

Chapter 2

Production of Membrane Proteins in *Escherichia coli* and *Lactococcus lactis*

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Abstract

As the equivalent to gatekeepers of the cell, membrane transport proteins perform a variety of critical functions. Progress on the functional and structural characterization of membrane proteins is slowed due to problems associated with their (heterologous) overexpression. Often, overexpression fails or leads to aggregated material from which the production of functionally refolded protein is challenging. It is still difficult to predict whether a given membrane protein can be overproduced in a functional competent state. As a result, the most straightforward strategy to set up an overexpression system is to screen a multitude of conditions, including the comparison of homologues, type and location of (affinity) tags, and distinct expression hosts. Here, we detail methodology to rapidly establish and optimize (membrane) protein expression in *Escherichia coli* and *Lactococcus lactis*.

Key words: Arabinose promoter, *Escherichia coli*, folding indicator, GFP, *Lactococcus lactis*, membrane proteins, nisin-controlled expression, overexpression, P_{BAD}

1. Introduction

Although for the elementary steps in the biogenesis of membrane proteins, which are membrane targeting, membrane insertion, and assembly and folding, the crucial components are known (reviewed in (1)), our current understanding of the overall process and the factors involved are far from complete. Predicting whether a given membrane protein can be overproduced in a functional competent state is currently not possible, and bottlenecks have not been identified that could guide a rational troubleshooting strategy should overexpression fail. To date, membrane protein biogenesis has been mostly studied from the perspective of the known components involved in targeting, membrane insertion, and folding. In general, this is done using a limited number of

small and simple model proteins, while systematic genomewide studies have indicated that overexpression of complex membrane proteins with multiple transmembrane segments is most challenging (2–4). Only recently efforts have been made to understand the complete process in the context of a cell by determining the physiological responses underlying (un)successful overproduction of (complex) membrane proteins (5,6).

As a consequence, if one wishes to obtain structural and functional information on a certain membrane protein that is not abundant in any natural source, a convenient strategy is to screen a wide range of conditions. Established initial variables involve the screening of homologues (7,8), varying the type and location of affinity tags and domains (9,10), and screening multiple-expression hosts (11,12). In practice, for prokaryotes screening is restricted to several *Escherichia coli* strains, which offers far less variation than the screening of different species.

The gram-positive bacterium *Lactococcus lactis* is an attractive alternative to *E. coli* and yeast-based membrane protein expression systems (13,14). Overexpression of membrane proteins in *L. lactis* leads mostly to well-folded, membrane-inserted material; inclusion bodies of aggregated material are rare. The lactococcal membrane is easily solubilized with a wide range of detergents. Growth of *L. lactis* proceeds rapidly at 30°C and does not require aeration. The nisin-inducible controlled expression (NICE) system (15) allows reproducible and modulatable expression from low to high levels. Previous difficulties associated with direct cloning in *L. lactis* (10) have been overcome by the high-throughput compatible vector backbone exchange (VBEx) procedure (16). As *L. lactis* is auxotrophic for multiple amino acids, adjustment of the lactococcal chemically defined medium (17) allows incorporation of labeled amino acid analogues into proteins (44, 45). Concerning membrane protein expression, there are five important differences between *E. coli* and *L. lactis*. First, the composition of the machinery involved in membrane protein insertion: *L. lactis* contains two paralogs of the Oxa/YidC/Alb family of membrane-inserted chaperones (18), whereas *E. coli* contains one (YidC). *Lactococcus lactis* does not contain homologues of the Sec translocon accessory proteins SecDF (19) and SecM (20). Finally, substantial differences in structure are observed between lactococcal and *E. coli* homologues of YidC and SecE (20,21). Second, the lipid composition of the membrane is very different: The major lipid component of the *E. coli* cytoplasmic membrane is phosphatidylethanolamine (PE), which is absent in *L. lactis*. Instead, anionic glycolipids and phosphoglycolipids are the major constituents in *L. lactis* (22). The third difference involves codon usage (23); *L. lactis* DNA is relatively AT rich (64% AT, compared to 50% AT in *E. coli*). The fourth difference is the absence of disulfide isomerase homologues in *L. lactis* (24), albeit that disulfides

are readily formed either spontaneously or via an alternative route in secreted proteins (25–27). The final difference is that some proteins toxic to one host do not affect the physiology of the other host (C. Mulligan and E.R. Geertsma, March 2007). In addition, although fewer attempts have been made than in *E. coli*, overexpression of eukaryotic membrane proteins in *L. lactis* is relatively successful (28).

1.1. High-Throughput Cloning in *E. coli* and *L. lactis*

To establish an overexpression system rapidly, several homologues, types and location of affinity tags, and expression hosts need to be screened. Due to the large number of plasmids required for this strategy, it will be advantageous to avoid low-throughput techniques and implement efficient cloning procedures such as ligation-independent cloning (LIC) (29). LIC is less restricted in the design of the sequences flanking the genes than other high-throughput cloning techniques, like Gateway (30) or the univector plasmid-fusion system (31). Therefore, the cloning-related sequences attached to the recombinant protein can be minimized, which might be advantageous for structural and functional analysis.

The LIC procedure described here, outlined in Fig. 2.1a, involves linearization of the vector by a unique *Sma*I site in the middle of the LIC cassette. Using the 3' to 5' exonuclease activity of T4 DNA polymerase, single-stranded overhangs are generated. By performing the exonuclease treatment in the presence of dCTP (deoxycytidine triphosphate), a nucleoside not present in the 3' sequences adjacent to the *Sma*I site, the removal of bases halts once the first dCMP (deoxycytidine monophosphate) is encountered, thereby creating overhangs of a defined length. Complementary single-stranded overhangs are created in a similar way in the polymerase chain reaction (PCR) product to be cloned (i.e., exonuclease treatment in the presence of dGTP (deoxyguanosine triphosphate)). On mixing the T4 DNA polymerase-treated vector and PCR product, a stable heteroduplex is formed, and the mixture can be transformed with high efficiency to *E. coli*.

