Chapter 8

Production of Recombinant *Trypanosoma cruzi* Antigens in *Leishmania tarentolae*

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Abstract

Trypanosomatids are unicellular organisms that colonize a wide diversity of environments and hosts. For instance, *Trypanosoma cruzi* is a human pathogen responsible for Chagas diseases, while *Leishmania tarentolae* infects amphibians and became a biotechnological tool suitable for recombinant protein expression. *T. cruzi* antigens are needed for the development of improved epitope-based methods for diagnosis and treatment of Chagas disease. Molecular cloning for the production of recombinant proteins offers the possibility to obtain *T. cruzi* antigens at high yield and purity. *L. tarentolae* appears as the ideal expression host to obtain recombinant *T. cruzi* antigens with a structure and posttranslational modifications typical of trypanosomatids. In this chapter, we present a protocol for the analytical to mid-scale production of recombinant *T. cruzi* antigens, using *L. tarentolae* as expression host (LEXSY® inducible system).

Key words *Trypanosoma cruzi*, Recombinant antigen, Eukaryotic expression system, *Leishmania tarentolae*

1 Introduction

Trypanosoma cruzi antigens play important roles as molecules that elicit host immune response as well as for the recognition and invasion of mammalian cells [1]. Hence, either entire antigenic proteins or short peptides are considered attractive targets for the development of new and improved epitope-based methods for diagnosis and treatment of Chagas disease [2]. As there is not a single immunological test considered the universal gold standard [3], the availability of complementary serological tests for Chagas disease may improve the diagnosis of infected patients. This is especially useful for patients with discordant diagnosis due to mixed parasitic infections or those coursing different phases of the disease [4–6]. In addition, several antigen-based immunogens are being studied as candidates in vaccine formulations, either for treatment [7–10] or disease prevention [11].

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Antigens can be (1) extracted and purified from the same organism of interest or (2) expressed in recombinant form in homologous, if available, or heterologous expression systems. Although the first strategy allows to obtain native material displaying high antigenicity and immunogenicity [12, 13], its main disadvantages are low yield, many purification steps, cross-reactivity, high production/purification costs, and biosafety issues. Among other factors, the production yield depends to a great extent on the relative abundance of the antigen in the cell, which in most cases is very low. Purification is usually tedious as it relies on multiple steps determined by the physicochemical properties of the antigen, requiring in some cases costly affinity-based methods, such as purification on a matrix coupled with an antigen-specific antibody. The need to supplement the culture medium with animal serum to support the growth of *T. cruzi* parasites also contributes to increase considerably the production costs. The production process becomes restrictive from the economic and biosafety point of view if the antigen is only expressed in the highly infective trypomastigote or amastigote forms of the parasite [14], as these will require the use of mammalian cell cultures and additional purification steps to separate parasites from host cells or proteins.

Molecular cloning for the production of recombinant proteins offers the possibility to obtain *T. cruzi* antigens at higher yields while avoiding the manipulation of pathogenic cultures. Furthermore, this methodology allows the design of chimeric proteins composed of several in tandem epitopes fused to affinity tags for standardized purification procedures [15]. Different organisms for cell-based expression of recombinant proteins are currently available. Systems based on prokaryotes, such as *Escherichia coli*, are probably the first choice to produce a recombinant antigen due to its relative low cost and the high biomass achieved [16, 17]. However, prokaryotic systems are not suitable for producing recombinant antigens whose immunogenicity depends almost exclusively on specific posttranslational modifications or folding [18]. Eukaryotic systems based on the use of yeast or plants as expression hosts can partially overcome this limitation, because they add posttranslational modifications to the recombinant antigen and their culture is still as affordable as *E. coli* [19]. However, the hyper modification of proteins with more complex branching sugar moieties makes yeast-based platforms unsuitable when such glycosylation pattern defines the antigenicity, a major requirement to elicit a specific immune response [20, 21]. It is worth mentioning that plant-based systems, on the other hand, need the setup of a laboratory greenhouse and similar infrastructure, which is time-consuming for labs without expertise. Recombinant antigen production in insect (baculovirus systems) or mammalian cells offers a wide range of posttranslational modifications and mechanisms of protein folding [22–24]. However, the low yields and high costs associated with

the use of these systems make them unsuitable and unaffordable for mid- to large-scale antigen production.

