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Heterologous expression of membrane proteins in *E. coli*

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Running title: Expression of membrane proteins in *E. coli*

Abstract

Over the decades, the bacterium *Escherichia coli* (*E. coli)* has become the cornerstone of recombinant protein production, used for heterologous synthesis of a variety of membrane proteins. Due to its rapid growth to high densities in cheap media, and its ease of manipulation and handling, *E. coli* is an excellent host cell for a range of membrane protein targets. Furthermore, its genetic tractability allows a variety of gene constructs to be screened for optimal expression conditions, resulting in relatively high yields of membrane protein in a short amount of time. Here, we describe the general workflow for the production of membrane proteins in *E. coli*. The protocols we provide show how the gene of interest is modified, transferred to an expression vector and host and how membrane protein yields can be optimised and analysed. The examples we illustrate are well suited for scientists who are starting their journey into the world of membrane protein production.

Keywords: Recombinant protein production, *Escherichia coli*, integral membrane proteins, outer membrane proteins.

1. Introduction

Membrane proteins are central to biological processes such as signal transduction, the import and export of nutrients and metabolites as well as cell communication [1,2]. Consequently, they are important drug targets although their potential remains largely untapped [3]. For structural and functional analysis, large quantities of pure, stable membrane proteins are required. Recently, we showed that over 46 % of unique eukaryotic membrane protein structures have been achieved with proteins expressed in bacterial expression systems [1] with the majority of them being *E. coli* strains [4]. Even though bacteria have different lipid environments and generally lack the ability to perform eukaryotic post translational modifications, their low cost, ease of manipulation and handling, as well as their short expression and processing times give them many advantages over other expression systems.

49 The abundance of natural membrane proteins in their native membranes is nearly always too
50 low for subsequent study [5]. Therefore, the majority of proteins are overexpressed using
51 strong promoters, which includes those controlled by the T7 RNA polymerase (T7 RNAP) from
52 T7 bacteriophage [1]. For T7-based expression, BL21(DE3) cells are the standard strain used.
53 However, overexpressing certain membrane proteins can be toxic to the cells and may inhibit
54 cell growth. This can be overcome by the use of strains such as C41(DE3) and C43(DE3),
55 weaker promoters such as the *lac* promoter, tightly regulated expression systems (e.g.
56 *paraBAD*) or the manipulation of expression conditions (e.g. decreasing the growth
57 temperature) [1,6,7]. Other options that may be trialled include the regulation of T7 RNAP
58 through the expression of T7 lysozyme in BL21(DE3) pLysS cells, using lower inducer
59 concentrations or the inhibition of *lac*-based promotor systems by the presence of glucose due
60 to catabolite repression. The expression rate and level of membrane proteins is of great
61 importance to avoid saturating the Sec translocon to obtain homogenous, active protein [6].

62 Most eukaryotic integral membrane proteins expressed in *E. coli* will be targeted to the inner
63 bacterial membrane. However, β-barrel containing membrane proteins (OMPs), which are
64 found in the outer membranes of Gram-negative bacteria, as well as in mitochondria and
65 chloroplasts, are targeted to the *E. coli* outer membrane. For an OMP to be inserted in the
66 outer membrane of *E. coli*, it must be expressed in the cytoplasm, traverse the inner
67 membrane and periplasmic space to be inserted into the outer membrane [8-10]. As for the
68 expression of integral membrane proteins, the gene of interest must carry a 5' signal sequence
69 for this to occur. Overloading of the cellular machinery responsible for these events (i.e. the
70 Sec translocon, the periplasmic chaperones, and the nascent Bam complex) will likely result
71 in toxicity and cell death [9-12]. Thus, like integral membrane proteins, the expression of
72 heterologous β-barrel containing membrane proteins can also be problematic.

73 After overexpression, recombinant membrane proteins must be extracted from the membrane
74 into a stable and soluble environment to avoid aggregation and allow purification. Information
75 about extraction and purification methods as well as different membrane mimetic

environments are described elsewhere in the literature [13-16]. For the purification of membrane proteins, protein tags are nearly always used. The position and properties of those tags can have a significant influence on protein expression and function. Therefore, it may be necessary to test tags at either terminus of the protein to achieve satisfactory protein yields and to add a cleavage site between the tag and the protein to ensure that the tag does not influence protein function.

In the following sections, we provide protocols for heterologous membrane protein production in *E. coli*. First, the gene of interest is extracted from genomic DNA or a plasmid containing the gene and modified with restriction sites, tags and potentially also signal sequences, typically by polymerase chain reaction (PCR). To save time, it is possible to purchase the synthesized gene of interest with modifications. Second, the gene is cloned into a suitable expression vector, followed by transformation of the plasmid into the *E. coli* expression strain of choice. Finally, expression conditions are tested to generate the highest yield of functional membrane protein. The general workflow of the process is visualized in Fig. 1.

2. Materials

All materials (tubes, tips or liquids [e.g. Milli-Q® water or medium]), should be autoclaved before their use in bacterial cultures to avoid contamination, unless otherwise stated.

2.1. Molecular cloning

2.1.1. Polymerase chain reaction (PCR)

1. DNA template containing the gene of interest (*see* **Note 1**).
2. PCR tubes (e.g. Axygen™).
3. High-Fidelity DNA polymerase and dedicated buffer (e.g. Q5 High-Fidelity DNA polymerase from New England Biolabs (NEB)).
4. dNTPs: Make a stock solution containing 10 mM of each nucleotide. Store aliquots at -20 ˚C.
5. Forward and reverse primers.

103 6. T100 Thermal Cycler (BIO-RAD).

104 7. Nanodrop 1000 (ThermoScientific).

105 8. DNA clean up kit (e.g. Monarch PCR and DNA kit from NEB)

106 *2.1.2. Restriction digestion*

107 1. DNA gel extraction kit (e.g. Monarch DNA gel extraction kit from NEB).

108 2. Agarose.

109 3. GelRed (Sigma-Aldrich).

110 4. 1x TAE buffer: 40 mM Tris Acetate, 2 mM EDTA, pH 8.5 (*see* **Note 2**).

111 5. Loading dye (e.g. 6× purple loading dye from NEB).

112 6. Chosen expression vector (e.g. pET15b).

113 7. Chosen restriction enzymes with adequate buffer (*see* **Note 3**).

114 8. Gel slicer / razor blades / scalpel.

115 *2.1.3. Ligation*

116 1. T4 DNA ligase and dedicated buffer.

117 2. Heat block.

118 *2.1.4. Heat-shock transformation*

119 1. Luria-Bertani (LB) medium: Dissolve 10 g tryptone, 5 g yeast extract, and 5 g NaCl in
120 1 L demineralized water. Sterilize by autoclaving (*see* **Note 4**).