The efficiencies for direct cloning in *L. lactis* are low, and LIC is virtually not possible. This bottleneck in cloning has been overcome by the VBEx procedure (16). Using VBEx, the initial LIC step can be performed with high efficiency in *E. coli* while the final expression vector that is generated can be kept devoid of alien *E. coli* elements. The VBEx procedure, outlined in Fig. 2.1b, relies on the bisection of a bona fide plasmid of the expression host into two parts, thereby separating the selection marker from the origin of replication. For *L. lactis*, pNZxLIC, a LIC-compatible derivative of the well-established expression vector pNZ8048 (15) was accordingly bisected. The segment containing the origin of replication was fused to a sequence coding for an erythromycin selection marker, yielding plasmid pERL, which can be maintained in *L. lactis*. The other segment of pNZxLIC,

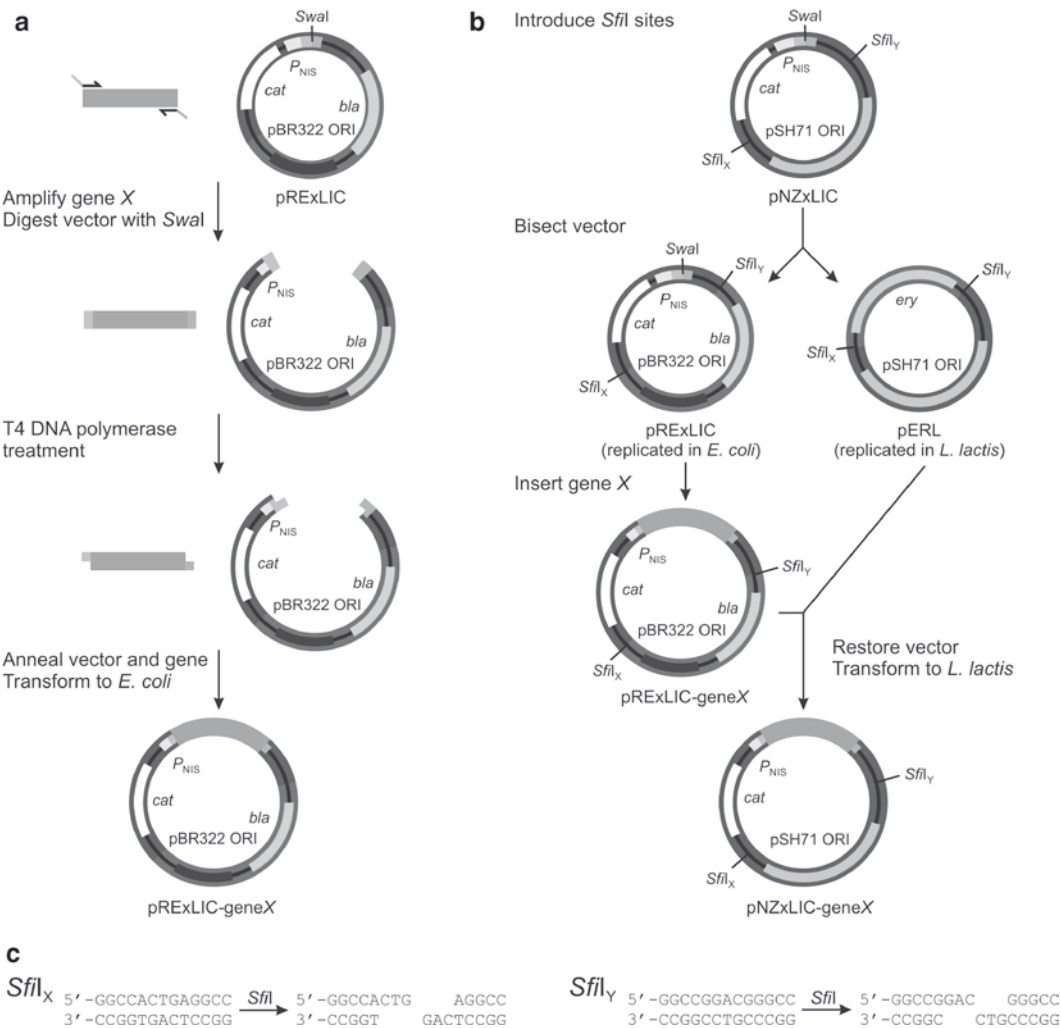


Fig. 2.1. High-throughput cloning in recalcitrant bacteria using LIC and VBEx. **(a)** Outline of the LIC procedure. Gene *X* is amplified using primers holding LIC-specific overhangs. The plasmid is linearized by *Swa*I restriction in the LIC cassette. Single-stranded overhangs on the PCR product and vector are generated using T4 DNA polymerase. The complementary overhangs of PCR product and vector anneal on mixing. The resulting heteroduplex is transformed efficiently to *Escherichia coli*. **(b)** Outline of the VBEx strategy. After introduction of two distinct *Sfi*I sites, the *Lactococcus lactis* expression vector pNZxLIC was bisected. Plasmid pERL received the pSH71 replicon, which was fused to the erythromycin marker. Plasmid pRExLIC received the chloramphenicol marker and LIC sequence, which were fused to the *E. coli* pBR322 replicon and β -lactamase marker. As these vectors are available, in practice the cloning involves the submission of vector pRExLIC to the LIC procedure (depicted in **a**). Subsequently, the VBEx procedure is applied: The pNZxLIC vector is restored by mixing pERL and pRExLIC-gene *X*, digestion with *Sfi*I, ligation, and selection on the ability to replicate in *L. lactis* (the presence of the pSH71 replicon) in the presence of chloramphenicol. **(c)** The *Sfi*I sites flanking both segments of the vectors yield different, nonpalindromic overhangs, thereby greatly reducing the number of possible ligation products. (Adapted from (16)).

containing the chloramphenicol resistance gene and LIC sequence, was fused to the backbone of an *E. coli* vector (containing an *E. coli* origin of replication and β -lactamase resistance gene). The resulting plasmid pRExLIC, allows the LIC manipulation to take place in *E. coli*.

Rapid and facile regeneration of the *L. lactis* expression vector is enabled as the relevant segments of the pRExLIC and pERL vectors are flanked with distinct *Sfi*I sites. DNA cleavage by *Sfi*I generates a 3' overhang that can be composed of any combination of three nucleotides. The two *Sfi*I sites used yield different, incompatible overhangs (Fig. 2.1c). Each segment of the pNZxLIC vector has unique selectable properties: (1) the ability to replicate a plasmid in *L. lactis* and (2) the resistance to chloramphenicol. Thus, on mixing a pRExLIC derivative and pERL, digestion by *Sfi*I, followed by ligation and transformation to *L. lactis* in combination with selection of clones using chloramphenicol, the pNZxLIC derivative can be exclusively recovered with high efficiencies. DNA sequences from virtually all sequenced genomes are compatible with VBEx as *Sfi*I sites are rare (16). Furthermore, genes containing internal *Sfi*I sites are not necessarily excluded as 64 different 3' overhangs can be generated after *Sfi*I digestion. Internal *Sfi*I sites with 3' overhangs not matching those of the vector will not form a bottleneck in the procedure.

