 % agarose gel electrophoresis and gel band purified using the GFX PCR DNA and gel band purification kit, according to the manufacturer's instructions. To obtain full-length mutated fragments, the two products from the first PCR are mixed in equimolar concentrations (approximately 0.4–0.8 pmol) and used as templates during the second PCR reaction, with the U and D primers. The reaction is performed using the same conditions as first PCR.

3.3 Cloning of M. hyopneumoniae Coding Sequences

1. The overlap extension PCR yields a full-length DNA fragment that is ligated into Champion pET200D/TOPO expression vector following the manufacturer's instructions (Fig. 1). The vector contains the T7 promoter and allows expression of the recombinant protein fused to His-tag at the N-terminus.
2. The ligation products are transformed (*see* **Note 1**) into *E. coli* TOP10 electro-competent cells following the manufacturer's instructions.
3. Recombinant clones are identified by lysis cells (*see* **Note 2**).
4. The identity of the inserts is determined by DNA sequencing (*see* **Note 3**) using the DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems—MegaBACE 500.

3.4 Expression of Recombinant Proteins and Solubility Testing

1. Recombinant plasmids are transformed into *E. coli* BL21(DE3) RIL expression competent cells by electroporation following a standard method (*see* **Note 1**).
2. One recombinant clone of each plasmid is used to inoculate 5 ml of Luria–Bertani (LB) medium containing 100 μg/ml kanamycin, incubated in shaker at 37 °C and 1 × *g* until $OD_{600} = 0.6$.
3. Expression of the recombinant proteins is induced with IPTG with a final concentration of 0.3 mM and the culture is grown at 37 °C for 3 h.
4. A volume of 500 μl of culture growth is centrifuged at 14,000 × *g* for 2 min, and the pellet is solubilized in 80 μl of 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing

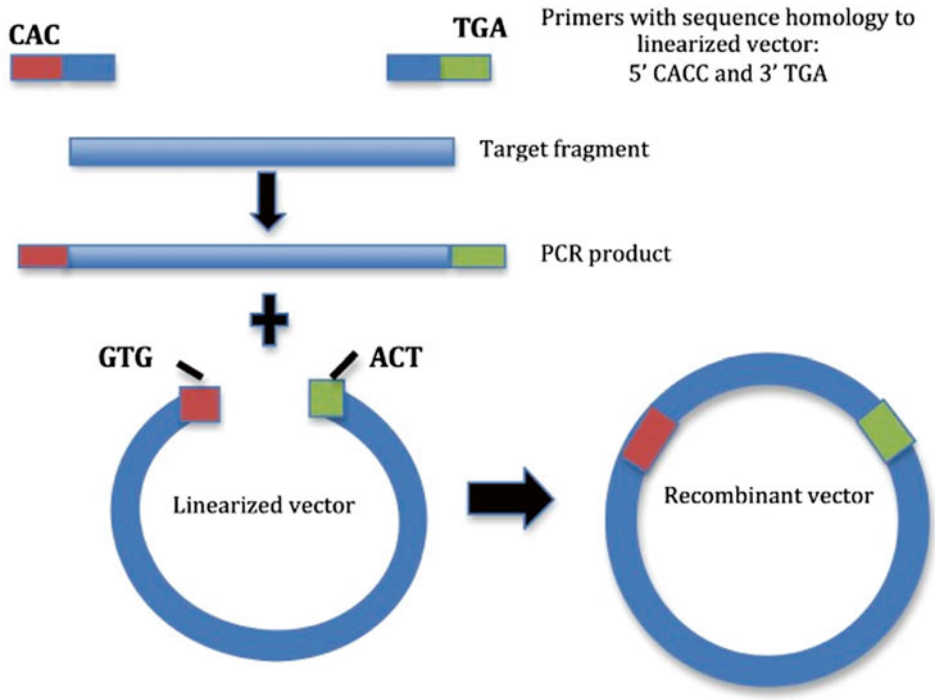

Fig. 1 Schematic representation of the cloning of *Mycoplasma hyopneumoniae* coding sequences into Champion pET200D/TOPO His-tag expression vector

20 µl of 5× loading buffer. After boiling for 10 min, a volume of 8 µl of the supernatant is submitted to 12 % SDS–PAGE (*see* **Note 4**).

5. Expression of recombinant proteins is identified by staining the gel with Coomassie Blue and visualization of an extra protein band of the predicted size.
6. Clones that expressed the recombinant protein are tested regarding the solubility of the protein. For that, a volume of 2 ml of culture growth is centrifuged at 14,000 × *g* for 2 min, and the pellet is solubilized in 500 µl of PBS containing 1 mg/ml lysozyme and 1 mM PMSF. Cells are lysed by sonication (6× 10 s pulses) in ice-water bath. The soluble and insoluble fractions are separated by centrifugation at 10,000 × *g* for 5 min at 4 °C. Soluble proteins remained in the supernatant and insoluble proteins are found in the pellet. Both fractions are submitted to 12 % SDS–PAGE (*see* **Note 4**) for identification of the protein localization.

