Leishmania tarentolæ as a promising tool for expressing polytopic and multi-transmembrane spans eukaryotic membrane proteins. The case of the multidrug ABC pump ABCG6.

Lucia Gonzalez-Lobato[1], Vincent Chaptal[1], Jennifer Molle[1] & Pierre Falson[2]

(1) These authors contributed equally.

Address: Drug Resistance and Membrane Proteins Lab, UMR CNRS-UCBL1 5086. IBCP, 7, passage du Vercors F-69367 Lyon

Correspondance to : pierre.falson@ibcp.fr

Abstract

The present chapter includes a practical method of membrane protein production in *Leishmania tarentolæ* parasites. 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 phospholipids 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.

Key words: ABC transporters, *Leishmania tarentolæ*, membrane proteins 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 requires 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 transporters 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. *Lactoccocus 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 (4). 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 down-regulation of protein expression via activation of transcriptional control mechanisms in the host. One alternative is using *Trypanosomatidæ* protozoa such as *L. tarentolæ* with a mammalian-type posttranslational modification of target proteins (13) and successfully used for the expression of other proteins (14). *L. tarentolæ* is a parasite of the gecko *Tarentolæ annularis* and has been developed as new eukaryotic expression system for the production 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).

The present chapter describes the experimental procedure to produce a membrane protein, ABCG6 from *L. braziliensis*, by using the pLEXSY system in *L. tarentolæ*. *lb*ABCG6 is expressed in the parasite plasma membrane and mediates phospholipids 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, non special bio-safety requirements and no cross-contamination with other cultures.

2. Materials

The material used for *lb*ABCG6 expression in *L. tarentolæ* described here is provided from Jena Bioscience and Lonza.

2.1. Proteins

1. The gene coding for *lb*ABCG6 (UniProtKB #A4HPF5) was synthetized 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.

2.2. Lab equipment

1. All the material needed for molecular biology experiments.
2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots are carried out using the Mini-Protean 3 apparatus and related devices from Bio-Rad.
3. Transfection and culture of the parasites 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.
4. Tissue culture T-25 and T-75 flasks (Falcon) are used for static cell cultures. Bigger cultures from 50 ml to 1 litre with 75 – 140 rpm agitation are carried out in erlen-meyer and baffled fernbach flasks respectively.

2.3. Cells

1. Eukaryotic protozoan parasite *L. tarentolæ* (Jena BioScience).
2. XL1-Blue chemically competent *E. coli* (NEB) or equivalent to generate the recombinant plasmid of interest.

2.4. Media

1. Parasites are grown in rich BHI media (Sigma). The powder is dissolved in deionized water (37 g/L), sterilized by filtration and stored at 4°C. Right before use, the media is supplemented with 0.5 % penicillin and streptomycin (PAA), 50 µg/ml G418-sulfate (PAA), 5 µg / ml Hemin (Sigma, stock solution at 0.25% in 50% triethanolamine, tube wrapped with foil to avoid light), 100 µg / ml nourseothricin (Jena BioScience) and 100 µg/ ml hygromycin (Euromedex). Media 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. Parasites also grow in Yeast Extract media containing 24 g/L of yeast extract (Sigma), 3 g/L glucose (Merck), 12.5 g/L K_2HPO_4 (Sigma) and 2.3 g/L KH_2PO_4 (Sigma), sterilized by filtration and stored at 4°C. Right before use, the media is supplemented with 1% fœtal bovine serum (FBS, Invitrogen), and the same supplements as for BHI media. Media is then stored at 4°C for a maximum of 15 days. Sterilization is achieved by filtration.
3. Bacteria are grown in 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 20 min at 120°C. Before use add 100 mg/L ampicillin. Store without antibiotics at room temperature.

2.5. Transfection

1. Reagent: Supplemented Nucleofector® solution (Basic Parasite Nucleofector Kit 1, Lonza).
2. Media: 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. The kits for small- (3–10 mg) and medium- (50–100 mg) scale plasmid (5–10 kbp) DNA preparations are NucleoSpin™ Plasmid from Macherey-Nagel.
3. Go Taq DNA polymerase used for polymerase chain reaction (PCR) was from Promega and NucleoSpin Extract II kit from Macherey-Nagel.
4. Other restriction and modifying enzymes were from New England Biolabs and Promega.