1.2. Optimization of Membrane Protein Overexpression

Following initial screening for expression, systematic optimization of the production conditions to increase the amount of well-folded protein inserted into the membrane is often required. As indicated, membrane protein biogenesis requires several additional steps and components beyond translation. Exceeding the capacity of the cell to process the nascent membrane protein correctly may reduce the final yield of well-folded material. This is especially true for *E. coli* as membrane protein production in this host is regularly accompanied by inclusion body formation. Adjustment of the expression rate is most easily done by varying the inducer concentration for well-tunable promoters, such as the *E. coli* P_{BAD} (32) or the lactococcal P_{nisA} (15). In addition, variation of the growth temperature during induction is an important parameter to affect the expression rate (33,34).

While traditionally optimization of functional expression involved time-consuming steps such as the isolation of membrane vesicles and activity assays, this process has been greatly accelerated by the use of green fluorescent protein (GFP), fused to the C-terminus of the target protein, as folding reporter. Proper maturation of GFP in *E. coli*, leading to a fluorescent species that maintains its folded state during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), depends highly on the correct folding of its fusion partner (35–38). Consequently, only correctly folded target yields a fluorescent fusion protein that has a higher electrophoretic mobility than the misfolded fusion protein. Plain SDS-PAGE and subsequent immunoblotting allow simultaneous quantification of both the well-folded and aggregated protein produced (Fig. 2.2) (38). This facilitates the assignment of bottlenecks limiting the functional overexpression. For instance, the absence of the upper, nonfluorescent band suggests that transcription or

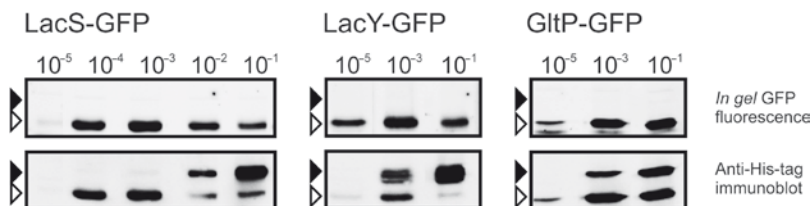


Fig. 2.2. SDS-PAGE analysis of membrane protein GFP fusions expressed to different levels. Expression of the GFP fusion proteins was controlled by the P_{BAD} , and *Escherichia coli* MC1061 cultures were induced with the percentage L-arabinose (w/v) indicated above the lanes. Upper panels represent the images obtained by *in gel* GFP fluorescence. Lower panels represent immunoblots of the same gels decorated with anti-His tag antibody. Black and white arrows indicate the positions of the aggregated nonfluorescent and well-folded fluorescent species of the GFP fusion proteins, respectively. (Adapted from (38)).

translation limits the functional expression, which can be resolved by increasing the inducer concentration. In contrast, the strong presence of the upper, nonfluorescent band suggests that the trajectory beyond transcription/translation is limiting. In addition to this application, the use of GFP fusions has additional advantages at the stages beyond membrane protein overexpression, such as the rapid screening of detergents for solubilization and stabilization (37,39).

2. Materials

2.1. Ligation-Independent Cloning Procedure

2.1.1. Preparation of the DNA Insert

1. Autoclaved TlowE: 10 mM Tris-HCl, pH 7.5, 0.2 mM $\text{Na}_2\text{-EDTA}$ (ethylenediaminetetraacetic acid).
2. Adhesive plate seals (e.g., plate seal AB-0580 from Thermo Scientific).
3. Phusion DNA polymerase and dedicated buffers (Finnzymes).
4. DNA gel extraction kit (e.g., the High Pure PCR Product Purification kit from Roche).

2.1.2. Preparation of the LIC Vector

1. LB-amp: Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L of demineralized water. Sterilize by autoclaving. Once the medium is cooled to room temperature, add 1 mL of a filter-sterilized solution of 100 mg/mL ampicillin dissolved in water.
2. Plasmid isolation kit (e.g., the Qiaprep Spin Miniprep kit from Qiagen).
3. 10% (w/v) SDS solution.
4. Proteinase K solution: Dissolve 20 mg/mL proteinase K in 10 mM Tris-HCl, pH 8.0, 1 mM $\text{Na}_2\text{-EDTA}$. Store aliquots at -20°C .
5. Phenol:chloroform:isoamyl alcohol (25:24:1) mixture. Phenol is extremely toxic; wear protective clothing and work in a fume hood.

6. Chloroform, analytical grade. Chloroform is irritating and a carcinogen; wear protective clothing and work in a fume hood.
7. 96% EtOH, analytical grade, precooled at -20°C .
8. 3M Na-acetate, pH 5.3; adjust the pH with concentrated acetic acid.
9. 70% EtOH, analytical grade, precooled at -20°C .
10. SpeedVac (Savant).
11. *Sma*I and dedicated buffer.
12. Chemically competent *E. coli* MC1061. Protocols for the preparation of chemically competent *E. coli* can be found in (40).
13. LB-amp agar plates: Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L of demineralized water and add 15 g agar. Sterilize by autoclaving. Once the medium is cooled to $50\text{--}60^{\circ}\text{C}$, add 1 mL of a filter-sterilized solution of 100 mg/mL ampicillin dissolved in water; mix well and pour the medium in sterile Petri dishes.

2.1.3. T4 DNA Polymerase Treatment of Vector and Insert

1. T4 DNA polymerase and dedicated buffer.
2. 25 mM dCTP: Dilute a 100 mM dCTP stock in sterile Milli-Q. Store aliquots at -20°C .
3. 25 mM dGTP: Dilute a 100 mM dGTP stock in sterile Milli-Q. Store aliquots at -20°C .
4. Heat block.

2.1.4. Verifying Clones by Colony PCR

1. Autoclaved Milli-Q.
2. Sterile toothpicks.
3. 96-well PCR plates.
4. Aluminum sealing films (e.g., PCR-AS-200 from Axygen).
5. Taq DNA polymerase and dedicated buffer.
6. dNTPs: Make a stock solution containing 10 mM of each nucleotide. Store aliquots at -20°C .
7. Generic primers (see Note 1).