From a biochemical point of view, nonpathogenic trypanosomatids are convenient eukaryotic hosts for the expression of recombinant *T. cruzi* antigens. Parasites from the genus *Phytomonas* and *Crithidia fasciculata*, which exclusively infect plants and invertebrates, respectively, had been proposed as expression hosts [25, 26]. In these systems, expression of foreign proteins occurs constitutively and relies on the activity of the endogenous RNA polymerases [27]. In the past decade, an inducible expression system based on *Leishmania tarentolae*, a trypanosomatid that infects amphibians, was developed and commercialized by Jena Bioscience (LEXSY®). This system consists of (1) an inducible expression vector (pLEXSY) where the expression of the gene of interest is under the control of a bacteriophage T7 promoter carrying a tetracycline (TET) operator and (2) a genetically modified *L. tarentolae* strain (named T7-TR) that constitutively expresses the bacteriophage T7 RNA polymerase (T7 RNA pol), which transcribes the target coding sequence, and a TET repressor protein (TR) that, by binding to the TET operator of the expression vector, inhibits gene transcription (Fig. 1). Thus, the expression of the recombinant protein in the *L. tarentolae* T7-TR/pLEXSY system is induced by the addition of tetracycline to the growth medium, which binds to and inactivates the TR repressor allowing gene transcription by T7 RNA pol [28]. pLEXSY vectors allow for the expression of target proteins either intracellularly or fused to a signal peptide for their secretion to the extracellular medium, simply by selecting the appropriate cloning site. Secreted recombinant proteins can be purified and/or concentrated from the culture medium [29].

The expression systems relying on nonpathogenic trypanosomatids are safe for manipulation due to the inability of the microorganisms to establish infection in mammals (biosafety level 1). Notably, cultivation of *L. tarentolae* is as easy to handle as that of *E. coli*, is significantly cheap compared to that of higher eukaryote systems, and reaches high densities without the supplementation with animal sera. Not less important, *L. tarentolae*'s gene expression machinery allows correct folding, processing, and posttranslational modification of most recombinantly expressed proteins of higher organisms, impacting positively in their functionality and antigenicity [30]. A number of proteins, whose production in other systems (bacteria, yeasts, insect cells) has been unsatisfactory due to poor yields, poor secretion, and/or improper folding (disulfide bond formation), have been produced in *L. tarentolae* at high yields as fully active molecules [31, 32]. *L. tarentolae* has been used to produce recombinant O- and N-glycosylated proteins [30]. Further, *L. tarentolae* has been shown to produce higher-eukaryote-like biantennary *N*-glycans [33] in contrast to most alternative