121 2. LB - ampicillin agar plates: LB medium with 15 g/L agar. Sterilize by autoclaving. Once
122 the medium, is cooled to 50 – 60 ˚C, add 1 mL per L medium of a filter-sterilized
123 solution of 100 mg/mL ampicillin (*see* **Note 5**) dissolved in water. Mix together gently
124 and pour the medium into sterile Petri dishes (*see* **Note 6**).

125 3. Heat block.

126 4. 1.5 mL sterile microcentrifuge tubes.

127 5. 50 % filter-sterilized glycerol.

128 **2.2 Small scale expression**

129 1. Autoclaved LB medium (*see* **Note 4**).

130 2. 100 mg/mL ampicillin (*see* **Note 5**).

131 3. 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) (*see* **Note 7**).

132 4. 50 mL centrifuge tubes (*see* **Note 8**).

133 5. A glycerol stock of the *E. coli* cells transformed with the expression plasmid (*see*
134 **Note 9**).

135 6. Three incubators at 37 °C, 30 °C and 25 °C (*see* **Note 10**).

136 7. A UV/Vis spectrophotometer which can measure the optical density at 600 nm (OD_{600}).

137 8. Eppendorf® centrifuge 5810R.

138 **2.3 SDS-PAGE and western blotting**

139 1. SDS-PAGE: For a 10 % resolving gel 10 % 40:1 acrylamide:bis-acrylamide Protogel
140 premix, 375 mM Tris-HCl pH 8.7, 1 % SDS, 6.68 mM 0.32 % APS. For a 5 % stacking
141 gel 5 % 40:1 acrylamide:bis-acrylamide Protogel premix, 125 mM Tris-HCl pH 6.9, 1 %
142 SDS, 6.68 mM TEMED, 0.8 % APS.

143 2. 6× SDS loading dye: 30 % 2-mercaptoehanol, 12 % SDS, 10 % glycerol, 0.1 %
144 bromophenol blue, 440 mM Tris-HCl pH 6.8.

145 3. 1× SDS running buffer: 250 mM Tris base, 1.92 M glycine, 1 % SDS.

146 4. Protein ladder (e.g. PageRuler from Invitrogen).

147 5. Coomassie protein staining (e.g. InstantBlue Ultrafast protein staining from Sigma-
148 Aldrich).

149 6. 1× TBS-T: 20 mM Tris-Cl pH 7.5, 500 mM NaCl, 0.05 % (w/v) Tween-20 (*see* **Note 11**).

150 7. 1× Transfer Buffer: 0.25 M Tris base, 1.92 M glycine, 10 % (v/v) methanol (*see*
151 **Note 12**)

152 8. Blocking buffer: 5 % (w/v) BSA in 1× TBS-T.

153 9. Penta-His HRP conjugate (Qiagen).

154 10. Amersham Hybon-N+ membrane.

155 11. Western blotting transfer system (e.g. BIO-RAD).

156 12. Electrophoresis chamber for SDS-PAGE (e.g. BIO-RAD).

157 13. Pierce™ Western blot signal enhancer kit from ThermoFisher.

158 **2.4 Cloning and expression of a toxic outer membrane protein**

159 1. Plasmids: pET22b/ PAR1 [12] and pET17b/ *bamA*$_{ENm}$ [11].

160 2. Restriction endonucleases: NdeI and XhoI.

161 3. Calf intestinal alkaline phosphatase (CIP) (NEB).

162 4. 0.8 % agarose gel containing 1× TAE and 1× TAE buffer.

163 5. 1 kb DNA ladder and 6× purple gel loading dye.

164 6. GelRed stain.

165 7. QIAquick Gel Extraction Kit (QIAGEN).

166 8. T4 DNA ligase.

167 9. Competent *E. coli* strains: JCB387 [17] for plasmid construction and BL21 (Novagen)

168 for recombinant protein over-expression.

169 10. LB broth and LB agar.

170 11. Lennox broth: 2 % (w/v) peptone (Oxoid), 1 % (w/v) yeast extract (Oxoid) and 170 mM

171 NaCl [18].

172 12. 50 mg/mL kanamycin sulphate solution: filter sterilized (store at -20 °C).

173 13. 40 % (w/v) glucose.

174 14. QIAprep Miniprep Kit (QIAGEN).

175 15. Sequencing primers: pET26b Forward (5`-GGTGATGTCGGCGATATAGG-3`) and

176 T7 Reverse (5`-GCTAGTTATTGCTCAGCG-3`)

177 16. 0.1 M IPTG solution: filter sterilized (store at -20 °C).

178 17. 2× Laemmli loading buffer.

179 18. 10 mM Tris-HCl, pH 7.4.

180 19. 200 mM phenylmethylsulfonyl fluoride (PMSF) in 100 % ethanol (store at -20 °C).

181 20. Emulsiflex C3 cell disruptor or suitable French press or sonication system.

182 21. BIO-RAD protein assay solution and 1 mg/ml BSA (bovine serum albumen) solution.

183 22. Broad range pre-stained colour protein standards.

184 23. BIO-RAD Mini-PROTEAN® 3 SDS-PAGE equipment, 10 % Tris-glycine SDS-PAGE

185 gel and running buffer solutions.

24. InstantBlue Ultrafast protein staining (Sigma-Aldrich).

3 Methods

Bacterial work should be done using aseptic techniques, either next to a Bunsen burner or in a laminar flow hood.

3.1. Molecular cloning

3.1.1 Polymerase chain reaction (PCR)

1. Design one set of Restriction Enzyme primers for the gene to be cloned. The primers should encode an N- or C-terminal His tag (*see* **Note 13**).
2. Perform PCR on the desired open reading frames using the Q5 High-fidelity DNA polymerase. Prepare a 50 µL reaction mix according to the manufacturer's protocol and add the polymerase just prior to starting the reaction. Place the sample in a thermal cycler and start the reaction. A good starting point for a PCR program is:
    a. 98 ˚C for 2 min.
    b. 98 ˚C for 10 sec.
    c. 50 – 72 ˚C (*see* **Note 14**) for 30 sec.
    d. 72 ˚C for 30 sec/kb.
    e. Repeat steps from 2.b. to 2.d. 35 times.
    f. 72 ˚C for 10 min.
    g. 4 ˚C hold.
3. Once the PCR is finished, add 4 µL of DNA loading buffer to the sample and analyse the material by a gel electrophoresis (*see* **Note 15**).
4. Purify the DNA using a commercial gel extraction kit according to the manufacturer's protocol, ensuring to cut out the smallest quantity of agarose possible (*see* **Note 16**).
5. Determine the DNA concentration using a Nanodrop or by comparison to markers of known concentration on an agarose gel. Store the DNA at -20 ˚C.

