

Rapid protein production pipeline in advanced inducible *Leishmania tarentolae* expression system

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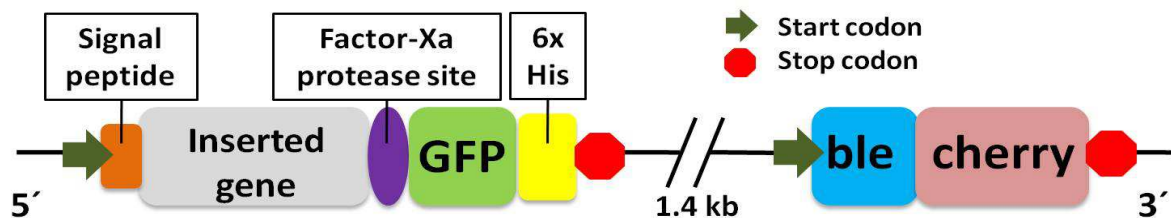
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Objectives: *Leishmania tarentolae* is a trypanosomatid protozoan host isolated from Moorish gecko *Tarentola mauritanica* [1], non-pathogenic to mammals, and can be used as a protein expression system. Proteins expressed by *Leishmania tarentolae* possess post-translation modifications close to mammalian-type, such as glycosylation, phosphorylation or prenylation. Moreover, these protozoa need similar handling as bacterial expression systems.

Aim of the study was to establish rapid pipeline for expression and purification of recombinant proteins from *Leishmania tarentolae* expression system.

Material and methods: Expression vector pLEXSY_I-blecherry3 (Jena Bioscience, Germany) was modified as depicted in Figure 1. The first, GFP fusion tag was inserted into the 3'-terminus of open-reading-frame (ORF) in the expression cassette. The second, to allow easy purification of overexpressed protein, polyhistidine tag was incorporated between GFP and stop codon. The third, factor-Xa protease site was inserted between protein of interest (ORF) and GFP tag. Modified plasmid was named as LBMI-LEXSY-GFP. Other features of this plasmid are discussed in the legend of Figure 1.

Figure 1. LBMI-LEXSY-GFP vector



Vector contains two major expression cassettes: 1. for expression of target protein, 2. co-expression of bleomycin resistance marker fused with cherry fluorescence protein to monitor protein expression in the day light). In many cases co-expression of mCherry protein does not assure expression of protein of interest in the first expression cassette. To overcome this problem GFP fusion tag was inserted in the first cassette. Signal peptide ensures extracellular transport of overexpressed protein; factor-Xa site can be used to get only protein of interest, cleaved from rest of the GFP-polyhistidine tag, for downstream applications like crystallography.

LBMI-LEXY-GFP vector was tested for overexpression of TNFR-Cys2 domain of CD40. In short, PCR amplified DNA of CD40 TNFR-Cys2 was ligated into LBMI-LEXSY-GFP and transformed into *E. coli* DH-5- α . Transformed colonies were selected on LB agar plates containing 50 μ g/ml carbenicillin. Plasmid DNA was isolated from overnight culture (16 hours at 30°C at 250 rpm shaking) of the single clone containing recombinant plasmid with the help of GenElute™ Plasmid Midi-Prep Kit (Sigma-Aldrich, Germany). Plasmid was linearized with SmaI enzyme digestion (1 hour at 37°C) for homologous recombination of plasmid DNA into *Leishmania* chromosome (linearized fragment is presented in Figure 1). Linearized vector was electrophoretically separated on a 0.7% agarose gel and extracted using QIAquick Gel Extraction Kit (QIAGEN, Germany).

