Leishmania tarentolae as a Promising Tool for Expressing Polytopic and Multi-Transmembrane Spans Eukaryotic Membrane Proteins: The Case of the ABC Pump ABCG6

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Abstract

This chapter includes a practical method of membrane protein production in *Leishmania tarentolae* cells. We routinely use it to express membrane proteins of the ABC (adenosine triphosphate-binding cassette) family, here exemplified with ABCG6 from *L. braziliensis*, implicated in phospholipid trafficking and drug efflux. The pLEXSY system used here allows membrane protein production with a mammalian-like *N*-glycosylation pattern, at high levels and at low costs. Also the effects of an N-terminal truncation of the protein are described. The method is described to allow any kind of membrane protein production.

Keywords

ABC transporters
Leishmania tarentolae
Membrane protein expression
Drug efflux

1. Introduction

Structural studies of membrane proteins are limited by the amount of fully functional protein that can be produced. This is especially vivid for mammalian membrane proteins, for which production in the milligram range ensuring correct folding and functionality remains challenging. In general, large-scale production and purification require a low-cost and effective expression system. Several approaches have been reported in mammalian cells [1], *Xenopus laevis* oocytes [2], bacteria [3, 4], *Spodoptera frugiperda Sf*9 insect cells [5], and yeast [6]. Up to now, mammalian cells are most often used in functional studies. ABCG2 is an ABC transporter rather difficult to produce for which several systems were evaluated. Oocyte system represents an easy system to study ABC transporter function; however, it produces a high background in efflux experiments due to nonspecific binding of hydrophobic ABCG2 substrates to intracellular

structures such as yolk granules, which represent around 50 % of cellular volume. *Lactococcus lactis* expression system allows quantifying sterol transport mediated by ABCG2, not possible in mammalian or insect cells where the membrane sterol content can reach up to 25 %. However, ABCG2 expression level remains too low for structural studies [7]. Overexpression in *Escherichia coli* provides a high yield of recombinant protein but devoid of drug efflux or ATPase activity [8]. The baculovirus-*Sf*9 expression system allows membrane protein expression in a quite high level in intact cells and membranes, being a good tool to measure ATPase activity and transport of fluorescence substrates [9]. Nevertheless, cholesterol content, crucial for ABCG2 function, is very low in insect cell membranes [10]. BTI-TN-5B1-4 High Five insect cells produce even higher levels of protein but in a heterogeneous manner. Membrane protein overexpression in *Pichia pastoris* yeast has been used to successfully express and purify large quantities of P-gp [11] and MRP1 [12]. However, this is not the case for ABCG2, which has been produced in comparable levels to the ones achieved in HEK cells but not yet purified. Previously cited expression methods constitute a useful tool for membrane protein expression leading to functional and structural studies but they are not suitable for all membrane proteins; that is the case of ABCG2 whose structure is still unsolved.

A fundamental problem in the production of heterologous proteins in prokaryotic systems is downregulation of protein expression via activation of transcriptional control mechanisms in the host. One alternative is using *Trypanosomatidae* protozoa such as *L. tarentolae* with a mammalian-type posttranslational modification of target proteins [13] and successfully used for the expression of other proteins [14]. *L. tarentolae* is a parasite of the gecko *Tarentola annularis* and has been developed as new eukaryotic system for expression of recombinant proteins with a mammalian-like *N*-glycosylation pattern [15]. This system has already been described to successfully express GFP protein in the parasite using pLEXSY vectors [16].

This chapter describes the experimental procedure to produce a membrane protein, ABCG6, from *L. braziliensis*, by using the pLEXSY system in *L. tarentolae. lb*ABCG6 is expressed in the plasma membrane of the parasite and mediates phospholipid trafficking and drug resistance [17]. It shares the highest similarity (28 %) with human ABCG2 among all the ABC transporters in *Leishmania* species. The latter protein confers resistance to anticancer drugs [18] and has been analyzed in multiple functional and comparative studies [19]. The expression system described below presents several advantages such as low cost, nonspecial biosafety requirements, and no cross-contamination with other cultures.