3.1.2 Restriction digestion

213 1. In 1.5 mL microcentrifuge tubes, set up the reactions for 20 µL of double digestions,
214 one for the insert and another one for the vector.
215 2. Digest samples according to the manufacturer's protocol using the designated
216 restriction enzymes (*see* **Note 17**).
217 3. Once the double digestion is finished, add 4 µL of DNA loading buffer to the sample
218 and analyse the material by agarose gel electrophoresis (*see* **Note 18**).
219 4. Purify the insert and vector band separately using a commercial gel extraction kit
220 according to the manufacturer's protocol (*see* **Note 16**).
221 5. Determine the DNA concentration, as in section 3.1.1, and store the DNA at -20 ˚C.
222 *3.1.3 Ligation*
223 1. In a 1.5 mL microcentrifuge tube, set up a 20 µL ligation reaction and leave the ligation
224 at 16 ˚C overnight according to the manufacturer's protocol (*see* **Note 19**). As a control,
225 perform a ligation reaction only with the vector.
226 2. Stop the reaction by heating the mixture for 10 min at 65 ˚C.
227 *3.1.4 Heat-shock transformation*
228 1. Thaw on ice 50 µL of competent cells DH5α.
229 2. Add 100 ng of ligated plasmid and incubate the cells on ice for 30 min (*see* **Note 20).**
230 3. Perform the heat-shock with an incubation of the cells at 42 ˚C for 90 sec and incubate
231 them on ice for 2 min immediately afterwards.
232 4. Add 250 µL of LB media and incubate at 37 ˚C, 180 rpm for 1 h.
233 5. Plate 100 µL onto LB-amp agar plates and incubate overnight at 37 ˚C.
234 *3.1.5 Verification of clones by diagnostic digestion*
235 1. Pick 3 or more colonies with sterile toothpicks or autoclaved micropipette tips into 5
236 mL of LB media supplemented with the correct antibiotic and incubate at 37 ˚C, at
237 180 rpm, overnight.
238 2. The following day use 500 µL to make glycerol stocks, store at -80 ˚C for several
239 months (*see* **Note 9**).

240 3. Use the remaining volume to isolate the plasmid DNA using a commercial Miniprep kit
241 according to the manufacturer's protocol. Store the material at -20 ˚C.
242 4. Perform steps from 3.1.2 for a double digestion and DNA visualization (Fig. 1).
243 5. Select one of the colonies that contained an insert of the expected size and send the
244 purified plasmid for sequencing. Discard the remaining glycerol stocks once the correct
245 gene insertion has been confirmed by sequencing.

246 **3.2 Small scale expression of a recombinant protein at different temperatures**

247 1. Add 5 µL of a 100 mg/mL ampicillin stock solution into 5 mL of LB medium in a 50 mL
248 centrifuge tube. Put a 200 µL tip, which was scratched over the top of the expression
249 glycerol stock, into the tube to inoculate the mixture, now called pre-culture (*see*
250 **Note 21**).
251 2. Incubate overnight at 37 °C and 180 rpm shaking.
252 3. Prepare six 50 mL centrifuge tubes containing 20 mL LB medium and 20 µL of
253 100 mg/mL ampicillin stock solution (*see* **Note 8** and **Note 22**).
254 4. Add 200 µL of pre-culture to each main culture.
255 5. Grow three cultures at 37 °C, two at 30 °C and one at 25 °C, all with shaking at 180
256 rpm, until an OD_{600} of 0.4 to 0.6 is reached (*see* **Note 23**).
257 6. Induce protein expression by adding 10 µL of a 1 M IPTG stock solution, resulting in a
258 final IPTG concentration of 0.5 mM (*see* **Note 24**).
259 7. For the cultures grown at 37 °C, continue to grow one of the cultures at 37 °C whereas
260 the other two are further incubated at either 30 °C or 25 °C after induction. Similarly,
261 continue growing one of the cultures grown at 30 °C at 30 °C and the other one at
262 25 °C. The 25 °C is kept at 25 °C (*see* **Note 25**).
263 8. Harvest the cultures by centrifuging them for 5 minutes at 4,000 x g and remove the
264 supernatant. Store the cell pellets at -80 °C if they cannot be processed immediately.
265 9. Resuspend the cell pellet in 5 mL water and perform Western Blot and/or SDS-PAGE
266 analysis on them (see section 3.3).

10. Compare the density of the designated protein bands by eye or using densitometry to determine the optimal condition for protein expression (Fig. 2 and *see* **Note 26** and **27**).

3.3 SDS-PAGE and western blotting

1. Prepare two 10 % SDS-PAGE gels.
2. Load onto each gel, 10 µL of each sample per lane mixed with 1× SDS-loading dye (2 µL 6× SDS-Loading dye). Include prestained molecular weight markers in an additional well and run the gel at 70 V until it passes the stacking gel (~15 - 20 min) and then increase the voltage to 140 V. Once the dye front has reached the bottom of the gel, disassemble the setup.
3. Soak one gel in Coomassie protein stain and gently agitate overnight. The next day, remove the Coomassie protein stain and soak the gel in Milli-Q® water for ~1 h with gentle agitation. The other gel will be used for the Western blot.
4. Soak the gel in cold transfer buffer ~5 min at 4 ˚C.
5. Cut the Amersham Hybon-N+ membrane into a suitable size for blotting (*see* **Note 28**). Wet the membrane in deionized water for 10 sec and equilibrate it in cold transfer buffer for ~5 min.
6. Assemble the blotting cassette as follows: Add sequentially one fibre pad, two filter papers, the gel, the Amersham Hybon-N+ membrane, two filter papers, and one fibre pad. Insert the sandwich into the holder cassette (the membrane should be placed beside the positive electrode) (Fig. 3).
7. Insert the cassette into the central core assembly unit (together with the cooling unit).
8. Perform the transfer for 1 h at 100 V in cold transfer buffer.

The following steps require agitation on a rocking plate at room temperature.

9. Recover the Amersham Hybon-N+ and wash the membrane for ~5 min with TBS-T.
10. Block the membrane with 5 % BSA containing TBS-T for 1 h (*see* **Note 29**).
11. Wash the membrane three times for ~5 min with TBS-T.

293 12. Incubate with penta-His HRP conjugate antibody diluted at 1/1500 in 5 % BSA
294 containing TBS-T for 1 h (*see* **Note 29**).
295 13. Wash the membrane three times for ~5 min with TBS-T.
296 14. Perform the detection using a commercial Pierce™ Western blot signal enhancer kit
297 according to the manufacturer's protocol and develop the blot.