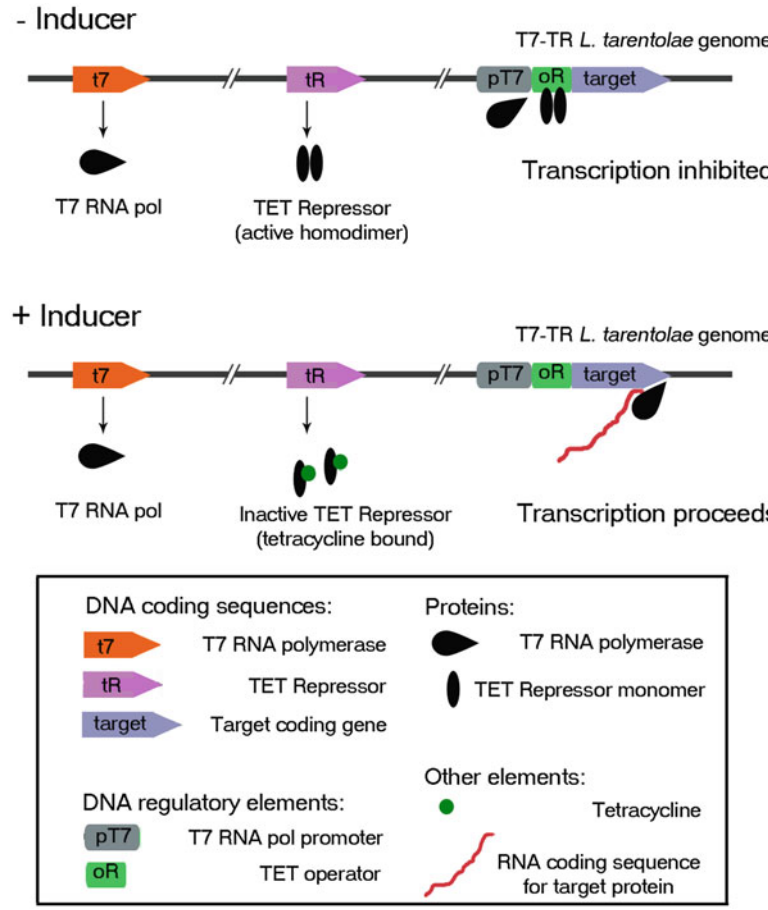

Fig. 1 Schematic representation of the *L. tarentolae* T7-TR/pLEXSY inducible expression system used in this work. After transfection, the recombinant pLEXSY expression vector containing the "pT7," "oR," and "target" elements (and also the resistance marker, not shown) integrates into the T7-TR *L. tarentolae* genome by homologous recombination. In the absence of tetracycline (− inducer), transcription of the target gene (*T. cruzi* coding sequence) is inhibited because the binding of the TET repressor protein (TR) to the TET operator prevents the association of T7 RNA polymerase with the T7 promoter. Tetracycline (+ inducer) avoids the formation of functional homodimers of the TET repressor. Inactive, tetracycline-bound TR monomers are unable to interact with the TET operator. Thus, T7 RNA pol can associate with the T7 promoter, allowing target gene transcription

eukaryotic expression hosts (yeast, insect cells). For the production of *T. cruzi* antigens, *L. tarentolae* appears as the ideal expression host to obtain recombinant proteins with a structure and posttranslational modifications identical to the native ones.

In this chapter, we provide a protocol for the expression of *T. cruzi* antigens using a version of the tetracycline-inducible commercial LEXSY system (https://www.jenabioscience.com/images/

ae3a4f50f1/EGE-1410.pdf). We describe how to grow *L. tarentolae* at the same temperature as *T. cruzi* epimastigotes and the use of an alternative, less expensive antibiotic for the selection of transgenic parasites. Moreover, a detailed protocol for the purification of recombinant antigens from the culture medium in their native form is provided. The protocol presented here proved to be suitable for the production of recombinant *T. cruzi* antigens in an analytical to mid-scale setting.

2 Materials

Use ultrapure deionized water and analytical grade reagents.

2.1 Reagents, Solutions, and Media

1. 5 mg/mL hemin sterile stock solution in 0.2 M NaOH.
2. 10 mg/mL tetracycline sterile stock solution in 70% ethanol.
3. 100 mg/mL zeocin sterile stock solution in 1 M HEPES buffer, pH 7.2.
4. 50 mg/mL ampicillin sterile stock solution in water.
5. Luria-Bertani (LB) broth (per liter): 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, water to 950 mL. Adjust pH to 7.5 and complete to 1 L with water. Sterilize by autoclaving.
6. LB agar: LB broth with the addition of 15 g/L agar. Sterilize by autoclaving.
7. Complete BHI: 37 g/L brain heart infusion sterile media, supplemented with 5 μg/mL hemin, 10 U/mL penicillin, 10 mg/L streptomycin, 100 μg/mL hygromycin, 100 μg/mL nourseothricin.
8. Nickel-nitrilotriacetic acid (Ni-NTA) coupled to agarose resin.
9. Lysis buffer: 50 mM NaH_2PO_4, 250 mM NaCl, 10 mM imidazole, pH 8.0.
10. Wash buffer: 50 mM NaH_2PO_4, 250 mM NaCl, 20 mM imidazole pH 8.0.
11. Elution buffer: 50 mM NaH_2PO_4, 250 mM NaCl, 250 mM imidazole pH 8.0.

2.2 Plasmids

Figure 2 shows the main characteristics for:

1. Cloning vector, suitable for the amplification of DNA sequences in *Escherichia coli* (*see* **Note 1**).
2. pLEXSY_I-ble3 expression vector (Jena Bioscience, Germany).

2.3 Bacteria

1. *E. coli* strain for amplification of the recombinant cloning vector (*see* **Note 2**).