3.5 Solubilization and Purification of Recombinant Proteins

1. Proteins expressed in *E. coli* as insoluble particles are solubilized with 8 M urea. For solubilization, a pellet obtained from a 500 ml culture (approximately 1 g cells) is solubilized in 30 ml of buffer A. After incubation at 4 °C for 60 min, the cell lysate is centrifuged at 14,000 × *g* for 60 min at 4 °C (*see* **Note 5**).

2. The recombinant proteins are purified from the supernatant by affinity chromatography using HisTrap™ HP 1 ml columns prepacked with precharged Ni Sepharose™ using the ÄKTAprime™ automated liquid chromatography system. The recombinant protein is eluted with a linear gradient from 5 to 500 mM imidazole. Fractions of 1 ml are collected and 5 μl is applied to 12 % SDS–PAGE (*see* **Note 4**).
3. Fractions of 10 ml containing the recombinant protein are pooled in a dialysis tubing membrane (Sigma-Aldrich, USA), with a concentration ranging from 0.6 to 16 mg and submitted to an extended stepwise dialysis in 10 l of PBS (pH 7.4) at 4 °C for 120 h, containing 0.05 % Triton X-100 to remove imidazole and urea and to promote protein refolding.
4. Purification of soluble proteins is performed under non-denaturing conditions with buffer A without urea or *N*-lauroylsarcosine.

3.6 Electrophoretic Transfer

1. Immediately following SDS–PAGE, when the dye front reaches the end of the gel, turn off the power supply. Separate the gel plates with the help of a spatula or similar tool. Remove the stacking gel.
2. Excise the gel with recombinant proteins such that there is one lane with the protein markers and one with the recombinant proteins.
3. Gently lay one nitrocellulose membrane, cut to the shape of the gel, on top of the gel (*see* **Note 6**).
4. Gently lift the gel–membrane sandwich from the glass plate and place it on a Whatman no. 3 filter (place membrane side directly on the filter paper and the exposed gel side on top) cut to the size of the gel.
5. Place a second Whatman no. 3 filter paper cut similarly (*see* **Note 6**).
6. Place the nitrocellulose–gel–filter paper sandwich between two mini-PROTEAN® 3 system running modules with transfer buffer.
7. Place this assembly in a Mini Trans-Blot and run at 37 °C for 60 min at 200 V. Remove the membrane for immunoblotting.

3.7 Western Blot Analysis

1. The molecular mass of the recombinant proteins expressed in *E. coli* is assessed by Western blot using anti-His antibody.
2. Purified recombinant proteins are separated by 12 % SDS–PAGE (*see* **Note 4**), electrotransferred into a nitrocellulose membrane, and blocked with 5 % nonfat dry milk in PBS at 37 °C for 2 h. After washing with PBST, the membrane is incubated with mouse peroxidase-conjugated monoclonal anti-6× His IgG.