298 **3.4 Cloning and expression of a toxic outer membrane protein**

299 1. The DNA encoding the $BamA_{ENm}$ chimeric outer membrane protein (*bamA*$_{ENm}$) was
300 cloned into pET26b/ PAR1, using NdeI and XhoI restriction sites [12,11] (*see* **Note 30**).
301 To do this, incubate plasmids pET26b/ PAR1 and pET17b/ *bamA*$_{ENm}$ with NdeI and
302 XhoI for 3 h at 37 °C. To prevent vector religation, remove the 5` phosphates form
303 pET26b/ PAR1 by adding CIP and incubate the sample for a further 1 h at 37 °C.
304 2. Mix the DNA samples with gel loading dye and run them on a 0.8 % agarose gel
305 containing 1× TAE, at 80 V constant voltage until the dye front reaches the bottom of
306 the gel. Also run 1 kb DNA ladder as a marker to correctly size the DNA fragments.
307 3. Stain the gel with GelRed and visualize the DNA (*see* **Note 31**).
308 4. Excise the pET26b/ PAR1 vector and the *bamA*$_{ENm}$ fragment from the gel with a razor
309 blade or scalpel. Purify the DNA using a QIAquick Gel Extraction Kit and elute them
310 from the QIAquick column with 50 µL of sterile deionized water.
311 5. Check 5 µL of purified pET26b/ PAR1 and *bamA*$_{ENm}$ fragment on a 0.8 % agarose gel
312 for band size and purity. Again visualize the DNA by staining the gel with GelRed.
313 6. Mix the vector and insert DNA in ~1:3 ratio with T4 DNA ligase and buffer (final volume
314 of 20 µL) and incubate over night at 16 °C.
315 7. Mix the ligation reactions with 100 µL of $CaCl_2$ competent JCB387 cells (on ice) and
316 transform cells as described in steps 3.1.4. Select for cells, containing plasmid DNA,
317 by plating them on LB agar supplemented with 50 µg/mL kanamycin.
318 8. Grow candidate colonies overnight in 5 mL LB broth with 50 µg/mL kanamycin and
319 isolate plasmid DNA using a QIAprep Miniprep Kit.

320 9. Verify the cloning of the *bamA*$_{ENm}$ fragment (generating plasmid pET26b/ PAR1/
321 *bamA*$_{ENm}$) by Sanger DNA sequencing, using primers pET26b Forward and T7
322 Reverse.
323 10. For protein overexpression, transform BL21 cells with pET26b/ PAR1/ *bamA*$_{ENm}$. In
324 the transformation reaction, mix 1 µL of plasmid DNA with 100 µL $CaCl_2$ competent
325 BL21 cells (on ice) as in steps 3.1.4. Select for colonies on LB agar with 50 µg/mL
326 kanamycin.
327 11. Grow cultures of BL21 pET26b/ PAR1/ *bamA*$_{ENm}$ overnight in 5 mL Lennox broth
328 (kanamycin 50 µg/ml), with shaking at 37 °C.
329 12. Dilute cells 1/100 into 50 mL of fresh pre-warmed Lennox broth (kanamycin
330 50 µg/mL), supplemented with 0.2 % glucose, where indicated, and grow with
331 shaking at 37 °C until they reach an optical density (OD_{600}) of 0.3 to 0.5 (*see* **Note 32**).
332 13. Induced protein over-expression by the addition of IPTG to final concentrations of
333 1 mM, 10 µM or 5 µM, and grow for a further 4 h (*see* **Note 32**) (Fig. 4b).
334 14. Prepare total protein samples for SDS PAGE electrophoresis as follows: spin 300 µL
335 of cells at full speed in a bench top micro-centrifuge, remove the supernatant and
336 resuspend the pellet in 2× Laemmli loading buffer (*see* **Note 33**). In this instance,
337 heat samples at 95 °C for 3 min and store at -20 °C.
338 15. For the preparation of membranes, isolate all cells from the remaining 50 mL cultures
339 by centrifugation at 6,000 x g and 4 °C for 10 min.
340 16. Wash the cell pellets with 20 mL of ice cold 10 mM Tris-HCl (pH 7.4) and centrifuge
341 again at 6,000 x g and 4 °C for 10 min.
342 17. Resuspend cells in 20 mL of cold 10 mM Tris-HCl (pH 7.4) containing 2 mM PMSF
343 protease inhibitor (*see* **Note 34**) and disrupt cells by continuous passage through an
344 Emulsiflex C3 for 5 min.
345 18. Remove unbroken cells and particulate material by centrifugation for 15 min at 10,000
346 x g and 4 °C.

347 19. Isolate the total membrane fraction (inner and outer membranes) by centrifuging the
348 supernatant for 1 h at 48,000 x g at 4 °C. Retain the soluble fraction, which contains
349 cytoplasmic and periplasmic proteins.
350 20. Wash the membranes by resuspending them in 1 mL of ice cold 10 mM Tris-HCl (pH
351 7.4) and re-isolate them by centrifugation at 48,000 x g at 4 °C for 30 min.
352 21. Resuspend the washed membranes in 400 µL of 10 mM Tris-HCl (pH 7.4) (*see*
353 **Note 35**).
354 22. Determine the protein concentration of the soluble fraction and membrane preps
355 using BIO-RAD protein assay solution with 1 mg/mL BSA solution as a standard (as
356 specified by the manufacturers) (*see* **Note 36**).
357 23. Resolve protein samples using reducing SDS-PAGE on a 10 % Tris-glycine gel in a
358 BIO-RAD Mini-PROTEAN® 3 gel system. For total protein samples (step 3.4.14) load
359 10 µL of sample. For soluble fractions (step 3.4.19) and membrane preparations (step
360 3.4.21) mix 5 µg and 3 µg of protein, respectively, with 10 µL of 2x Laemmli loading
361 buffer. Heat samples at 95 °C for 3 min and centrifuge prior to loading.
362 24. Run the gel at 150 V (constant voltage) until the dye front reaches the bottom of the
363 gel and calibrate it by loading 6 µL of broad range protein standards
364 25. Stain the gel with InstantBlue Ultrafast protein stain (*see* **Note 37**) (Fig. 4c and d).
365