2.2. Vector Backbone Exchange Procedure

2.2.1. Preparation of pERL

1. GM17-ery: Dissolve 37.25 g of M17 broth (Oxoid) (41) in 1 L of demineralized water. Sterilize by autoclaving. Once the medium is cooled to room temperature, add 25 mL sterile 20% (w/v) glucose and 1 mL of a filter-sterilized solution of 5 mg/mL erythromycin dissolved in water.
2. Resuspension buffer: 10 mM Tris-HCl, pH 8.1, 10 mM $\text{Na}_2\text{-EDTA}$, 50 mM NaCl, and 20% (w/v) sucrose. Autoclave and store at 4°C . Supplement with 20 mg/mL lysozyme prior to use.

2.2.2. Vector Backbone Exchange

1. *Sfi*I and dedicated buffer.
2. 50 mM ATP stock: 50 mM Na₂-ATP, 50 mM MgSO₄, and 50 mM phosphate buffer, pH 7.0. Adjust the pH to 6.5–7.0 with NaOH. Store aliquots at –20°C.
3. T4 DNA ligase and dedicated buffer.

2.2.3. Electrotransformation of *L. lactis*

1. Sterile electroporation cuvetts (2 mm gap between the electrodes). The cuvetts can be reused several times. Clean the cuvetts after use with 70% EtOH and wash extensively with Milli-Q. Allow the cuvetts to dry and sterilize the cuvetts by 20 min exposure to an ultraviolet (UV) source. Immediately close the cuvetts with the supplied cap.
2. Electroporation device.
3. Rescue medium: Mix 1 volume of sterile M17 medium with 1 volume of sterile 1M sucrose, 1% (w/v) glucose, 40 mM MgCl₂, 4 mM CaCl₂.
4. SGM17-cam agar plates: Mix 37.25 g of M17 broth (Oxoid) and 15 g agar in 750 mL of demineralized water. Sterilize by autoclaving. Once the medium is cooled to about 60°C, add 250 mL sterile 2M sucrose, 25 mL 20% (w/v) glucose, and 1 mL of a filter-sterilized solution of 5 mg/mL chloramphenicol dissolved in EtOH. Mix well and pour the medium in sterile Petri dishes.
5. Parafilm.

2.3. Initial Characterization and Optimization of Protein Production

2.3.1. Cultivation and Induction of MP Overexpression in *E. coli*

1. Sterile 87% (w/v) glycerol.
2. Cryovials.
3. TB-amp: Dissolve 24 g tryptone, 48 g yeast extract, and 17.2 mL 87% (w/v) glycerol in 0.9 L of demineralized water and autoclave. In addition, autoclave 100 mL of 170 mM KH₂PO₄, 720 mM K₂HPO₄. Mix the solutions prior to use and add 1 mL of 100 mg/mL ampicillin.
4. L-Arabinose: Filter-sterilize a 20% (w/v) L-arabinose stock.
5. Screw-cap tubes compatible with the FastPrep-24 (e.g., screw-cap microtube 72.693 from Sarstedt).

2.3.2. Cultivation and Induction of MP Overexpression in *L. lactis*

1. GM17-cam: M17 medium supplemented with 0.5% (w/v) glucose and 5 µg/mL chloramphenicol after sterilization.
2. Nisin-containing supernatant of a *L. lactis* NZ9700 culture: Use a 1% (v/v) inoculum of a preculture of *L. lactis* NZ9700 on GM17 to inoculate 200 mL GM17 and incubate overnight at 30°C. Next day, pellet the cells, filtrate the supernatant using a 0.2-µm filter, and store the aliquots at –20°C.

2.3.3. Cell Disruption and Sample Preparation

1. Glass beads with a diameter of about 0.1 μm (Sigma-Aldrich).
2. Disruption buffer: 50 mM phosphate buffer, pH 7.5, 10% (w/v) glycerol, and 1 mM MgSO_4 . Precool the solution on ice. Add 1 mM PMSF (phenylmethylsulfonyl fluoride) (from a 200 mM stock in EtOH (ethanol)) and 0.1 mg/mL DNase (deoxyribonuclease) prior to use.
3. FastPrep-24 device (MP Biomedicals).
4. 5X protein sample buffer: 120 mM Tris-HCl, pH 6.8, 50% (w/v) glycerol, 100 mM dithiothreitol (DTT), 2% (w/v) SDS, and 0.1% (w/v) bromophenol blue (see Note 2). Store aliquots at -20°C .

2.3.4. SDS-PAGE, In Gel GFP Fluorescence, and Western Blotting

1. SDS-PA gel: For a 10% running gel, mix 3.38 mL 30% acrylamide/bis solution (cross-linker ratio 37.5:1), 2.54 mL 1.5M Tris-HCl, pH 8.8, 4.15 mL Milli-Q, 100 μL 10% (w/v) SDS, 40 μL 10% (w/v) APS (ammonium persulfate), and 10 μL TEMED. For the stacking gel, mix 0.68 mL 30% acrylamide/bis solution (cross-linker ratio 37.5:1), 1.13 mL 0.5M Tris-HCl, pH 6.8, 2.25 mL Milli-Q, 50 μL 10% (w/v) SDS, 30 μL 10% (w/v) APS, and 5 μL TEMED (see Note 2).
2. Precision Plus Protein Dual Color standard (Bio-Rad).
3. LAS-3000 imaging system (Fujifilm).
4. Electroblothing and immunodetection system with primary antibody directed against a His-tag.

3. Methods

3.1. Ligation-Independent Cloning Procedure

3.1.1. Preparation of the DNA Insert

1. Design two sets of LIC primers for each gene to be cloned. The nLIC and cLIC primer sets will finally produce proteins with an N- or C-terminal affinity tag, respectively. The LIC strategy requires the presence of dedicated 5' extensions on the primers (indicated in Table 2.1). In addition, the gene-specific part of the primer should be designed so that it (1) does not contain the endogenous start or stop codon of the target gene; (2) is sufficiently long to anneal in a stable and selective way with the target DNA (in general, 20–25 nucleotides suffice; see Note 3). The primer should not contain strong secondary structure elements that could interfere with the PCR (see Note 4). Keep the total length of the

Table 2.1
Primer extensions needed for ligation-independent cloning

Primer type	5' Extensions of primers
nLIC forward	5' AT GGT GAG AAT TTA TAT TTT CAA GGT
nLIC reverse	5' T GGG AGG GTG GGA TTT TCA TTA
cLIC forward	5' ATG GGT GGT GGA TTT GCT
cLIC reverse	5 TTG GAA GTA TAA ATT TTC

primers below 50 residues to ensure sufficient quality and cost-efficiency.