2. Materials

2.1. Proteins

The gene coding for *lb*ABCG6 (UniProtKB #A4HPF5) was synthesized by GENEART (Life Technologies SAS). Two *Bam*HI restriction sites were added, at the beginning and right before the nucleotide-binding domain, allowing N-terminal truncation by molecular biology methods (*lb*ABCG6ΔN). Also an N-terminal 6xHis-tag was added to allow protein purification. The material used for *lb*ABCG6 expression in *L. tarentolae* described here is provided from Jena Bioscience and Lonza.

2.2. Lab Equipment

1. All the material needed for molecular biology experiments.
2.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots are carried out using the Mini-Protean 3 apparatus and related devices from Bio-Rad.

3. Microfluidizer M-110P (Microfluidics IDEX CORPORATION).

4. Transfection and culture of the cells are achieved in a class I-type room (*see* **Note 1**) equipped with a Steril Bio Ban 48, an incubator Heraeus BK6160 with a H + P Biomag Biomodule 40B, a microscope Olympus CKX31, and a low-speed centrifuge handling 15/30 mL Falcon-type tubes.

5. Tissue culture T-25 and T-75 flasks are used for static cell cultures. Bigger cultures from 50 mL to 1 L with 75–140 rpm agitation are carried out in Erlenmeyer and baffled Fernbach flasks, respectively.

2.3. Cells

1. Eukaryotic protozoan parasite *L. tarentolae* (Jena BioScience).

2. XL1-Blue chemically competent *E. coli* or equivalent to generate the recombinant plasmid of interest.

2.4. Media

1. BHI medium: The powder is dissolved in deionized water (37 g/L), sterilized by filtration, and stored at 4 °C. Right before use, the medium is supplemented with 0.5 % penicillin and streptomycin, 50 μg/mL G418-sulfate, 5 μg/mL hemin (stock solution at 0.25 % in 50 % triethanolamine, tube wrapped with foil to avoid light), 100 μg/mL nourseothricin, and 100 μg/mL hygromycin. Medium is then stored at 4 °C up to 15 days. Sterilization is achieved by filtration as autoclaving leads to partial degradation of nutriments varying from batch to batch, to which *L. tarentolae* cells are sensitive.

2. Tetracycline 10 mg/mL.

3. Yeast extract medium: 24 g/L of yeast extract, 3 g/L glucose, 12.5 g/L K_2HPO_4, and 2.3 g/L KH_2PO_4, sterilized by filtration and stored at 4 °C until use. Right before use, the medium is supplemented with 1 % fetal bovine serum (FBS), and the same supplements as for BHI medium. Medium is then stored at 4 °C for a maximum of 15 days. Sterilization is achieved by filtration.

4. Luria-Bertani medium: 10 g/L Bactotryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, supplemented with 15 g/L agar for plates. Autoclave for 20 min at 120 °C. Before use add 100 mg/L ampicillin (*see* **Note 2**). Store without antibiotics at room temperature.

5. Hepes buffer: 50 mM Hepes-NaOH pH 7.5, 200 mM NaCl.

2.5. Transfection

1.

Reagent: Supplemented Nucleofector® solution (Basic Parasite Nucleofector Kit 1, Lonza).

2. Medium: BHI.

3. Equipment: Nucleofector II-S®(Lonza) and Lonza-certified cuvettes.

2.6. Molecular Biology

1. The plasmid used here is the pLEXSY-I-neo3 (Jena Bioscience) with the neo marker gene allowing selection of recombinant LEXSY strains with G418, and designed for inducible expression of target genes in LEXSY host T7-TR.

2. Kits for small- (3–10 mg) and medium- (50–100 mg) scale plasmid (5–10 kbp) DNA preparations (NucleoSpin™ Plasmid, Macherey-Nagel).

3. Go Taq DNA polymerase (Promega).

4. NucleoSpin Extract II kit (Macherey-Nagel).

5. Restriction enzymes *Nco*I, *Xba*I, and *Swa*I.

2.7. SDS-PAGE

1. Separating buffer stock solution: 1.5 M Tris–HCl, pH 8.8.

2. Stacking buffer stock solution: 1 M Tris–HCl, pH 6.8.

3. 10 % SDS.