366 **4 Notes**

367 1. DNA template can be obtained from genomic DNA by using genomic DNA extraction
368 kits (e.g. PureLink Microbiome DNA purification kit from ThermoFisher); or from
369 plasmids that contain the gene of interest. Plasmids can be bought as glycerol stocks,
370 e.g. from Addgene (https://www.addgene.org/browse/).
371 2. To save time this buffer is normally made from concentrated stocks (e.g. Tris acetate-
372 EDTA 50× from ThermoFisher) or TAE buffer 50× stock recipe: 242 g Tris base,
373 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA solution (pH 8.0) and adjust the

374 volume to 1 L. EDTA must be constantly pH adjusted to 8 with NaOH to allow it to
375 dissolve.
376 3. The restriction enzymes recognition sequence must be present in the multiple cloning
377 site (MCS) of the chosen plasmid and absent in the gene of interest. Try choosing
378 different restriction enzymes with at least one with a “sticky” end, which will reduce re-
379 ligation or nonspecific ligation issues.
380 4. Luria-Bertani media is the most referenced rich media because it allows fast growth
381 and maintenance of recombinant *E. coli* strains. Other types of medium can be used
382 in the same way.
383 5. Ampicillin is used in these examples because of the vectors used (pET15b and
384 pBAT4). Care must be taken when using ampicillin not to incubate plates for >16 h to
385 avoid the outgrowth of satellite colonies, which do not carry the plasmid and therefore
386 are not resistant to ampicillin. Note that the appropriate antibiotic must be used for the
387 chosen vector.
388 6. Usually between 20 – 30 mL of LB agar are needed per plate. LB-agar without
389 antibiotic can be stored at RT and melted down when needed. Unused LB-amp agar
390 plates can be stored at 4 ˚C.
391 7. Produced in larger batches and frozen in 1 mL stocks at -20 °C. Store for up to 6
392 months.
393 8. If possible, reusable, small Erlenmeyer flasks should be used to reduce plastic
394 consumption. Ensure that they are clean and sterile before use.
395 9. Glycerol stocks are made by adding 500 µL of the overnight culture into 500 µL of 50 %
396 sterile glycerol for a final glycerol content of 25 % into sterile 1.5 mL centrifuge tubes
397 or cryovials.
398 10. Lower temperatures can be tested as well. However, some incubators do not have the
399 ability to cool below room temperature. Therefore, the room temperature often defines
400 the lowest temperature possible. Depending on the season, instruments and
401 conditions, the laboratory temperature can increase significantly and vary throughout

402 the day – as a consequence, laboratories are often cooler overnight than in the
403 daytime.

404 11. To save time, prepare the buffer from a concentrated stock of 20× TBS: 48.4 g Tris-
405 base, 160 g NaCl. Adjust the pH to 7.6 with HCl and make up to a final volume of 1 L.

406 12. To save time, prepare the buffer from a concentrated stock of 10× Transfer buffer:
407 2.5 M Tris base, 19.2 M glycine.

408 13. The Restriction Enzyme cloning strategy requires the presence of the same restriction
409 enzymes in the designed primers and the chosen vector. Therefore, the primers should
410 also include the appropriate restriction sites in their 5' end. The primers should not
411 contain strong secondary structure that could interfere with the PCR.
412 For proteins targeted to inner membrane, the tag should be inside the cell in the
413 cytoplasm. If both the N- and C-terminal ends are located inside the cell, a C-terminal
414 His tag is recommended to reduce interference with membrane protein targeting and
415 localisation. If the N-terminus is chosen, the tag should start with the Met AUG initiation
416 codon. If the C-terminus is chosen, the stop codon of the protein should be removed
417 and the DNA encoding the tag added at the end of the gene.
418 For ordering primers, select the lowest synthesis scale offered by the supplier with
419 desalting as the purification step. Higher grades of primers are not generally necessary
420 for PCR and cloning applications.
421 On delivery, spin down the material within the tube, dissolve the primers in Milli-Q®
422 water to a final concentration of 100 μM, and make a working stock solution of 10 μM.
423 Primers can also be dissolved in TE buffer but EDTA can sometimes interfere with
424 downstream applications.
425 Primer stocks can be stored for several months or years at -20 ˚C.

426 14. The annealing temperature of the PCR depends on the melting temperature of the
427 primer, which relates to the GC content. A good starting temperature is generally 50 to
428 60 ˚C. If the GC content of the primer is higher, then a higher annealing temperature
429 is required for the PCR, though, this should not exceed 68 ˚C.

430 15. Loading 10 µL of each PCR product is recommended first. If a single band of the
431 expected size is observed, there is no need to run a second gel and purification of the
432 PCR product can be performed.
433 16. Elute your DNA with Milli-Q water, TE buffer can be used but the EDTA can interfere
434 with downstream applications.
435 17. Some restriction enzymes are capable of cleaving similar sequences to their defined
436 recognition sequence, which is known as "star activity". High concentrations of
437 enzyme or DNA, non-optimal buffer conditions and prolonged incubation times can all
438 contribute to star activity. Read carefully the manufacturer's advice to avoid this.
439 18. As a control, reactions for insert and vector with only one restriction enzyme or without
440 restriction enzymes can be set up and loaded onto the gel, alongside the double
441 digestions reactions.
442 19. Usually, a 3:1 molar ratio (insert to vector) is recommended for a ligation. Ratios 1:1
443 and 5:1 can also be tested. It is also possible to ligate for a shorter length of time, e.g.
444 1 h.
445 20. For a positive control, cells can be transformed with the empty plasmid or a plasmid
446 that has been transformed successfully in the past. As a negative control, the
447 transformation can be done with competent cells without plasmid, which can be plated
448 onto LB-agar plates with and without antibiotics.
449 21. Instead of using a glycerol stock, single colonies from an agar plate can be used.
450 22. The tubes will be used for the main cultures which are grown at different temperatures.
451 Labelling the tubes is essential at this point so that they are not mixed up later on. Be
452 sure to write the temperature used for the growth of the culture and the induction of
453 the protein expression onto them.
454 23. After the main cultures have reached the desired OD_{600}, they will be induced at different
455 temperatures. Cultures grown at 30 °C and 25 °C will not be induced at higher
456 temperatures. Therefore, only two or one culture respectively needs to be incubated
457 at those temperatures. The cultures should reach approximately the same OD_{600} to