2. For ordering, select the lowest synthesis scale offered by the supplier and mere desalting as the purification step. For large sets (>48 primers), order the primers in a 96-well plate.
3. On delivery, spin down the material and dissolve the primers to 100 μM in TlowE and make a substock of 5 μM . Use a transparent sticker to seal the plates. The 100 μM stock can be stored for several months at $-20^{\circ}C$. Store the 5 μM stock at $4^{\circ}C$. Spin down the material before use.
4. PCR the desired open reading frames using the Phusion DNA polymerase, which combines high fidelity with high production (see Notes 5 and 6). Prepare a 50 μL reaction mix according to the manufacturer's protocol and add the polymerase just prior to the start of the reaction. Place the sample in a PCR machine preheated to $98^{\circ}C$ and start the reaction. A good starting point for a touchdown PCR program (see Note 7) is
 - a. 60 s at $98^{\circ}C$.
 - b. 10 s at $98^{\circ}C$.
 - c. 15 s at $58.5^{\circ}C$ (decrease $0.5^{\circ}C$ per cycle).
 - d. XX s at $72^{\circ}C$ (15–30 s/kb).

Repeat steps 4a–4d 14 times.

- e. 10 s at $98^{\circ}C$.
- f. 15 s at $51^{\circ}C$.
- g. XX s at $72^{\circ}C$ (15–30 s/kb).

Repeat steps 4e–4g 14 times.

- h. 300 s at $72^{\circ}C$.
- i. Forever at $10^{\circ}C$.

Once the PCR is finished, add DNA loading buffer to the sample and analyze all the material by TAE (Tris-acetate-EDTA) gel electrophoresis (see Note 8). Gel purify the band using a

Table 2.2
Basic and additional LIC vectors

Vector name	Protein sequence	Protein sequence after TEV protease cleavage	Expression host
pBADnLIC	M-His ₁₀ -G-TEV site-protein	G protein	<i>Escherichia coli</i>
pBADcLIC	MGGGFA-protein-TEV site-His ₁₀	MGGGFA-protein-ENLYFQ	<i>E. coli</i>
pBADcLIC-GFP	MGGGFA-protein-TEV site-GFP-His ₁₀	MGGGFA-protein-ENLYFQ	<i>E. coli</i>
pBAD-OmpA-nLIC	M-ssOmpA ^a -His ₁₀ -G-TEV site-protein	G-protein	<i>E. coli</i>
pREnLIC	M-His ₁₀ -G-TEV site-protein	G-protein	<i>Lactococcus lactis</i>
pREcLIC	MGGGFA-protein-TEV site-His ₁₀	MGGGFA-protein-ENLYFQ	<i>L. lactis</i>
pREcLIC-GFP	MGGGFA-protein-TEV site-GFP-His ₁₀	MGGGFA-protein-ENLYFQ	<i>L. lactis</i>
pRE-USP45-nLIC	M-ssUSP45 ^b -His ₁₀ -G-TEV site-protein	G-protein	<i>L. lactis</i>

^assOmpA indicates the signal sequence of the *E. coli* OmpA protein. The pBAD-OmpA-nLIC vector is used if the N-terminus of the membrane protein of interest is predicted to be on the outside of the cytoplasmic membrane or to replace the signal sequence of the protein of interest with the ssOmpA

^bssUSP45 indicates the signal sequence of the *L. lactis* USP45 protein. The pRE-USP45 -nLIC vector is used if the N-terminus of the membrane protein of interest is predicted to be on the outside of the cytoplasmic membrane or to replace the signal sequence of the protein of interest with the ssUSP4

commercial gel extraction kit according to the manufacturer's protocol. Determine the DNA concentration spectrophotometrically (see Note 9). Store the material at -20°C.

3.1.2. Preparation of the LIC Vector

1. Inoculate approximately 20 mL LB-amp with *E. coli* MC1061 containing one of the pBAD- or pRE-derived LIC vectors (Table 2.2). Cultivate overnight at 37°C with vigorous shaking.
2. Isolate the plasmid using a commercial plasmid isolation kit and elute the plasmid with TlowE. Remove remaining proteins by combining a proteinase K treatment with a phenol:chloroform extraction as described in steps 3–5.
3. Combine several fractions and adjust the volume to 300 µL. Supplement the sample with SDS to a final concentration of 0.5% and add proteinase K to a final concentration of 40 µg/mL. Incubate for 30 min at 60°C to digest remaining proteins.

4. In a fume hood, add 1 volume of phenol:chloroform and vortex shortly. After 2 min, the sample is vortexed again and centrifuged for 2 min at full speed in a table centrifuge. Collect most of the top phase without disturbing the (white) interface and transfer to a new cup. Add 1 volume of chloroform and mix by vortexing. Centrifuge for 2 min at full speed in a table centrifuge and transfer the top phase without disturbing the interface to a new cup.
5. Add 2.5 volume of ice-cold 96% EtOH and 0.1 volume of 3M Na-acetate, pH 5.3. Vortex the sample and precipitate the DNA for 30 min at -80°C . Next, centrifuge the sample at 4°C for 15 min at full speed in a table centrifuge. Remove the supernatant and add 0.9 mL of ice-cold 70% EtOH. Centrifuge 5 min at full speed at room temperature in a table centrifuge. Remove the supernatant and dry the pellets shortly in a SpeedVac. Dissolve the pellet in 20 μL TlowE, determine the DNA concentration, and verify the integrity of the plasmid by restriction analysis.
6. Take 5 μg plasmid and digest the plasmid in a total volume of 40 μL containing 20 U *Sma*I according to the manufacturer's protocol (see Note 10). After 3 h, supplement the reaction with 10 U *Sma*I and extend the reaction for another 3 h. Analyze the full sample on 0.8% (w/v) agarose TAE gel using combs with wide wells. Isolate the linearized vector using a gel purification kit and determine the concentration. Store the material at -20°C .
7. To test the completeness of the digestion, transform 15 ng of the linearized plasmid to chemically competent *E. coli* MC1061 according to standard procedures. After the transformation procedure and 1 h incubation at 37°C with LB medium, sediment the cells by centrifugation (7,600g, 5 min), remove most of the supernatant, and plate the resuspended material on LB-amp agar plates. After overnight incubation, the colony count should be low (<100 CFU (colony-forming units)).