4. Acrylamide/bis solution: 40 %, 37.5:1 with 2.6 % C (*see* **Note 3**).

5. 10 % Ammonium persulfate (stored at 4 °C up to 15 days).

6. *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED).

7. Running buffer: Dilute Tris-glycine-SDS 10× (Euromedex), can be stored at room temperature.

8. Laemmli-type loading buffer (5×) “5×U”: 100 mM Tris–HCl, pH 8.0, 8 M urea, 4 % SDS, 1.4 M β-mercaptoethanol, 0.0025 % bromophenol blue. The solution is stored at −20 °C and aliquoted to freeze/thaw ten times maximum [20].

9. Pre-stained molecular weight markers: Kaleidoscope markers (Bio-Rad).

10. Staining solution: Dissolve 1 g of Coomassie Brilliant Blue (Bio-Rad) in 1 L of 50 % [v/v] ethanol, 10 % [v/v] glacial acetic acid, 40 % H_2O; stir the solution until complete solubilization and then filter through Whatman filter paper; store at room temperature; do not reuse.

2.8. Western Blotting

1. Transfer buffer: Dilute 10× Tris-glycine (Euromedex), 20 % methanol (*see* **Note 4**). Prepare fresh and use cold, with a cooling ice bag during transfer.
2. Nitrocellulose membrane and 3 MM chromatography paper (Whatman).
3. Tris-buffered saline with tween and triton (TBS-TT): Dilute 10× TBS stock (Euromedex) with water, add 0.05 % Tween-20 and 0.2 % triton.
4. Blocking solution: 0.5 % Blocking reagent (Qiagen) in TBS, 0.1 % Tween-20.
5. Antibody anti-His HRP conjugated (Qiagen) is used 1/20,000 diluted in blocking solution.
6. Enhanced chemiluminescent (ECL) reagents and autoradiography films are used for revelation.

3. Methods

3.1. Molecular Biology

Cloning of *lb*ABCG6 and the truncated *lb*ABCG6ΔN into pLESXY plasmid (*see* restriction map in Fig. 1) is achieved using classical methods of molecular biology described in the LESXY kit and in [20]. *lb*ABCG6 was cloned between the *Nco*I and *Xba*I restriction sites inside the multiple cloning sites controlled by the T7 RNA polymerase promoter. Utr1, utr2, and utr3 are optimized non-translated gene-flanking regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in the LEXSY host. The following plasmids including the different constructs were generated: *pLEXSY-lbabcg6* and *pLEXSY-lbabcg6ΔN*. Once constructed, each plasmid was checked by sequencing and digested with *Swa*I to remove the *E. coli* fragment (Fig. 1). Each construction was extracted from agarose gel using the kit NucleoSpinExtract II and used in the nucleofection of *L. tarentolae*.

Fig. 1

Cloning strategy of *lb*ABCG6 based on pLEXSY system. *lb*ABCG6 was cloned between the *Nco*I and *Xba*I restriction sites inside the multiple cloning site. Then, *E. coli* fragment was removed by *Swa*I digestion

3.2. *L. tarentolae* Growth

1. Cells are grown at 26 °C in the dark, under the promastigote shape with flagella allowing them to swim in the medium.
2. Healthy cells tend to aggregate as cell density increases, forming larger aggregates at higher cell densities (*see* **Note 5**). Ideally, cells are amplified by dilution in fresh medium when they are in the exponential phase, OD^{600} = 1.4–2 (6–8 × 10^7 cells/mL).

3. *L. tarentolae* is maintained in BHI medium in static T-25 flasks. Typically, a 50-fold dilution in 10 mL fresh BHI medium is carried out at time 0, and then cells reach a sufficient density for a 20-fold dilution 5 days later (*see* **Notes 6 – 8**).