458 ensure the comparability of protein expression later on. For BL21(DE3) cells reaching
459 the desired OD_{600} of 0.4 to 0.6 can take up to 2 to 2 ½ hours at 37 °C. However, this
460 can change depending on the overall expression system used and should be checked
461 every half an hour to avoid overgrowth of cultures.
462 24. Final IPTG concentrations depend on the protein and plasmid used. Toxic proteins or
463 high concentrations of IPTG lead to low cell growth and/or an overload of the
464 expression and translocation machinery and ultimately in protein aggregation. Lower
465 IPTG concentrations or shorter expression times should be tested. For example,
466 concentrations of 0 mM, 0.25 mM, 0.5 mM, 0.75 mM and 1 M of IPTG can be used for
467 initial test conditions.
468 25. Sometimes longer incubation times, overnight for example, are needed to reach
469 satisfactory amounts of expressed protein. However, this can result in the formation of
470 inclusion bodies of incorrectly folded membrane protein. The functionality of the protein
471 always needs to be tested after purification.
472 26. Once the optimal conditions have been found, the expression can be scaled up to
473 larger culture volumes such as 1 L or 2 L. Large scale expression work in the same
474 way as small scale expressions. For example, add 1 mL of a 100 mg/mL ampicillin
475 stock solution to a 1 L culture to reach a final concentration of 100 mg/L. If protein
476 expression is induced with 0.5 mM IPTG, then 0.5 mL IPTG of a 1 M stock needs to
477 be added. Cell harvesting can be done with an Avanti JXN-26 in combination with a
478 JLA8.1000 rotor (Beckmann coulter).
479 27. As a general rule, membrane protein samples should not be heated before performing
480 SDS-PAGE because there is the possibility that no protein band will be seen on the
481 gel. Furthermore, membrane proteins often migrate at a different molecular weight to
482 that expected [19]. Western blotting with antibodies, which specifically bind to the
483 protein of interest, and/or mass spectrometry can be used to verify the expression of
484 the protein.
485 28. Cutting a corner off the membrane (e.g. the upper right) will help in orienting the blot.

486 29. This step can also be carried out overnight at 4 ˚C on a rocking platform.

487 30. The *Neisseria meningitidis* chimeric OMP protein ($BamA_{ENm}$) is a potential vaccine
488 candidate against *N. meningitidis*. The DNA encoding the $BamA_{ENm}$ chimeric outer
489 membrane protein (*bamA*$_{ENm}$) was codon optimized for expression in *E. coli* and
490 chemically synthesised by Genscript (www.genscript.com) [11]. The construct consists
491 of the signal sequence of *E. coli* BamA, which targets the protein to the periplasm via
492 the Sec translocon, the five periplasmic POTRA (polypeptide transport associated)
493 domains of *E. coli* BamA and the β-barrel domain of *N. meningitidis* BamA, which
494 anchors the protein into the outer membrane [11].

495 31. In addition to using commercial DNA stains such as GelRed, gels can also be stained
496 with a 100 mL of 0.1 mg/mL ethidium bromide solution and DNA visualized using a UV
497 light box. As ethidium bromide is a carcinogen, care and appropriate PPE (*e.g.* lab
498 coat and gloves) should be used when handling both solutions and stained gels.
499 Similarly, when using a UV light box, PPE and a full-face UV-absorbing shield should
500 be worn.

501 32. High-level expression of the recombinant $BamA_{ENm}$ OMP is toxic to *E. coli* and so
502 suitable precautionary steps must be taken to control its expression level. In this
503 instance, the pET26b/ PAR1 expression vector carries a tightly-regulated *lac* operon
504 promoter, PAR1 [13]. As expression from the *lac* promoter can be modulated by
505 growing cells in the presence of glucose (catabolite repression), glucose repression
506 and/ or lowering the inducer concentration (IPTG) is used to control the expression of
507 $BamA_{ENm}$, such that product accumulates without toxicity (Fig. 4)

508 33. The volume of 2× Laemmli loading buffer used is determined by multiplying the OD_{600}
509 of the sample by a 1/10th of the volume taken, *e.g.* in this case OD_{600} of the sample x
510 30.

511 34. PMSF is a protease inhibitor and toxic. Care should be taken when weighing out solid
512 and appropriate PPE (*e.g.* lab coat and gloves) worn when handling solid and
513 solutions.

514 35. Membrane preparations can be store at -20 °C for short-term storage or -80 °C for
515 longer.
516 36. Ensure membrane preps are thoroughly mixed before pipetting as they tend to
517 sediment over time. Adding the membrane sample directly into the BIO-RAD protein
518 assay solution prevents the membranes from clumping during the assay. Deionized
519 water can then be added to obtain the volume required for the assay.
520 37. In addition to using commercial gel stains, such as Ultrafast protein stain, standard
521 Coomassie blue can also be used (0.025 % (w/v) Coomassie brilliant blue R250, 50 %
522 (v/v) methanol, 10 % (v/v) acetic acid). Stain the gel for ~2 h, remove excess stain with
523 fast (40 % (v/v) methanol, 10 % (v/v) acetic acid) and then slow destain (10 % (v/v)
524 methanol, 10 % (v/v) acetic acid) solutions. Store the stained gel in 10 % (v/v) acetic
525 acid.
526

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533

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604

605 **Figure Legends**

606 **Fig. 1.** General workflow for the synthesis of a recombinant membrane protein. The workflow
607 starts with the modification of the gene of interest so that it can be inserted into the expression
608 vector, continues with the verification of successful cloning and ends with the small scale
609 production of the membrane protein and its analysis by western blotting.

610

611 **Fig. 2.** Small scale synthesis of the recombinant chimeric potassium ion channel KcsA1.3 [20]
612 in *E. coli* using different temperatures (b) and IPTG concentrations (c). BL21(DE3) cells in
613 combination with the T7-controlled pBAT4 vector have been chosen as the expression system.
614 Changing the temperature from 30 °C pre induction to 25 °C post induction (overnight) results
615 in the highest protein amounts as seen by western blotting (b). Using 0.5 mM IPTG seems to
616 be optimal for the expression of the chimera because the densest protein band is found at this
617 concentration (c). Due to the relatively weak T7*lac* repressor system, the protein is expressed
618 even with no added IPTG. A truncated version of the KcsA tetramer (PDB code: 1BL8 [21]) is
619 shown in (a) where each monomer consists of two alpha helices forming the pore region on
620 the extracellular membrane side. The figure was generated using ChimeraX [22].