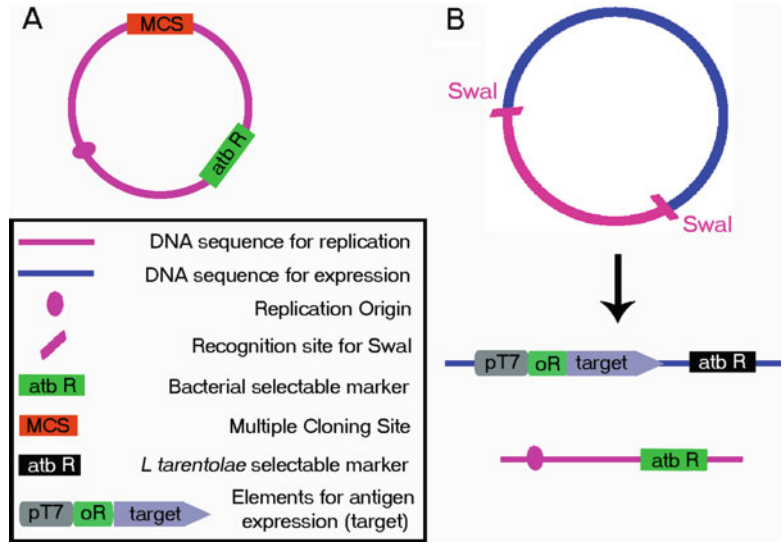

Fig. 2 (**a**) Cloning vector to amplify the *T. cruzi* antigen coding sequences in *E. coli*. The multiple cloning site (MCS) includes at least one recognition site for a restriction enzyme which leaves blunt ends. For replication and maintenance in *E. coli*, the cloning vector bears a bacterial origin of replication and a drug resistance gene (selectable marker), which confers antibiotic resistance (atbR). (**b**) Expression vector to produce recombinant *T. cruzi* antigens in the *L. tarentolae* T7-TR/pLEXSY system. The pLEXSY vector is organized in two major fragments (flanked by SwaI restriction sites): (i) one containing the elements for amplification in *E. coli* (blue line), such as an origin of replication and a selectable marker, and (ii) the other containing the elements for the inducible expression of the recombinant protein (T7 promoter, pT7; TET operator, oR) and homologous recombination into the T7-TR *L. tarentolae* genome (pink line). This fragment also harbors a selection marker for antibiotic resistance (atbR) in *L. tarentolae* and the untranslated regions to fully process the DNA coding sequences into mature mRNAs (not shown) [28]. Vector linearization with *SwaI* separates both fragments and exposes the DNA sequences that will guide the integration into the *L. tarentolae* genome

2. *E. coli* strain for amplification of the recombinant pLEXSY_I-ble3 vector (*see* **Note 3**).

2.4 Parasites

Leishmania tarentolae strain T7-TR (Jena Bioscience).

2.5 Specific Equipment for Parasite Culturing

1. Static incubator device set at 28 °C.
2. Gene Pulser II Electroporator with Pulse Controller II and Capacitance Extender Plus (Bio-Rad, USA).

3 Methods

3.1 Primer Design and T. cruzi Antigen Sequence Amplification in Cloning Vectors

This protocol describes the steps to clone and amplify the *T. cruzi* antigen coding sequence in an *E. coli* vector, prior cloning into the *L. tarentolae* expression vector (*see* **Note 4**).

1. Design forward and reverse primers containing the restriction sites at the 5′and 3′ ends for amplification of the *T. cruzi* coding sequence, which upon insertion in the *L. tarentolae* expression plasmid allows the in-frame fusion with the signal peptide for secretion into the culture medium and the hexa-histidine tag for affinity purification (*see* **Notes 5** and **6**).
2. Check that the sequence of interest does not contain a *Swa*I restriction site, which is used for plasmid linearization prior transfection to *L. tarentolae* or for the restriction enzymes to be used for cloning into pLEXSY_I-ble3 (see **Note 7**).
3. Amplify the intronless coding sequence of *T. cruzi* antigen from genomic DNA by PCR, using a *Pfu* DNA polymerase which leaves blunt ends (*see* **Note 8**).
4. Purify the amplicon (PCR product of the sequence coding for the *T. cruzi* antigen) with a commercial kit for DNA gel extraction.
5. Cut the cloning vector with a restriction enzyme that leaves blunt ends. Run the digestion reaction on an agarose gel, and purify the digested vector using a commercial kit for DNA gel extraction.
6. Set up DNA ligation reactions with the amplicon and the digested cloning vector optimizing the insert to vector molar ratio. Add T4 DNA ligase and incubate reactions overnight at 16 °C. Transform competent *E. coli*, and select recombinant clones plating cells on LB agar Petri dishes supplemented with the selection antibiotic (conferred by the drug resistance gene present in the cloning vector).
7. Perform the screening on several transformed *E. coli* colonies by mini-preparations of plasmid DNA and subsequent analysis of restriction digestion and double-strand sequencing (using primers annealing on each side of the insert in the cloning vector).