4. Yeast extract medium is used for scale-up. A culture in BHI medium in exponential phase is diluted 20-fold in yeast extract medium (pre-culture). When the culture reaches the exponential phase, a baffled Fernbach flask of 500 mL of yeast extract medium is inoculated to a final OD^{600} of 0.1–0.2 and incubated in the dark, 26 °C and 75–90 rpm (higher cell densities can be obtained in agitated flasks compared to static cultures) (*see* **Notes 9** and **10**). The exponential phase will be reached after 36 h.

3.3. Cryo-Conservation of *L. tarentolae*

1. Cryo-conservation of *L. tarentolae* should be realized for a culture in exponential phase in BHI medium.

2. Add sterile glycerol to the cells in BHI medium, to a final concentration of 20 %, and aliquot by 1.6 mL in sterile cryotubes.

3. Incubate the cells for 10 min at room temperature, and then transfer the tubes to a precooled (4 °C) isopropanol cryobox for 10 min. Transfer the cryobox to −80 °C and incubate overnight. Store at −80 °C or in liquid nitrogen.

4. To reactivate frozen stocks, thaw a cryotube on ice, and then pour the content of the tube into 10 mL of fresh BHI medium in a T-25 flask. Check that the cells are vital by direct observation under a microscope. Incubate at 26 °C until OD^{600} = 1.4–2, which usually takes 2–3 days. Then dilute tenfold for allowing cells to fully recover from the freezing, and proceed to normal dilution.

3.4. *L. tarentolae* Nucleofection

1. The best efficiency of transfection is obtained for *L. tarentolae* in the exponential phase. Grow 10 mL of cells in BHI medium until OD^{600} = 1.4 and ensure by microscopy that they are vital and of drop-like shape grouping in aggregates.

2. Spin cells for 3 min, 2000 × *g*, at room temperature and suspend pellet in 100 µL of supplemented Nucleofector® solution. Add 4 µg of DNA and transfer to an electroporation cuvette. Electroporate according to the Basic Parasite Nucleofector Kit 1 (Lonza), using the program U-033.

3. Transfer electroporated cells to 10 mL of LEXSY BHI medium in a ventilated flask. Incubate for 24 h as static suspension culture (Fig. 2). Proceed to a clonal selection.

Fig. 2

Protein expression based on pLEXSY system. (**a**) Cells of the parasite are harvested and diluted to 6.10^7 cells/mL and OD^{600} ~1.4. (**b**) After spinning, cells are suspended in 100 µl of supplemented Nucleofector solution and mixed with 4 µg DNA. (**c**) All the mix is transferred to an electroporation cuvette and electroporated according to the Basic

Parasite Nucleofector Kit 1 (Lonza), using the program U-033. (**d**) Electroporated cells are transferred to BHI media in a ventilated flask. After 24-h incubation, proceed with clonal selection. (**e**) Genomic DNA is extracted from cells and integration of *lb*ABCG6 is verified by PCR. *Lane M* molecular weight marker, *line 1* genomic DNA from *lb*ABCG6 transfected cells giving a PCR product of 2300 pb

3.5. Monoclonal Selection of Recombinant Cells

1. 24 h after transfection, harvest 2 mL from the transfected 10 mL culture obtained by the electroporation protocol.
2. Pellet cells for 3 min at 2000 × *g* at room temperature. Remove supernatant and suspend the cells in the residual medium left in the tube, approximately 50 μL.
3. Carefully spread the suspended cells onto freshly prepared BHI agar supplemented with the selective markers: G418, nourseothricin, hygromycin, and penicillin-streptomycin.
4. Seal plates with parafilm and incubate them covering up.
5. 5–7 days after plating, small defined colonies begin to appear. After these colonies have grown up to 1–2 mm diameter, they can be transferred to 0.2 mL of selective growth medium in a 96-well plate using a pipette tip.
6. After 24-h incubation at 26 °C, these clones must be expanded into 1 mL selective medium in a 12-well plate and incubated under agitation (140 rpm).
7. After 48-h incubation at 26 °C, the cultures are expanded into 10 mL selective medium in T-25 flasks and can be used for evaluation.