621

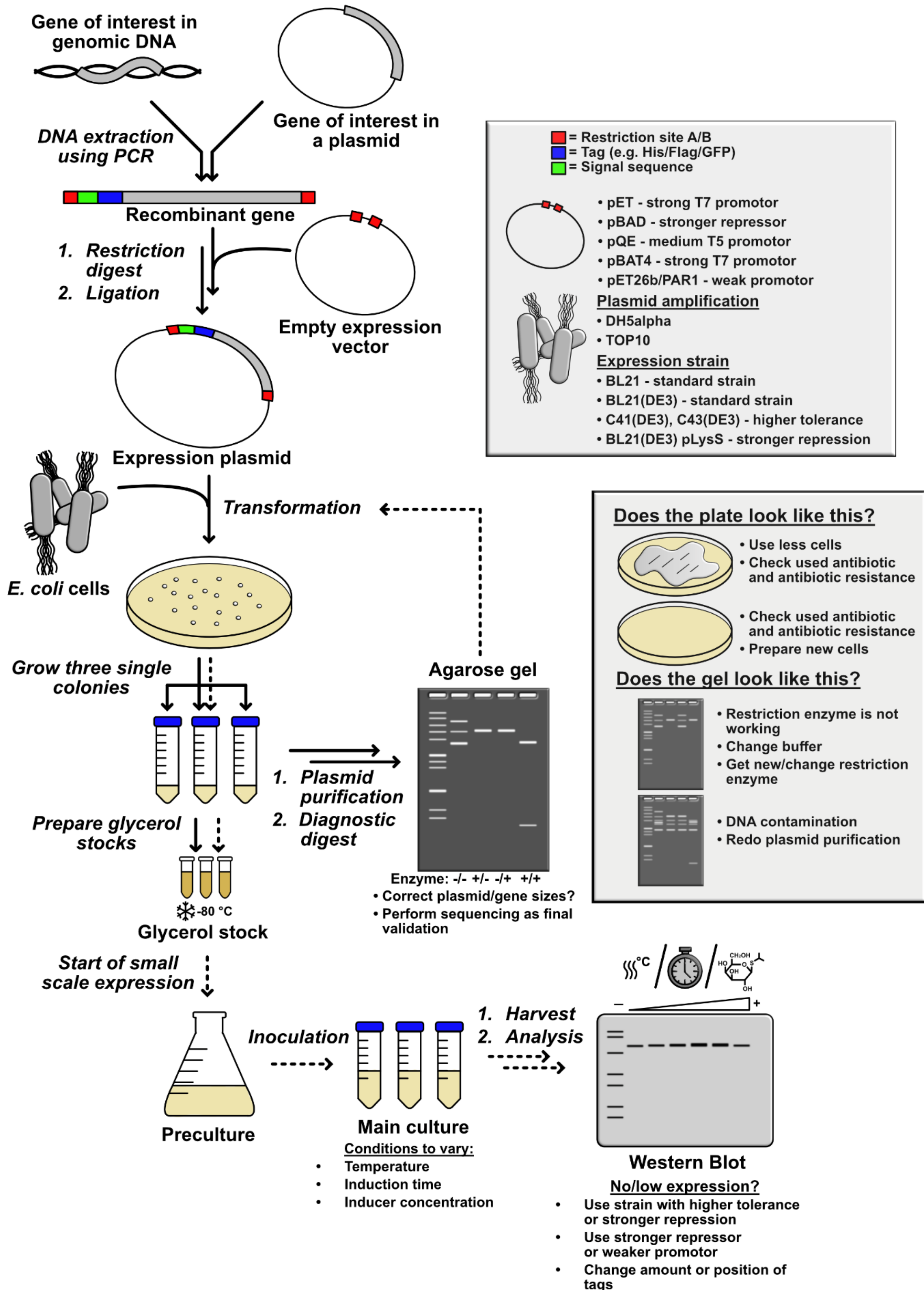
622 **Fig. 3.** Schematic picture of Western Blot Assemble cassette. Starting over the black frame
623 (anode, negative electrode) => fibre pad => two filter papers => gel => membrane => two
624 filter papers => fibre pad => red frame (cathode, positive electrode).

625

626 **Fig. 4.** Production of the *N. meningitidis* $BamA_{ENm}$ chimera protein in *E. coli* BL21 cells. (a)
627 The panel shows the structure of the *E. coli* OmpW (PDB: 2F1V) as an example of a bacterial
628 OMP, highlighting the β-strands, periplasmic turns and external loops [23]. The figure was

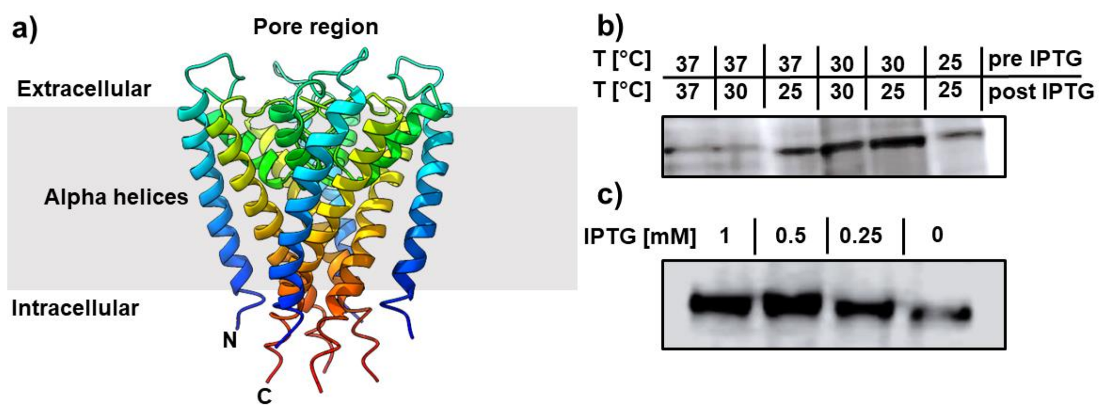
629 generated using PyMOL [24]. (b) The panel shows growth of the *E. coli* BL21 cells carrying
630 either pET26b/ PAR1 empty vector (EV) or pET26b/ PAR1/ $BamA_{ENm}$ in Lennox broth,
631 supplemented with 0.2 % glucose (Glu 0.2 %), where indicated. Expression of $BamA_{ENm}$ was
632 induced after 3 h growth by the addition of IPTG at either 1 mM, 10 μM or 5 μM, where
633 indicated. (c) Detection of $BamA_{ENm}$ chimera expression. The panel shows a Coomassie blue
634 stained SDS PAGE gel of normalised total cell protein from the BL21 cells in panel (b), carrying
635 either pET26b/ PAR1 empty vector or pET26b/ PAR1/ $BamA_{ENm}$ after 4 h induction. Culture
636 numbering in panel (b) is the same for the loading of samples in panel (c) (denoted as *). The
637 gel was loaded as follows: lane 1, BL21 pET26b/ PAR1 (EV); lane 2, BL21/ pET26b PAR1/
638 $BamA_{ENm}$ uninduced; lane 3, pET26b/ PAR1/ $BamA_{ENm}$ induced with 1 mM IPTG; lane 4, BL21
639 pET26b/ PAR1/ $BamA_{ENm}$ induced with 10 μM IPTG; lane 5, BL21/ pET22b PAR1/ $BamA_{ENm}$
640 induced with 5 μM IPTG; lane 6, BL21 pET22b/ PAR1/ $BamA_{ENm}$ induced with 1 mM IPTG and
641 supplemented with 0.2 % glucose. (d) Detection of $BamA_{ENm}$ in membrane fractions. The panel
642 shows a Coomassie blue stained gel of soluble (S) and membrane (M) fractions from the BL21
643 cells expressing $BamA_{ENm}$ after 4 h induction. 5 μg of soluble and 3 μg of membrane protein,
644 respectively, was loaded.