3.2 Cloning of T. cruzi Antigen Sequences in the L. tarentolae Expression Vector

1. Choose one of the sequence-checked DNA minipreps and digest the recombinant vector containing the coding sequence of *T. cruzi* antigen with the restriction enzymes that have been previously chosen for directional cloning into *L. tarentolae* plasmid pLEXSY_I-ble3.
2. After reaction completion, run the digestion products on an agarose gel and purify the released fragment (corresponding to

the coding sequence of *T. cruzi* antigen) using a commercial kit for DNA gel extraction.

3. On parallel, digest and purify the plasmid pLEXSY_I-ble3 with the same restriction enzymes, as in **steps 1** and **2**.
4. Set up DNA ligation reactions with both the digested *T. cruzi* antigen coding sequence and the *L. tarentolae* expression plasmid optimizing the insert to vector molar ratio. Include a ligation reaction lacking the digested *T. cruzi* antigen coding sequence to test the efficiency of the digestions (undigested plasmid and self-ligation of plasmid digested with only one enzyme). Add T4 DNA Ligase and incubate reactions overnight at 16 °C.
5. Use 5–10 μL of the different ligation reactions to transform *E. coli* competent cells that tolerate eukaryotic DNA.
6. After DNA transformation, recover the transformed *E. coli* cells adding 600 μL of LB medium without antibiotics and incubate for 45 min at 30 °C with shaking (*see* **Note 9**).
7. Select the recombinant clones by plating 100 μL of recovered *E. coli* cells on LB agar Petri dishes supplemented with the selection antibiotic ampicillin (50 μg/mL). Incubate plates at 30 °C for 24–30 h (*see* **Note 10**).
8. Pick 5–10 single colonies from the vector-insert plate, and inoculate them in liquid LB medium supplemented with 50 μg/mL ampicillin. Let them grow for 16 h with shaking at 30 °C, centrifuge cultures at 4000 × *g* for 5 min, and perform mini-preparations of DNA from each *E. coli* pellet.
9. Run PCR reactions of the DNA minipreps using forward and reverse primers complementary to the *L. tarentolae* expression plasmid (5′CCGACTGCAACAAGGTGTAG 3′) and to the *T. cruzi* coding sequence, respectively. Confirm the identity of the cloned fragment by double-strand DNA sequencing with the two oligonucleotides indicated above.
10. Pick one of the *E. coli* colonies whose plasmid resulted positive in the PCR screening described above, and inoculate a culture flask containing 50 mL of LB medium supplemented with 50 μg/mL ampicillin. Grow the culture overnight at 30 °C with shaking. Pellet cells, and perform midi preparations of the recombinant pLEXSY_I-ble3, using a commercial midiprep kit to obtain a high quality and quantity of plasmidic DNA, suitable for transfection of eukaryotic cells (*see* **Note 11**).

3.3 Transfection and Selection of Recombinant L. tarentolae

Growth of T7-TR *L. tarentolae* is carried out at 28 °C as static culture in the dark. Plastic tissue culture or glass conical Erlenmeyer sterile flasks with volume capacity ranging from 5 mL to 2 L are suitable for culturing this strain. *L. tarentolae* cells must be maintained as a fresh culture with continuous 1:30 dilution every 3 days

in complete BHI medium (*see* **Note 12**). On the day of transfection with recombinant pLEXSY_I-ble3 vectors, check the morphology of the parasites under a light microscope. Parasites in logarithmic phase have a drop-like form and are optimal for transfection. *L. tarentolae* with enlarged and thin morphology are observed in starved cultures and should not be used for transfection.