3.6. Verification of lbABCG6 Gene Integration into Leishmania Genome

After genomic DNA extraction from the cells, the integration of genes of interest into the *L. tarentolae* genome is verified by PCR using GoTaq polymerase (*see* Fig. 2e). The following primers were used, recognizing the pLEXY-I-neo3 sequence flanking the inserted gene so that the same primers can be used for all the clones:

Fwd 5′- CCGACTGCAACAAGGTGTAG and Rev 5′- GAGATGTTCCTGACCGACC.

3.7. Analysis of Protein Expression by SDS-PAGE and Western Blot

The expression of the protein can be checked rapidly after cell transfection. Cells grown to an OD^{600} = 1.4 in a T-25 flask and protein expression are induced with 10 μg/mL tetracycline for 24 h. One milliliter of culture is harvested by centrifugation and suspended in 50 μL of 50 mM Hepes-NaOH pH 7.5 and cells are broken by three cycles of freeze/thaw in liquid nitrogen/warm water. Fifteen microliters of broken cells are mixed with 5 μL of loading buffer 5×U, followed by analysis on SDS-PAGE and Western blot (Fig. 3).

Fig. 3

Expression of *lb*ABCG6 (**a**) and *lb*ABCG6ΔN (**b**) in *L. tarentolae* cells as a function of time and culture media, under agitation. Expression of *lb*ABCG6/*lb*ABCG6ΔN is carried out as described in Subheading 3.5. Transfected cells were maintained under antibiotic selection for 2 weeks in BHI and yeast extract medium. Then, protein expression is induced by adding 10 μg/mL tetracycline and cells harvested after 24, 48, and 72 h. *lb*ABCG6/*lb*ABCG6ΔN expression was analyzed by SDS-PAGE and Western blot loading 15 μl of samples normalized to OD^{600} ~1.4 onto a 10 % SDS-PAGE. (**c**) Membrane expression of *lb*ABCG6 and *lb*ABCG6ΔN in *L. tarentolae* cells. Expression and membrane preparation of *lb*ABCG6/*lb*ABCG6ΔN are carried out as described in Subheading 3.8. Protein expression was analyzed by SDS-PAGE (Coomassie blue-stained Western blot) loading 20 μg and 10 μg of samples, respectively, onto a 10 % SDS-PAGE. *NI*, protein expression without induction as negative control

1. Generate the separating gel (4 mL) of a 10 % SDS-PAGE by mixing 1.92 mL of water, 1 mL of 40 % acrylamide bisacrylamide solution, 1 mL of 1.5 M Tris–HCl pH 8.8, 40 μL of 10 % SDS, 40 μL 10 % ammonium persulfate, and 1.6 μL TEMED. Pour the Bio-Rad Mini-Protean 3 device to be 8 mm under the bottom of the wells. Add 200 μL of water at the surface of the gel for preventing the formation of waves (*see* **Note 11**). Polymerization occurs in 30 min at room temperature (22 °C).

2. Generate the 5 % stacking gel by mixing 1.46 mL of water, 0.25 mL of 40 % acrylamide bisacrylamide solution, 0.25 mL of 1.5 M Tris–HCl pH 6.8, 20 μL of 10 % SDS, 20 μL 10 % ammonium persulfate, and 2 μL TEMED.

3. Load 20 μL samples onto the stacking gel and run for about 1.5 h at 120 V at room temperature.

4. After electrophoresis, proteins are either stained with Coomassie blue or transferred onto nitrocellulose membrane (*see* below).

For Coomassie Blue staining, incubate the gel for 30 min in 50 mL of staining solution, and then wash it three times in 10 % [v/v] glacial acetic acid and 90 % H_2O.

When proteins are transferred from the SDS-PAGE to the nitrocellulose membrane, proceed as follows:

1. Incubate the gel in 5 mL of cold transfer buffer for 5 min.

2. Wet the nitrocellulose membrane for 5 min in the cold transfer buffer.

3. Prepare the transfer sandwich built by superposing successively two paper sheets briefly wet in the transfer buffer, the acrylamide gel, the nitrocellulose membrane, and again two wet paper sheets.

4. Add the ice cube to the Mini-Protean 3 transfer device and a magnetic stirrer and transfer for 2 h at 100 V under agitation to optimize cooling.