2.7. SDS-PAGE

1. Prepare stock solutions (room temperature) of separating buffer (1.5 M Tris-HCl, pH 8.8), stacking buffer (1 M Tris-HCl, pH 6.8), 10% SDS, acrylamide/bis (see Note 3) solution (40%, 37.5:1 with 2.6% C) and 10% ammonium persulphate (stored at 4°C up to 15 days). N,N,N,N-tetramethylethylenediamine (TEMED) is used pure. All products come from Bio-Rad.
2. 1x Running buffer: dilute Tris-glycine-SDS 10x (Euromedex), can be stored at room temperature.

3. Laemmli-type loading buffer “5xU”: (5x) 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).
4. Pre-stained molecular weight markers: Kaleidoscope markers (Bio-Rad).

2.8. Western-blotting

1. 1x Transfer buffer: dilute 10x 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. 1x Tris-buffered saline with tween and triton (TBS-TT): Dilute 10x 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 1/20000 diluted in blocking solution.
6. Enhanced chemiluminescent (ECL) reagents (GE healthcare) and Autoradiography films (Genesee Scientific) 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 Figure 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 post transcriptional 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 (Figure 1). Each construction was extracted from agarose gel using the kit NucleoSpinExtract II and used in the nucleofection of *L. tarentolæ*.

3.2. L. tarentolæ growth

1. Parasites are always grown at 26°C in the dark. Cells grow under the promastigote shape with a flagella allowing them to swim in the media.
2. Healthy cells tend to aggregate as cell density increases, forming larger aggregates at higher cell densities. Care should be taken not to dilute too much upon passages, as isolated cells do not divide well. Ideally, cells are amplified by dilution in fresh media when they are in the exponential phase, OD^{600} =1.4-2 (6-8x 10^7 cells/ml).
3. *L. tarentolæ* cells are maintained in BHI media in non-agitated T-25 flasks. Typically, a 50-fold dilution in 10 ml fresh BHI media is carried out on day 0, and cells reach a sufficient density for a 20-fold dilution on Day 5. A 100-fold dilution is the limit of good growth and should be kept occasional.
4. Parasites can be kept in culture for up to 3 months, after which a new frozen stock should be used.
5. As the number of passages increases, cells can reach higher cell densities.
6. Yeast extract media is used for scale-up. A culture in BHI media in exponential phase is diluted 20-fold in yeast-extract media (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. The exponential phase will be reached after 36 h. To reduce costs, nourseothricin is not added in large cultures, without incidence on protein expression.

7. Cultures can also be carried out in erlen-meyer flasks for volumes ranging from 50 to 500 ml, under agitation of 100-140 rpm. Higher cell densities can be obtained in agitated flasks compared to static cultures.

3.3. Cryo-conservation of L. tarentolæ

1. Cryo-conservation of parasites should be realized for a culture in exponential phase in BHI media.
2. Add sterile glycerol to the cells in BHI media, to a final concentration of 20%, and aliquot by 1.6 ml in sterile cryotubes.
3. Incubate 10 min. at room temperature, then transfer the tubes to a pre-cooled (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 media in a T-25 flask. Check that the parasites 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 10 fold for allowing cells to fully recover from the freezing, and proceed to normal dilution.

3.4. L. tarentolæ nucleofection

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

2. Spin cells 3 min, 2000xg 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 media in a ventilated flask. Incubate 24h as static suspension culture (Figure 2). Proceed with clonal selection.

3.5. Monoclonal selection of recombinant parasites

1. 24 hours after transfection, harvest 2 ml from the transfected 10 ml culture obtained by the electroporation protocol.
2. Pellet cells for 3 min at 2000xg 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 cover 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 hours 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 hours 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 parasites (NucleoSpin Tissue, Macherey-Nagel), the integration of genes of interest into the *L. tarentolæ* genome is verified by PCR using GoTaq polymerase (see Figure 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 cells transfection. Cells grown to an OD^{600} = 1.4 in a T-25 flask and protein expression is induced with 10 µg/mL tetracycline for 24 h. One milliliter of culture is harvested by centrifugation, suspended in 50 µl of 50 mM Hepes-NaOH pH 7.5 and cells are broken by 3 cycles of freeze/thaw in liquid-nitrogen/warm water. Fifteen microliters of broken cells are mixed with 5 µL of loading buffer 5xU, followed by analysis on SDS-PAGE and Western-blot (Figure 3).