645 **Figure 1**



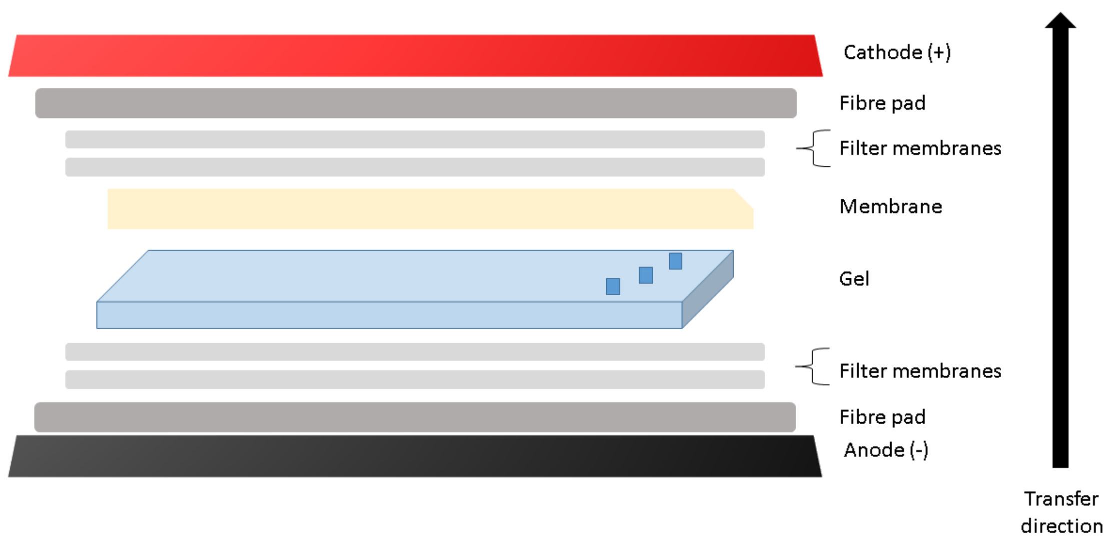
646

647 **Figure 2**



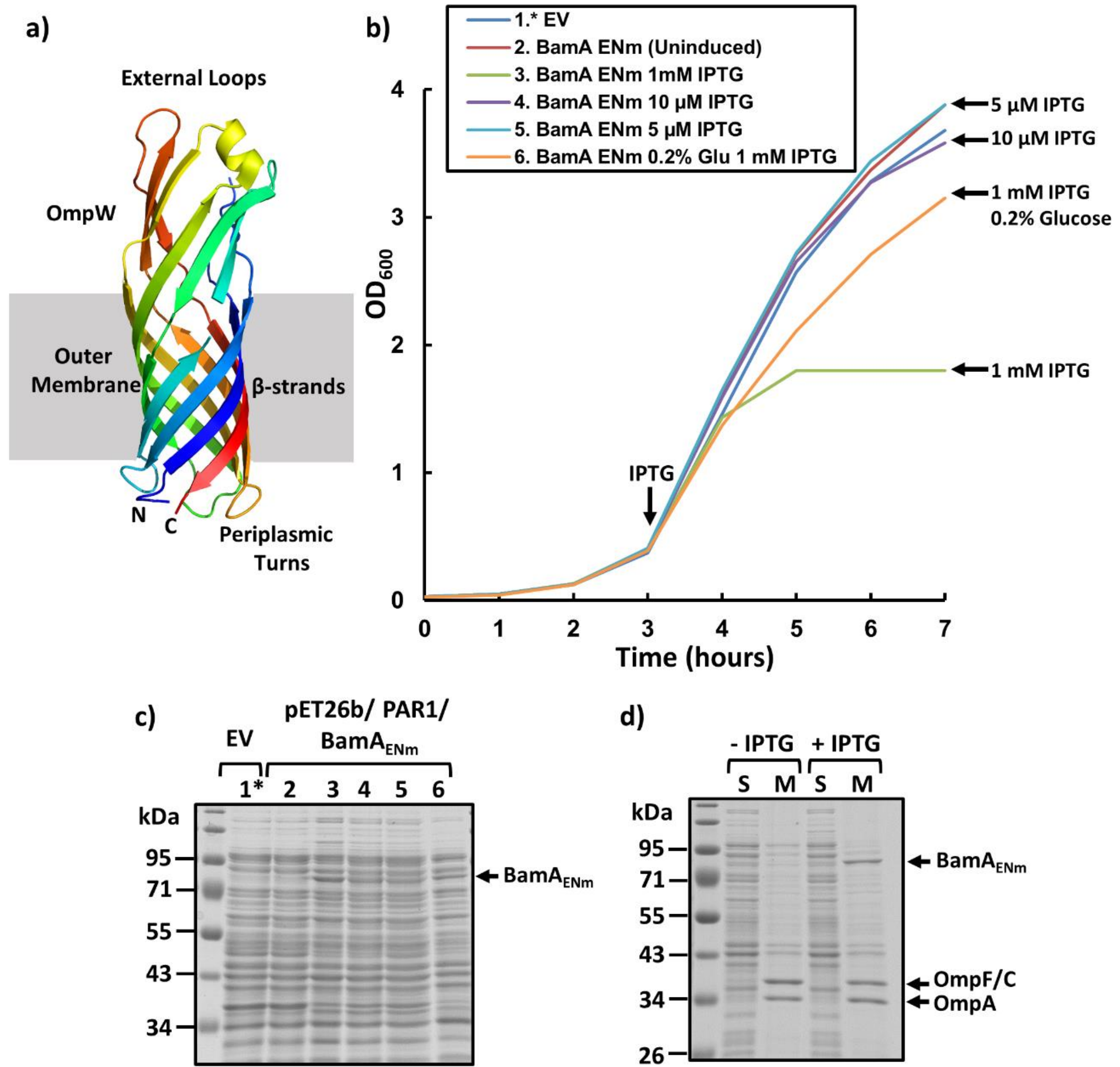
648

649 **Figure 3**



650

651 **Figure 4**



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