3.1.3. T4 DNA Polymerase Treatment of Vector and Insert

1. Take 200 ng of the *Sma*I-digested and gel-purified vector and adjust the volume to 10 μL . Add 3 μL of 5X T4 DNA polymerase buffer, 1.5 μL of 25 mM dCTP, and 0.5 μL of T4 DNA polymerase (0.5 U) (see Note 11). Mix and incubate for 30 min at room temperature. Immediately heat inactivate the T4 DNA polymerase by incubating for 20 min at 75°C and store the T4 DNA polymerase-treated vector at -20°C .
2. Take an amount of gel-purified insert equimolar to 200 ng vector (see Note 12) and adjust the volume to 10 μL . Add 3 μL of 5X T4 DNA polymerase buffer, 1.5 μL of 25 mM dGTP,

and 0.5 μL of T4 DNA polymerase (0.5 U) (see Note 11). Mix and incubate for 30 min at room temperature. Immediately heat inactivate the T4 DNA polymerase by incubating for 20 min at 75°C and store the T4 DNA polymerase-treated vector at -20°C.

3.1.4. Ligation-Independent Cloning

Mix 3 μL of the treated insert with 1 μL of the vector and incubate for 5 min at room temperature. Place the cup on ice and after 5 min add chemically competent *E. coli* MC1061. Transform the cells using standard procedures. Plate 10% and 90% of the transformed cells on LB-amp (see Note 13). Incubate overnight at 37°C.

3.1.5. Verifying Clones by Colony PCR

1. Divide the bottom plate of an empty LB-amp agar plate in 32 numbered squares with a marker. Fill each well in a 96-well PCR plate with 12.5 μL sterile Milli-Q.
2. Use a sterile toothpick to pick a colony and set a small streak on the agar plate (see Note 14). This plate will be temporarily used for archiving the clones. Subsequently, dip the toothpick in the appropriate well of the 96-well PCR plate and discard it. A set of four clones per construct usually suffices to finally obtain at least three positive clones.
3. Once the clones have been picked, seal the 96-well plate thoroughly with an aluminum sealing film (see Note 15) and lyse the cells for 15 min at 98°C in a PCR machine.
4. Allow the plate to cool to room temperature and supplement each well with 12.5 μL of a twofold concentrated PCR mix containing double the amount of Taq DNA polymerase, buffer, dNTPs, primers (see Note 1), and other additives as detailed in the manufacturer's protocol. After sealing the plate with a fresh aluminum sticker, continue the PCR according to
 - a. 30 s at 94°C.
 - b. 30 s at 94°C.
 - c. 60 s at 54°C.
 - d. XX s at 72°C (~45 s/kb).Repeat steps 4a–4d 30 times.
 - e. 300 s at 72°C.
 - f. Forever at 10°C.

Add DNA loading buffer to the sample and analyze all the material by TAE gel electrophoresis. Positive clones of the pBADxLIC vectors can be used for the expression analysis described in Section 2.3.3. immediately. Positive clones of the pRExLIC vectors first need to be converted into pNZxLIC vectors using the VBEx procedure described next. If positive clones are not sequenced at this stage, continue with three positive clones to spread the risk of PCR errors.

3.2. Vector Backbone Exchange Procedure

3.2.1 Isolation of pRExLIC Plasmids Containing Inserts

1. Inoculate 5 mL LB-amp with a positive streak from the agar plate containing the streak archive from the colony PCR. Incubate overnight at 37°C with vigorous aeration.
2. Isolate the plasmid using a commercial miniprep kit and elute the DNA in TlowE buffer. Determine the concentration (see Note 16) and store the sample at -20°C.

3.2.2. Preparation of pERL

1. Inoculate 20 mL of GM17-ery with NZ9000/pERL. Incubate overnight at 30°C (see Note 17).
2. Pellet the cells and resuspend the cell pellet in resuspension buffer. Incubate the cells at 55°C for 15 min to disrupt the cell wall (see Note 18).
3. Proceed with the plasmid isolation using a commercial miniprep kit, starting at the addition of the cell lysis buffer. Elute the plasmid in TlowE.
4. Remove remaining proteins by proteinase K digestion and Phenol:chloroform extraction as described in Section 2.3.1.2., steps 3–5. Dissolve the pellet of the EtOH precipitation in 20 µL TlowE, determine the DNA concentration. and verify the integrity of the plasmid by restriction analysis. Store at -20°C.

3.2.3. Vector Backbone Exchange

1. In a PCR cup, mix about 125 ng of pERL, about 125 ng of pRExLIC vector containing the insert, 1 µL of 10X *Sfi*I buffer, sufficient Milli-Q to adjust the volume to 9.5 µL, and 0.5 µL of *Sfi*I (5 U; see Note 19).
2. Digest the plasmids for 1 h at 50°C in a PCR machine with heated lid to avoid excessive evaporation. Subsequently, inactivate *Sfi*I by heating for 20 min at 80°C (see Note 19).
3. Allow the sample to reach room temperature and add 1.5 µL of 8 mM ATP and 0.5 µL of T4 DNA ligase (0.5 U; see Note 20). Incubate for 1 h at room temperature and inactivate the ligase in a PCR machine by heating for 20 min at 65°C. Store the sample at -20°C.

*3.2.4. Electrotransformation of *L. lactis**

1. Thaw electrocompetent *L. lactis* NZ9000 (see Note 21) on ice.
2. Transfer 50 µL cells to an ice-cold electroporation cuvet and add 2 µL of the sample resulting from the VBEx procedure (Section 2.3.2.3, step 3). Mix by pipetting up and down.
3. Expose the cells to a single electrical pulse with a field strength of 2.0 kV/2 mm, 25 µF capacitance, and 200 Ω resistance. Immediately following discharge, add 1 mL ice-cold rescue medium and leave the cells on ice for 10 min.
4. Incubate the cells, kept in the electroporation cuvet, for 2 h at 30°C.

5. Plate 10% and 90% of the transformed cells on SGM17-cam agar (see Note 13). Seal the plates with Parafilm (see Note 22) and incubate overnight at 30°C.
6. The colonies of *L. lactis* NZ9000/pNZxLIC holding the insert obtained in this step can be used for the expression analysis described in Section 2.3.3.