1. Generate the separating gel (3.4 µL) of a 10% SDS-PAGE by mixing 1.92 µL of water, 1 µL 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 persulphate 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. Do not use organic solvent for this step as membrane proteins have a tendency to interact with it. Polymerization occurs in 30 min at room temperature (22°C).

2. Generate the 5% stacking gel by mixing 1.46 µL of water, 0.25 µL of 40% acrylamide bisacrylamide solution, 0.25 µL of 1.5 M Tris-HCl pH 6.8, 20 µL of 10% SDS, 20 µL 10% ammonium persulphate and 2 µL TEMED.
3. Load 20 µL sample onto the stacking gel and run for about 1.5 h at 120 V at room temperature.
4. After electrophoresis, transfer the protein from the SDS-PAGE to the nitrocellulose membrane as follows:
    a. Incubate the gel in 5 mL of cold transfer buffer for 5 min.
    b. Wet the nitrocellullose membrane for 5 min in the cold transfer buffer.
    c. 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.
    d. 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.
    e. After transfer, block the membrane for 1 h into 20 mL of TBS containing 0.5% blocking reagent and 0.1% Tween 20.
    f. Add the primary antibody to the solution and incubate for an additional 1 h.
    g. Wash three times with 20 mL of TBS-TT buffer.
    h. Wash once with 20 mL of TBS 1x buffer.
    i. 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

3.8.1. Optimization of culture media and time of expression

1. To evaluate the type of media and the influence of protein expression induction time, transfected *L. tarentolæ* parasites were grown in BHI or yeast extract media in a T-25 flask as described above.
2. When parasites reach $OD^{600} = 1.4$, add 10 µg/ml tetracycline to induce protein expression.
3. At 24, 48 and 72 hours, harvest an aliquot of cell culture and check for *lb*ABCG6 expression by SDS-PAGE and Western blot as described in 3.7.
4. 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 hours post-induction. This result permits the scale up of parasite culture, which will grant the quantities of protein required for structural studies.

3.8.2. Scale-up in fernbach flasks

To achieve the yields needed for protein production and purification for structural studies, larger cultures are necessary.

1. Grow parasites in a T-25 flask (10 ml), used to seed a pre-culture in a T-75 flask (30 ml), both in BHI media.
2. Once the pre-culture reached a suitable cell density, inoculate a 1 L baffled fernbach flask filled with 500 ml of yeast extract media, to a final $OD^{600} = 0.2$.
3. When parasites reach $OD^{600} = 1.4$, add 10 µg/ml tetracycline to induce protein expression.

4. Protein expression is continued for 48 hours, and cells reach an OD^{600} around 4. Harvest the cells by centrifugation 10 min at 7500xg, 4°C. Take the pellet in 50 ml buffer (50 mM Hepes-NaOH pH 7.5, 200 mM NaCl) per liter of culture and break the cells by 2 passages at 15,000 psi on a microfluidizer M-110P (Microfluidics IDEX CORPORATION). Centrifuge 30 min at 15,000xg, 4°C, discard the pellet and centrifuge the supernatant 1 h at 180,000xg, 4°C, to collect the membranes. Routinely, 1 L of cell culture yields 0.5 g of dry membrane.

3.8.3. *Effect of ΔN truncation*

As observed in Fig. 3c, the first 30 residues of the protein are not very relevant in terms of expression. There are no remarkable differences in expression levels between *lb*ABCG6ΔN and *lb*ABCG6 constructs when analyzing membrane samples both by western blot and coommasie gel. Differences shown in Fig. 3a-b, using whole cells, might be due to an artefact in western blot.