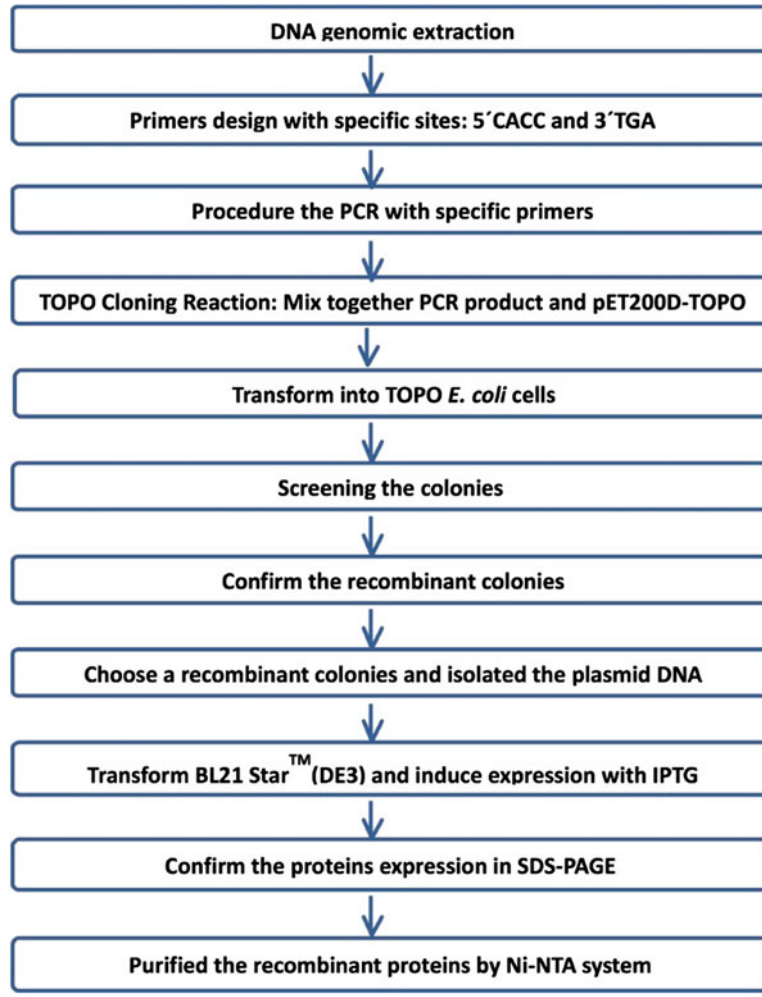

Fig. 2 Step-by-step recombinant protein vaccine production

3. Immunoreactive protein bands are detected with 0.005 % (w/v) 4-chloro-1-naphthol and 0.015 % (v/v) hydrogen peroxidase in PBS (0.005 % (w/v) of 4-chloro-1-naphthol in 10 ml Tris–HCl 50 mM pH 7.5). Add 10 μl H_2O_2 just before adding to the membrane.
4. Figure 2 shows step-by-step procedure for the generation of recombinant protein vaccines.

3.8 Vaccine Formulation

1. BCA™ Protein Assay kit is used to measure the recombinant protein concentrations according to the instructions provided by the manufacturer.
2. Recombinant proteins are used in a concentration ranging between 20 and 50 μg in 15 % aluminum hydroxide or similar adjuvants.

4 Notes

1. Set up electroporator Genepulser II (Bio-Rad, USA) for bacterial transformation following the manufacturer's instructions. Add 1–2 μl of each ligation reaction to the volume of cells recommended by the manufacturer (may be less than 50 μl). Mix gently with pipette tip. Do not mix by pipetting up and down. Transfer the cells to the chilled electroporation cuvette (0.1 or 0.2 cm) on ice. Electroporate the cells as per the manufacturer's recommended protocol. Quickly add 250 μl room temperature LB medium and mix gently. Transfer the solution to a 15 ml snap-cap tube (i.e., Falcon) and shake for at least 1 h at 37 °C to allow expression of the antibiotic resistance gene. Spread 10–150 μl from each transformation on a pre-warmed LB plate containing kanamycin (100 μg/ml). The remaining transformation mix may be stored at 4 °C and plated out the next day, if desired. Incubate the plates overnight at 37 °C. Select colonies and lyse the cells for plasmid isolation.
2. Identification and selection of colonies on the plate. To the Eppendorf tube, add 15 μl of phenol–chloroform solution and 15 μl of lysis buffer. Add one identified colony to this tube, shake and centrifuge at 14,000×*g* for 4 min. Apply the upper phase (approximately 15 μl) in agarose gel 0.8 %. It is important to use as a control the plasmid DNA from the vector in the gel to differentiate DNA recombinant from not recombinant clones.
3. Assemble each sequencing reaction as follows: template DNA (0.1–0.2 pmol), primer (5 pmol), sequencing reagent premix (8 μl—DYEnamic ET Terminator Cycle Sequencing Kit, GE Healthcare, USA), and water to a total volume of 20 μl. For sequencing with this DYEnamic ET Terminator Cycle Sequencing Kit, a sequencing reagent premix is combined with template DNA and primer and thermally cycled. The reaction products are then precipitated with ethanol to remove unincorporated dye-labeled terminators. Samples are finally dissolved in an appropriate loading solution for separation and detection using the MegaBACE DNA Analysis Systems—MegaBACE 500 (GE Healthcare, USA) sequence instruments.
4. Mix the recombinant protein 4:1 with the sample buffer (10 % w/v SDS; 10 mM dithiothreitol, or beta-mercaptoethanol; 20 % v/v glycerol; 0.2 M Tris–HCl, pH 6.8; 0.05 % w/v bromophenol blue). Heat the samples at 95 °C for 5–10 min. Clamp in your gel and fill both buffer chambers with gel running buffer (25 mM Tris–HCl; 200 mM glycine; 0.1 % w/v SDS) according to the instructions for your specific apparatus. Add the sample into the gel adjusting the volume according to the amount of protein. Be sure to include a lane with molecular weight standards. Electrophorese at 15 mA

until the sample has entered the gel and then continue at 25 mA till the dye front reaches the bottom of the gel. Remove the gel for the power supply and process further. Visualize your proteins using Coomassie Blue or any of the other protein stains.

5. N-lauroylsarcosine (0.2 %) is used to replace 8 M urea for solubilization of insoluble proteins that reaggregated during dialysis. In this case, the inclusion bodies are solubilized in buffer A, containing 0.2 % N-lauroylsarcosine instead of 8 M urea, incubated at 4 °C for 72 h for complete solubilization. Purification and dialysis are performed as described above.
6. Hold the two top corners of the membranes with each hand. Lower the bottom part of the membrane first on the lower part of the gel and gently release the membrane slowly to lay the complete membrane on the gel. This will prevent trapping of bubbles in between the gel and the membrane.

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