3.3. Initial Characterization and Optimization of Protein Production

3.3.1. Cultivation and Induction of MP Overexpression in *E. coli*

1. Inoculate 3 mL LB-amp from a verified streak obtained in Section 2.3.1.5. and incubate overnight at 37°C with vigorous aeration.
2. Mix 770 μ L of culture with 230 μ L of 87% (w/v) glycerol in a cryovial. Snap-freeze the vial in liquid nitrogen and archive the frozen stock at -80°C.
3. Use 60 μ L of the remaining culture to inoculate a culture tube containing 6 mL TB-amp (see Note 23). Incubate the cells at 37°C and 200 rpm and follow the optical density of the culture (see Note 24).
4. At $OD_{660} = \sim 0.2$, place the tubes in a water bath at room temperature for 15 min and subsequently continue growth at 25°C.
5. After 30 min, induce the cultures with 60 μ L of $5 \cdot 10^{-1}\%$ (w/v) L-arabinose stock (100-fold concentrated) to obtain a final L-arabinose concentration of $5 \cdot 10^{-3}\%$ (w/v). Incubate an additional 4 h.
6. Place the tubes in an ice-water bath and determine the OD_{660} of all cultures. Assuming that $OD_{660} = 1$ corresponds to a total protein concentration of 0.3 mg/mL, pellet the amount of culture equivalent to 1 mg protein in a FastPrep-compatible tube. Completely remove the supernatant, snap-freeze the pellet in liquid nitrogen, and store the sample at -20°C.

3.3.2. Cultivation and Induction of MP Overexpression in *L. lactis*

1. Inoculate 3 mL of GM17-cam with a single colony obtained in Section 2.3.2.4. and incubate overnight at 30°C.
2. Mix 770 μ L of culture with 230 μ L of 87% (w/v) glycerol in a cryovial. Snap-freeze the vial in liquid nitrogen and archive the frozen stock at -80°C.
3. Use 80 μ L of the remaining culture to inoculate a culture tube containing 8 mL of GM17-cam (see Note 23). Incubate the cells at 30°C and follow the optical density of the culture (see Note 24).
4. The nisin A present in the supernatant of an overnight culture of the nisin-producing strain *L. lactis* NZ9700 is used to induce the cells. Dilute an aliquot of this supernatant 50-fold in GM17-cam to obtain a 100X concentrated stock. At $OD_{660} = \sim 0.5$, induce the cells with 80 μ L of the 50-fold diluted

supernatant to obtain a final dilution of 5,000-fold. Incubate an additional 2.5 h.

5. Place the tubes in an ice-water bath and determine the OD_{660} of all cultures. Assuming that $OD_{660} = 1$ corresponds to a total protein concentration of 0.2 mg/mL, pellet the amount of culture equivalent to 1 mg protein in a FastPrep-compatible tube. Completely remove the supernatant, snap-freeze the pellet in liquid nitrogen, and store the sample at -20°C .

3.3.3. Cell Disruption and Sample Preparation

1. Add about 300 mg of glass beads and 400 μL of ice-cold disruption buffer to the frozen cell pellets.
2. Disrupt the cells by two rounds of bead-beating in the FastPrep-24 device at force 6 for 20 s (for *E. coli*) or 30 s (for *L. lactis*). Place the cups in an ice-water bath for 5 min after each round (see Note 25).
3. Allow the glass beads to sediment for about 1 min and mix a 100 μL fraction from the supernatant in a new cup with 25 μL of 5X sample buffer (see Note 2). Do not boil the sample as this will lead to aggregation of the membrane protein and denaturation of GFP fusion proteins. Store the sample at -20°C or proceed immediately with the SDS-PAGE.

3.3.4. SDS-PAGE, In Gel GFP Fluorescence, and Western Blotting

1. Load 10 μL of each sample per lane on an SDS-polyacrylamide gel (see Note 2). Include the Precision Plus Protein dual-color prestained molecular weight marker and run the gel at 150 V. Once the dye front has reached the bottom of the gel, disassemble the setup and soak the gel in Milli-Q.
2. For GFP fusion proteins, determine the *in gel* fluorescence. Place the gel on the black imaging tray of the LAS-3000 imaging system. Adjust the focus of lens to the gel and expose the gel to 460 nm light from the blue LED (light-emitting diode) and use a 515 nm emission filter (see Note 26). The fluorescent bands of the marker at 25 and 75 kDa should be clearly visible.
3. Submit the gel to immunoblotting to detect the His-tagged proteins. Use the LAS-3000 in chemiluminescence mode to measure the signal (see Note 26). The 75 kDa band of the marker should give a clear signal. Once a satisfactory exposure has been obtained, without moving the gel, use the top white light mode of the LAS-3000 to obtain an image of the membrane to locate the prestained marker bands.
4. Use the marker bands detected during the *in gel* fluorescence, chemiluminescence, and the white light image to estimate the intensity and the molecular weight of the detected bands and correlate the different images (see Note 27).

3.3.5. Expression Optimization

1. Optimize the expression by varying the protein expression rate over a wide range. For the P_{BAD} system, use inducer concentrations between 1.10⁻⁵% and 1.10⁻¹% (w/v) of L-arabinose; vary the induction temperature between 37°C, 25°C, and 17°C; and induce for 1, 2, 4, and 16 h. Determine the effect on expression using small cultures as described in Sections 2.3.3.1.–2.3.3.4. If possible, make use of the GFP fusion protein to guide the optimization procedure in *E. coli*. The presence of a strong, nonfluorescent band, indicative for aggregated protein, suggests too high an expression rate; the absence of a nonfluorescent band suggests that the capacity of the targeting, insertion, and folding pipeline is not yet saturated.
2. For the lactococcal P_{nisA} system, use dilutions of the nisin-containing supernatant ranging from 100,000- to 500-fold; vary the induction temperature between 30°C, 25°C, and 17°C; and induce for 1, 2, 4, and 16 h. Determine the effect on expression using small cultures as described in Sections 2.3.3.1.–2.3.3.4.

4. Notes

1. Use generic primers instead of gene-specific primers. This facilitates the analysis of several genes and provides a positive control for the PCR because for empty vectors a band will be produced as well. Generic primers for the pBADxLIC derivatives are 5'ctctactgtttctccatacccg and 5'gctgaaaatct-tctctcatccg. Generic primers for the pRExLIC derivatives are 5'cgcgagcataataaacggc and 5'ctgctgctttttggctatcaatc.
2. Deviations in the composition from the here-described buffers for sample preparation and gel electrophoresis might result in a decrease in the intensity of the *in gel* GFP fluorescence.
3. The initial steps of primer design can be easily automated using a spreadsheet program. This will speed up the design process and reduce the number of errors. A spreadsheet that combines the terminal 20 nucleotides of a set of DNA sequences with the nLIC and cLIC extensions to produce a series of initial primers that might require additional corrections can be obtained from the authors' Web site (<http://www.rug.nl/gbb/research/researchgroups/enzymology/index>).
4. Removal of secondary structure elements is often best done by making silent mutations in the gene-specific part of the primer while preventing the use of rare codons as much as possible. Do not make any changes in the primer extensions required for LIC.