4. Conclusion

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

5. Notes

1. *Leishmania tarentolœ* 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-polymerised, thus handle with gloves.
4. Methanol is neurotoxic.

References

1. Robey RW, Honjo Y, Morisaki K, Nadjem TA, Runge S, Risbood M, Poruchynsky MS, Bates SE (2003) Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. Br J Cancer 89: 1971-1978
2. Nakanishi T, Doyle LA, Hassel B, Wei Y, Bauer KS, Wu S, Pumplin DW, Fang HB, Ross DD (2003) Functional characterization of human breast cancer resistance protein (BCRP, ABCG2) expressed in the oocytes of *Xenopus laevis*. Mol Pharmacol 64: 1452-1462.
3. Béjà O, Bibi E (1996) Functional expression of mouse Mdr1 in an outer membrane permeability mutant of *Escherichia coli*. Proc Natl Acad Sci U S A 93:5969-74.
4. Trometer C, Falson P (2010) Mammalian membrane protein expression in baculovirus-infected insect cells. Methods Mol Biol 601:105-17.
5. Sarkadi B, Price EM, Boucher RC, Germann UA and Scarborough GA (1992) Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. J Biol Chem 267: 4854-4858.

6. Mao Q, Conseil G, Gupta A, Cole SP, Unadkat JD (2004) Functional expression of the human breast cancer resistance protein in *Pichia pastoris*. Biochem Biophys Res Commun 320: 730-7.
7. Janvilisri T, Venter H, Shahi S, Reuter G, Balakrishnan L, van Veen HW (2003) Sterol transport by the human breast cancer resistance protein (ABCG2) expressed in *Lactococcus lactis*. J Biol Chem 278: 20645-20651.
8. Pozza A, Perez-Victoria JM, Di Pietro A (2010) Insect cell versus bacterial overexpressed membrane proteins: an example, the human ABCG2 transporter. Methods Mol Biol 654:47-75.
9. Ozvegy C, Varadi A, Sarkadi B (2002) Characterization of drug transport, ATP hydrolysis, and nucleotide trapping by the human ABCG2 multidrug transporter. Modulation of substrate specificity by a point mutation. J Biol Chem 277: 47980-47990.
10. Telbisz A, Müller M, Ozvegy-Laczka C, Homolya L, Szente L, Váradi A, Sarkadi B (2007) Membrane cholesterol selectively modulates the activity of the human ABCG2 multidrug transporter. Biochim Biophys Acta 1768:2698-713.
11. Lerner-Marmarosh N, Gimi K, Urbatsch IL, Gros P, Senior AE (1999) Large scale purification of detergent-soluble P-glycoprotein from *Pichia pastoris* cells and characterization of nucleotide binding properties of wild-type, Walker A, and Walker B mutant proteins. J Biol Chem 274: 34711–34718.
12. Cai J, Daoud R, Georges E, Gros P (2001) Functional expression of multidrug resistance protein 1 in *Pichia pastoris*. Biochemistry 40: 8307–8316.
13. Niimi T (2012) Recombinant protein production in the eukaryotic protozoan parasite *Leishmania tarentolae*: a review. Methods Mol Biol 824:307-15.