The LEXSY T7-TR strain (Jena Biosciences, Germany) of *Leishmania tarentolae* was cultivated as a static suspension in the dark at 26°C in BHI medium supplemented with hemin at final concentration 5 µg/ml (essential for *Leishmania*), nourseothricin and hygromycin at final concentration 100 µg/ml each (for maintaining T7 polymerase and TET repressor genes in the host genome), and with penicillin and streptomycin (to prevent bacterial infections). Cells in mid-log phase were centrifuged for 3 min at 2000 x g at room temperature and a half of the medium was removed and cells were resuspended in remaining medium to get a suspension 10⁸ cells/ml. Cells were chilled on wet ice for 10 min and mixed with linearized DNA. Electroporation was performed using Gene Pulser MXcell™ (Bio Rad, USA) set at 450 V and 450 µF in pre-chilled 2 mm gap electroporation cuvette resulting in a ~6 ms long pulse. Cuvette was put back on ice for 10 min and subsequently the cells were transferred to a fresh BHI medium with antibiotics and cultivated in the dark at 26°C overnight. To select recombinant *Leishmania*, bleomycin at final concentration 100 µg/ml was added and incubation was continued at 26°C for another 5 days. Recombinant *Leishmania* were passaged further and expression of TNFR-Cys2 GFP fused protein was induced with tetracycline at the final concentration 10 µg/ml. The expression of GFP fused protein and co-expressed mCherry protein was checked under microscope at 488nm and 590 nm, respectively, at 24 and 48 hours after induction (Figure 2). After full protein expression (48 hours) cells were centrifuged for 3 min at 2000 x g at room temperature. The supernatant was collected and used for direct protein purification. In short, 20 µl of Talon metal affinity resin (Clontech) was washed twice with native wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole). To the resin 100 µl of BHI medium supernatant obtained from overexpression of protein and 900 µl of wash buffer was added. Binding of tagged protein to the resin was allowed for 10 min at 4°C. Resin was then centrifuged at 10000 x g for 1 min and washed two times with wash buffer. Small amount (1 µl) of affinity resin was placed on glass slide and presence of GFP fused protein bound on agarose beads was checked under microscope at 488nm (Figure 3). Protein bound on agarose beads was eluted with 20 µl of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 200 mM imidazole). Elutions were desalted with ZipTip_{C4} pipette tips (Millipore, MA, USA) according to manufacturer's instructions and protein was identified with MALDI-TOF analysis (Microflex, Bruker-Daltonics, Germany) by mixing 1 µl of elute with 1 µl of sDHB matrix. Mass spectra were recorded in the linear, positive mode at a laser frequency of 60 Hz (250 shots total, Figure 4). The spectra were calibrated using the protein Standard II (20.000 to 70.0000 kDa range) from Bruker-Daltonics.

Results and discussion: The successful transformation, selection and expression of target protein in *Leishmania* were confirmed directly in living cells as the green and red fluorescence under a fluorescent microscope (Figure 2). The presence of secreted GFP fused CD40 TNFR-Cys2 protein in cultivation medium was detected upon green fluorescence of silica beads (Figure 3). Finally, the result of MALDI-TOF analysis showed the present of a ~36 kDa protein (Figure 4), which corresponds to the mass of CD40 TNFR-Cys2 fused with GFP.

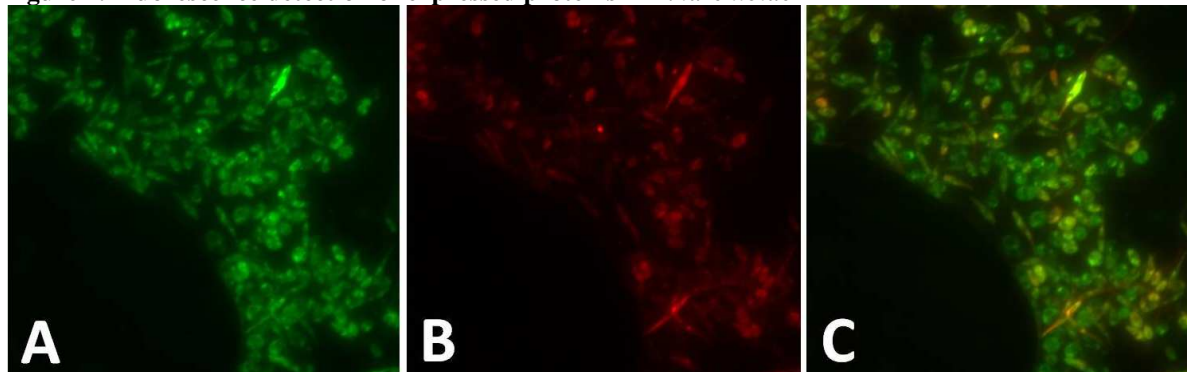
In summary, a rapid pipeline for overexpression of protein with LBMI-LEXY-GFP vector in *L. tarentolae* established in the present work has following advantages:

1. GFP fusion tag allowed easy and rapid assessment of the level of expression during the induction of target protein directly in living cells.
2. Polyhistidine tag served to capture and detect target protein, in its native state, directly from BHI medium (within 10 min), while GFP tag allowed detection of protein directly on agarose beads (less than a minute). Detection of target protein with classical methods like precipitation with TCA followed by SDS-PAGE separation and western blotting needs at least 2 days.