5. After transfer, block the membrane for 1 h into 20 mL of TBS containing 0.5 % blocking reagent and 0.1 % Tween 20.
6. Add the primary antibody to the solution and incubate for an additional 1 h.
7. Wash three times with 20 mL of TBS-TT buffer.
8. Wash once with 20 mL of TBS buffer.
9. Withdraw the buffer and incubate with a 1:1 mix of 2 mL ECL solutions A and B for 5 min and expose onto a sensitive film for 1–20 min depending on the antibodies.

A typical result is illustrated in Fig. 3 .

3.8. Optimization of lbABCG6 Expression

For optimization of culture medium and time of expression, proceed as follows:

1. To evaluate the type of medium and the influence of protein expression induction time, transfected *L. tarentolae* cells were grown in BHI or yeast extract media in a T-25 flask as described above.
2. When cells reach OD^{600} = 1.4, add 10 μg/mL tetracycline to induce protein expression.
3. At 24, 48, and 72 h, harvest an aliquot of cell culture and check for *lb*ABCG6 expression by SDS-PAGE and Western blot as described in Subheading 3.7 .

The result is illustrated in Fig. 3 . It shows that the expression is higher with yeast extract medium and in both cases it is better at 48 h post-induction. This result permits the scale-up of cell culture, which will grant the quantities of protein required for structural studies.

Scale-up in Fernbach flasks to achieve the yields needed for protein production and purification for structural studies:

1. Grow cells in a T-25 flask (10 mL) used to seed a pre-culture in a T-75 flask (30 mL), both in BHI medium.
2. Once the pre-culture reached a suitable cell density, inoculate a 1 L baffled Fernbach flask filled with 500 mL of yeast extract medium to a final OD^{600} = 0.2.
3. When cells reach OD^{600} = 1.4, add 10 μg/mL tetracycline to induce protein expression. Protein expression is continued for 48 h, and cells reach an OD^{600} around 4.
4. Harvest the cells by centrifugation for 10 min at 7500 × *g*, 4 °C.
5. Take the pellet in 50 mL Hepes buffer per liter of culture and break the cells by two passages at 15,000 psi in a microfluidizer.
6. Centrifuge for 30 min at 15,000 × *g*, 4 °C, discard the pellet, and centrifuge the supernatant for 1 h at 180,000 × *g*, 4 °C, to collect the membranes.

Routinely, 1 L of cell culture yields 0.5 g of dry membrane.

As observed in Fig. 3c, the first 30 residues of the protein are not very relevant in terms of expression. There is no remarkable difference in expression levels between *lb*ABCG6ΔN and *lb*ABCG6 constructs when analyzing membrane samples both by Western blot and Coommassie blue-stained SDS-PAGE. Differences shown in Fig. 3a, b, using whole cells, might be due to an artifact in Western blot.

The detailed methodology described above to express membrane proteins using the pLEXSY system in *L. tarentolae* represents an interesting alternative for structural studies. Indeed, culture medium optimization allows cost reduction while maintaining high protein expression levels. In addition, *L. tarentolae* cell culture does not require special biosafety requirements and cross-contamination with other cultures is very low.

4. Notes

1. *Leishmania tarentolae* is not infectious for human; its culture can be done in a class I culture room.
2. Ampicillin should be prepared fresh to a maximal efficiency.
3. Acrylamide is neurotoxic when non-polymerized; thus handle with gloves.
4. Methanol is neurotoxic.
5. Care should be taken not to dilute too much upon passages, as isolated cells do not divide well.
6. A 100-fold dilution is the limit of good growth and should be kept occasionally.
7. *L. tarentolae* can be kept in culture for up to 3 months, after which a new frozen stock should be used.
8. As the number of passages increases, *L. tarentolae* can reach higher cell densities.
9. To reduce costs, nourseothricin is not added in large cultures, without incidence on protein expression.
10. Cultures can also be carried out in Erlenmeyer flasks for volumes ranging from 50 to 500 mL, under agitation of 100–140 rpm.
11. Do not use organic solvent for this step as membrane proteins have a tendency to interact with it.

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