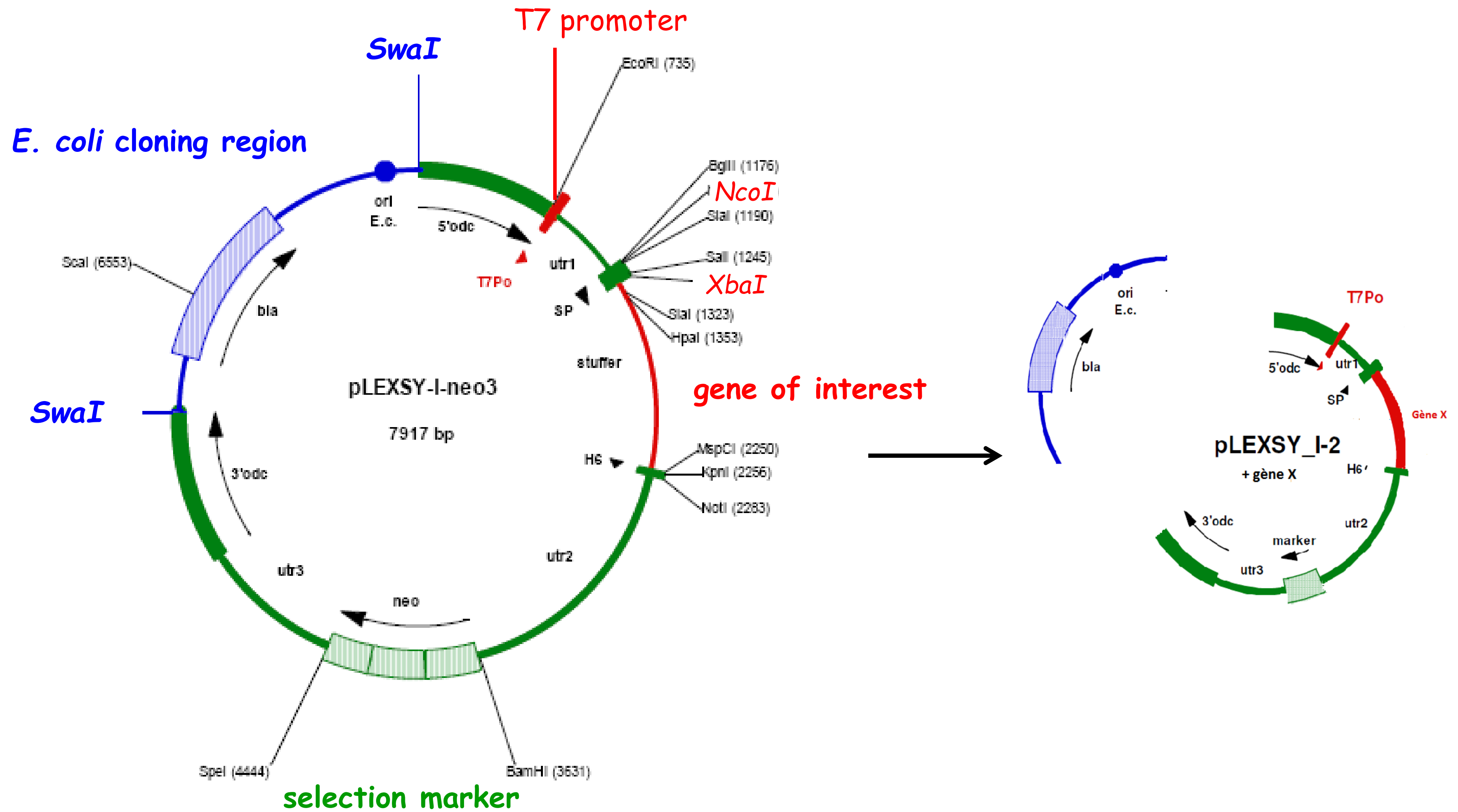
14. Kushnir S, Gase K, Breitling R, Alexandrov K (2005) Development of an inducible protein expression system based on the protozoan host *Leishmania tarentolæ*. Protein Expr Purif 42:37-46.
15. Breitling R, Klingner S, Callewaert N, Pietrucha R, Geyer A, Ehrlich G, Hartung R, Müller A, Contreras R, Beverley SM, Alexandrov K (2002) Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. Prot Expr Purific 25: 209-218.
16. Bolhassani A, Taheri T, Taslimi Y, Zamanilui S, Zahedifard F, Seyed N, Torkashvand F, Vaziri B, Rafati S (2011) Fluorescent *Leishmania* species: development of stable GFP expression and its application for in vitro and in vivo studies. Exp Parasitol 127:637-45.
17. Castanys-Muñoz E, Pérez-Victoria JM, Gamarro F, Castanys S (2008) Characterization of an ABCG-like transporter from the protozoan parasite Leishmania with a role in drug resistance and transbilayer lipid movement. Antimicrob Agents Chemother 52:3573-9.
18. Litman T, Brangi M, Hudson E, Fetsch P, Abati A, Ross DD, Miyake K, Resau JH, Bates SE (2000) The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J Cell Sci 113:2011-21.
19. Liu XL, Tee HW, Go ML (2008) Functionalized chalcones as selective inhibitors of P-glycoprotein and breast cancer resistance protein. Bioorg Med Chem 16:171-80.
20. Lenoir G, Menguy T, Corre F, Montigny C, Pedersen PA, Thinès D, le Maire M, Falson P (2002) Overproduction in yeast and rapid and efficient purification of the rabbit SERCA1a Ca(2+)-ATPase. Biochim Biophys Acta 1560:67-83.
21. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York