3. Capture of the tagged protein with metal affinity beads directly in BHI medium can be up-scaled to purify protein of interest in its native state. This is one of the major advantages against precipitation techniques, as many proteins may denature and lose their function. Proteins purified with metal affinity directly in BHI medium can be the fastest and safest method for downstream applications like in protein:protein interaction assays or study of co-enzyme activities.

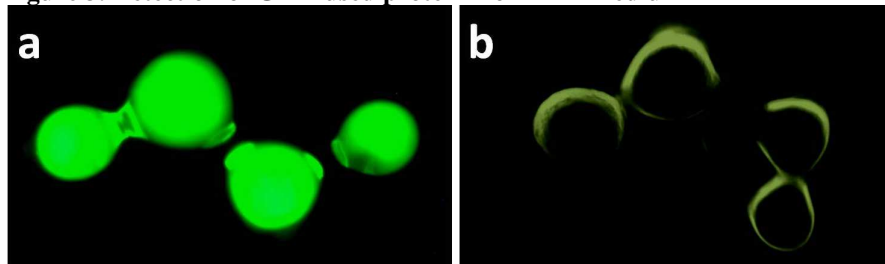
4. Factor-Xa cleavage site allows separation of protein of interest from GFP and His tags. Such cleaved proteins are the most suitable for downstream applications like crystallography.

Figure 2. Fluorescence detection of expressed proteins in *L. tarentolae*



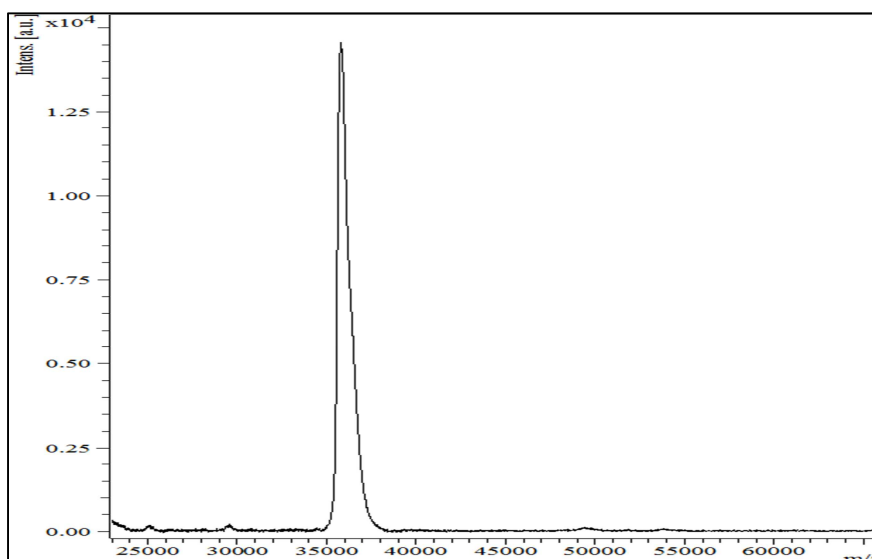
A) green fluorescence of the fused GFP protein at 488 nm; B) red fluorescence of the co-expressed mCherry protein at 590 nm; C) merge of GFP and mCherry fluorescence.

Figure 3. Detection of GFP fused protein from BHI medium



Metal affinity agarose beads with GFP fused CD40 TNFR-Cys2 protein (a), agarose beads without GFP fusion protein (b).

Figure 4. MALDI-TOF MS spectrum of purified GFP fused CD40 TNFR-Cys2 protein (~36 kDa)



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References:

1. Wallbanks, K.R. et al. (1985): The identity of *Leishmania tarentolae* Wenyon 1921. Parasitology 90, 67–78