5. Genomic DNA is easily compromised by repetitive freeze/thawing, vortexing, or vigorous pipetting and thus requires careful handling; storage in sterile tubes at 4°C is preferred.
6. Combine PCRs for targets of similar length and template type (plasmid or genomic) as much as possible instead of performing a PCR for each gene separately. This will speed up the whole process and allows more time for optimizing PCRs of “difficult genes.”
7. Touchdown PCR ensures a more specific product formation (42), and as a range of annealing temperatures is used, it is a good alternative to optimizing the reaction conditions for each target on an individual basis.
8. Should the DNA template that was used for the PCR reaction be a plasmid that confers resistance to β -lactam antibiotics such as ampicillin and carbenicillin, then the PCR mix should be incubated for 30 min at 37°C with 1 μ L *DpnI* prior to the gel electrophoresis to remove the template. As the pBADxLIC and pRExLIC vectors used for the LIC procedure carry the β -lactamase marker, traces of template plasmids that carry the same marker will lead to a high number of background colonies.
9. Should the PCR not yield the desired product in sufficient amounts, consider the use of alternative buffers supplied by the manufacturer, the addition of dimethyl sulfoxide (DMSO), or replacement of the template. More detailed suggestions can be found in the *Molecular Cloning* protocol series (40).
10. Depending on the producer of *SwaI*, the suggested incubation temperature is 25°C or 30°C. Condensation at the lid, leading to suboptimal reaction conditions, can be prevented by performing the reaction in an incubator or PCR machine with heated lid.
11. The quality of the dNTP stocks and the T4 DNA polymerase is a critical parameter for the success of the procedure. Prevent repetitive freezing/thawing of the nucleotides by preparing small aliquots and label the aliquots appropriately to prevent mixing up dCTP (used for the vector) with dGTP (used for the insert). Transport the T4 DNA polymerase in a cooling tray and keep the enzyme cold under all conditions.
12. The use of equimolar amounts of vector and insert ensures that both are treated with T4 DNA polymerase to a similar extent. The amount of the purified PCR product needed can be calculated using the following: Amount insert needed (ng) = $((200 \text{ ng} * \text{Insert size (bp)}) / \text{Vector size (bp)})$.
13. Especially for large numbers of samples, spreading the transformed cells over the plate is most easily done by making use

- of glass beads (diameter 5 mm). In addition, this prevents cross contamination.
14. Use of toothpicks over an inoculation needle prevents cross contamination when analyzing multiple samples at one time. A cross-locking tweezer facilitates handling the toothpicks for prolonged periods.
 15. Take care to properly seal the plate, especially around the sides. Incomplete sealing will lead to evaporation.
 16. Especially for large numbers of plasmid samples, determining the DNA concentration can be time consuming. As the VBEx reaction only requires an approximate estimation of the DNA concentration, it suffices to determine the concentration of a small subset (~5 samples) and use the average DNA concentration as representative for this batch.
 17. As *L. lactis* does not require aeration, small cultures are not shaken and can be maintained in closed glass or plastic containers.
 18. The extent of the degradation of the cell wall depends on the incubation time and the amount of cells and lysozyme used; insufficient degradation leads to lower plasmid yields. Whether the lysozyme treatment was sufficient can be easily determined by mixing a 10 μL aliquot of the cell suspension with 10 μL of the supplied lysis buffer. Adequate disruption of the cell wall will lead to complete cell lysis within 5 min. If needed, extend the incubation time or use a higher lysozyme concentration.
 19. For the overall procedure, it is critical that the *Sfi*I enzyme can be heat inactivated. For unknown reasons, this property seems to depend on the supplier. We have had a good experience with *Sfi*I obtained from Fermentas.
 20. For a large number of samples, prepare a premix containing 6 mM ATP, 0.25 U/ μL T4 DNA ligase, and 1X T4 DNA ligase buffer. Use ice-cold Milli-Q and prepare the mix just prior to use.
 21. Preparation of electrocompetent *L. lactis* NZ9000 was essentially done as described (43), but with minor modifications. Briefly, cells were grown in M17 supplemented with 0.5% glucose, 0.5M sucrose, and 2% glycine at 30°C to $\text{OD}_{600} = \sim 0.5$. Cells were harvested by centrifugation at 5,000g for 15 min at 4°C. Following washes with 1 volume of ice-cold solution A (0.5M sucrose and 10% glycerol, prepared in Milli-Q), 0.5 volume of solution A supplemented with 50 mM Na-EDTA, pH 7.5, and 0.25 volume of solution A, cells were resuspended in 0.01 volume of solution A. Aliquots of 50 μL were snap-frozen in liquid nitrogen and stored at -80°C until use.
 22. Sealing the agar plates with Parafilm is not essential but allows faster appearance of *L. lactis* colonies.

23. Preheat the medium without the antibiotics overnight at 37°C or 30°C for *E. coli* and *L. lactis*, respectively. This prevents a slow start of the cultivation due to a small temperature shock.
24. When handling a large set of samples, use an additional dedicated set of approximately six reference cultures to follow the OD₆₆₀ and use their average OD₆₆₀ to time the moment of cooling or induction.
25. Alternative methodologies for cell disruption, such as sonication and vortexing in the presence of glass beads, can be used as well but might be less efficient. This is especially true for *L. lactis*, whose thicker and stable cell wall makes it more difficult to disrupt than *E. coli*. French press disruption is comparable to cell disruption by a FastPrep but requires larger volumes and is therefore less suited. For *L. lactis*, treatment with lysozyme to digest the peptidoglycan layer prior to disruption greatly enhances the efficiency. This step is not required for FastPrep-mediated cell disruption.
26. The use of the same tray height for *in gel* fluorescence and chemiluminescence imaging prevents rescaling of images, thus facilitating the correlation of the images.
27. The transfer efficiencies from the gel to the PVDF (polyvinylidene difluoride) membrane will differ for different membrane proteins. This complicates selection of promising clones. In contrast, the *in gel* fluorescence of GFP fusion proteins can be compared directly.

Acknowledgments

We thank G.K. Schuurman-Wolters, M.B. Tol, M. Groeneveld, D.J. Slotboom, and S. Henstra for their contribution in establishing the methodologies discussed. This work was funded by the European Membrane Protein Consortium (grant 504601), a FEBS fellowship (to E.R.G.), and the Netherlands Proteomics Centre.

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<http://www.springer.com/978-1-60761-343-5>

Heterologous Expression of Membrane Proteins

Methods and Protocols

Mus-Veteau, I. (Ed.)

2010, XI, 272 p., Hardcover

ISBN: 978-1-60761-343-5

A product of Humana Press