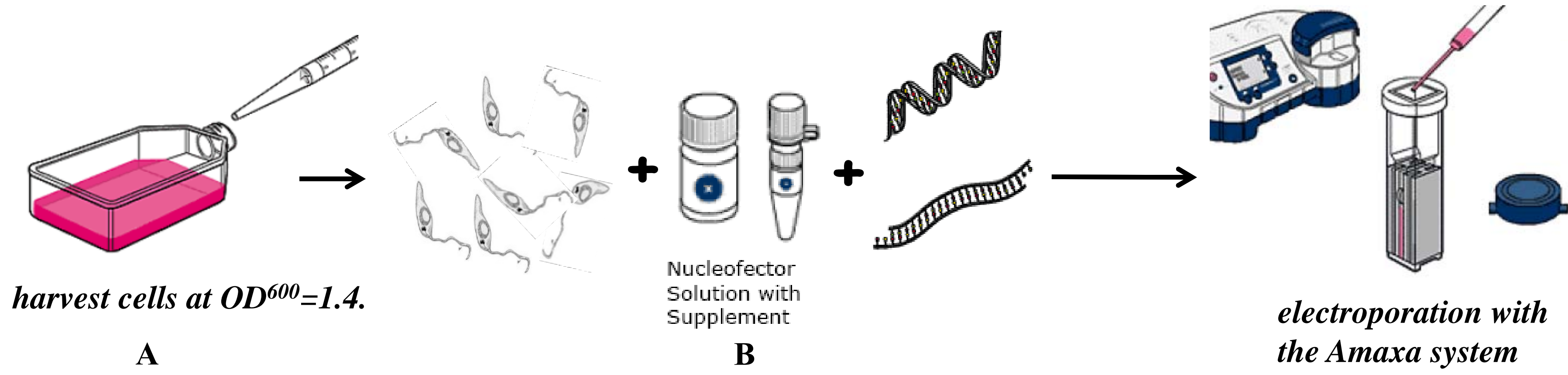
Fig. 1. Cloning strategy of *lb*ABCG6 based on pLEXSY system. *lb*ABCG6 was cloned between the *Nco*I and *Xba*I restriction sites inside the multiple cloning site. Then, *E.coli* fragment was removed by *Swa*I digestion.

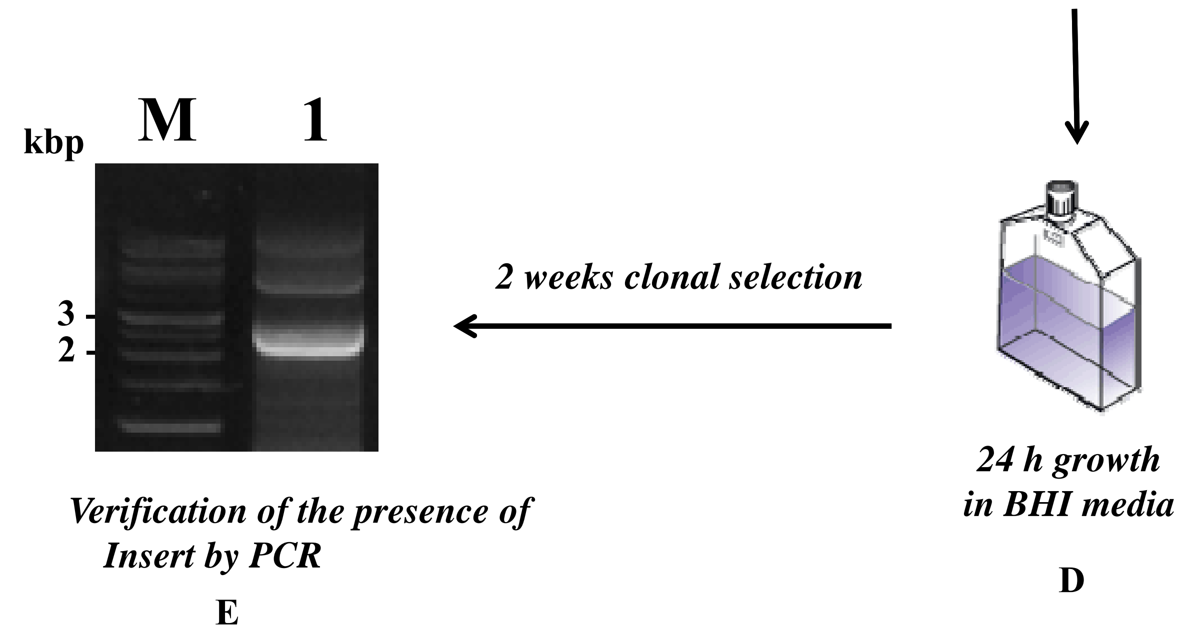
Fig. 2. Protein expression based on pLEXSY system. (**A**) Parasites are harvested and diluted to 6.10^{7} cells/ml and OD^{600} ~1.4. (**B**) After spinning, parasites are suspended in 100 µl of supplemented Nucleofector solution and mixed with 4 µg DNA. (**C**) All the mix is transferred to an electroporation cuvette and electroporated according to the Basic Parasite Nucleofector Kit 1 (Lonza), using the program U-033. (**D**) Electroporated parasites are transferred to BHI media in a ventilated flask. After 24 hours incubation, proceed with clonal selection. (**E**) Genomic DNA is extracted from parasites and integration of *lb*ABCG6 is verified by PCR. *Lane M* molecular weight marker, *line 1* genomic DNA from *lb*ABCG6 transfected parasites giving a PCR product of 2300 pb.

Fig. 3. Expression of *lb*ABCG6 (**A**) and *lb*ABCG6ΔN (**B**) in *L. tarentolæ* parasites as a function of time and culture media, under agitation. Expression of *lb*ABCG6/ *lb*ABCG6ΔN is carried out as described in *section 3.5*. Transfected parasites were maintained under antibiotic selection for 2 weeks in BHI and Yeast extract media. Then, protein expression is induced by adding 10 µg/ml tetracycline and parasites harvested after 24, 48 and 72 hours. *lb*ABCG6/ *lb*ABCG6ΔN expression was analyzed by SDS-PAGE and Western blot loading 15µl of samples, normalized to OD^{600} ~1.4, onto a 10% SDS-PAGE. (**c**) Membrane expression of *lb*ABCG6 and *lb*ABCG6ΔN in *L. tarentolæ* parasites. Expression and membrane preparation of

*lb*ABCG6/ *lb*ABCG6ΔN is carried out as described in *section 3.8.3*. 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.


T7 promoter
SwaI
EcoRI (735)
E. coli cloning region
BglII (1176)
NcoI
SlaI (1190)
SalI (1245)
XbaI
SlaI (1323)
HpaI (1353)
ori
E.c.
5'odc
T7Po
utr1
SP
ScaI (6553)
bla
stuffer
pLEXSY-I-neo3
7917 bp
gene of interest
SwaI
H6
MspCI (2250)
KpnI (2256)
NotI (2283)
3'odc
utr2
utr3
neo
SpeI (4444)
BamHI (3631)
selection marker
ori
E.c.
bla
T7Po
5'odc
utr1
SP
Gène X
pLEXSY_I-2
+ gène X
H6
3'odc
utr2
marker
utr3


harvest cells at OD600=1.4.
A
Nucleofector
Solution with
Supplement
B
electroporation with
the Amaxa system


kbp
M
1
3
2
2 weeks clonal selection
Verification of the presence of
Insert by PCR
E
24 h growth
in BHI media
D

The author has requested enhancement of the downloaded file. All in-text references underlined in blue are linked to publications on ResearchGate.