1. Linearize the recombinant pLEXSY_I-ble3 plasmid containing the *T. cruzi* antigen coding sequence by digestion with *Swa*I to allow homologous recombination into the *L. tarentolae* genome. Add 10 units of enzyme per microgram of plasmid, and incubate the reaction in the appropriate buffer at 25 °C for 2–6 h. Check the complete digestion running an aliquot of DNA in an agarose gel (*see* **Note 13**).
2. Purify the DNA fragment containing the *T. cruzi* antigen coding sequence (upper band in gel) using a commercial solid-phase purification kit. Elute the DNA with sterile, deionized water. Concentrate the linearized plasmid at least until 0.2 micrograms per μL, using a speed vacuum concentrator or ethanol precipitation.
3. Dilute a culture of early-logarithmic-phase *L. tarentolae* in a fresh medium (1:10 dilution), 16–20 h before transfection.
4. Check the drop-like shape of the parasites under the microscope on the day of transfection, and dispense 15 mL of culture containing 1×10^7 parasites/mL in a Falcon tube.
5. Centrifuge for 10 min at 1500 $\times g$ at room temperature. Wash the cells once with one volume of BHI without supplements and discard supernatant.
6. Resuspend the cell pellet in 300–350 μL of cold BHI, and add 50–100 μL of the linearized recombinant plasmid containing a minimum of 10 μg of DNA (25 μg is optimal). Include positive (linear, empty pLEXSY_I-ble3) and negative (no plasmid DNA) transfection controls.
7. Transfer the mixtures into a 0.2 cm gap sterile electroporation cuvette, and incubate on ice for 10 min. Electroporate cells with 1 pulse of 450 μF and 450 V. Check for the absence of voltaic arc, and immediately place the cuvettes on ice for 5 min.
8. Transfer the cells to a tissue culture flask, and add 5–10 mL of complete BHI medium. Incubate for 12 h before adding 75 μg/mL of the selection antibiotic zeocin. From this point on, dilute the cultures 1:10 every 2 days by adding fresh complete BHI medium containing zeocin. Selection is achieved when no visual growth is observed in the negative control cultures.
9. Once selection of recombinant *L. tarentolae* is achieved, grow the zeocin-resistant parasites in BHI complete medium plus

antibiotics, and dilute the culture 1:30 with fresh medium every 3 days. For cryopreservation of recombinant *L. tarentolae* storage, add ice-cold 20% glycerol to a logarithmic growth phase culture, and freeze at −80 °C (*see* **Note 14**).

10. Induce the expression of the recombinant *T. cruzi* antigen by adding once 12 μg/mL of tetracycline to the *L. tarentolae* culture, and incubate for 96 h (*see* **Note 15**).

3.4 Affinity Purification of Recombinant T. cruzi Antigens

All steps must be carried out at low temperature to avoid protein degradation. Keep all buffers and reagents on ice and the centrifuges set at 4 °C all over the process.

1. Centrifuge the tetracycline-induced culture of *L. tarentolae* for 10 min at 1500 ×*g*, and separate supernatant from the cell pellet.
2. Add immediately a protease inhibitor cocktail (EDTA-free) to the supernatant.
3. Clarify the supernatant containing the secreted *T. cruzi* antigen by centrifugation at 12,000 × *g* for 30 min, to remove the remaining cellular debris, precipitated salts, and excess hemin.
4. Dialyze the supernatant with 10 volumes of binding buffer overnight, using a magnetic bar and continuous stirring to avoid solute concentration gradients. Concentrate dialyzed supernatant 10× by diafiltration on a centrifuge using a Vivaspin® filter device.
5. Add 0.1 volumes of Ni-NTA resin previously equilibrated with binding buffer to the concentrated supernatant. Incubate the supernatant-resin mixture in a rotary shaker 1–3 h.
6. Centrifuge the supernatant-resin mixture for 1 min at 900 × g and then remove the supernatant. Wash the resin six times with 5 volumes of wash buffer, centrifuging 1 min at 900 × g between steps. Save aliquots of 20–30 μL from the supernatant, and wash fractions for SDS-PAGE and Western blot analysis.
7. Elute the recombinant *T. cruzi* antigen four times with one-bed resin volume of elution buffer each time. Measure the absorbance at 280 nm of the elution fractions in a spectrophotometer, and pool the fractions containing higher protein content (*see* **Note 16**).
8. Dialyze the eluted fractions/pool against phosphate or the desired buffer to remove imidazole from the purified antigens.
9. Analyze the performance of the procedure and the purity of the recombinant antigens by subjecting samples from the different expression/purification steps to SDS-PAGE gel electrophoresis.
10. Store the purified antigens in aliquots at −80 °C adding 50% glycerol.

4 Notes

1. Any pUC derivative [34] or the plasmid pZErO-2 (Thermo Fisher Scientific, USA) can be used as cloning vectors. If the pZErO-2 vector is used to clone the *T. cruzi* coding sequence, manufacturer's instruction can be found at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/zeroblunt_man.pdf.
2. DH5 or DH10B *E. coli* strains are suitable hosts either for pUC or pZErO-2 vectors.
3. pLEXSY vectors are quite unstable in *E. coli*, due to the presence of repetitive and homopolymeric DNA stretches within the non-translated regions (UTR). Therefore, it is recommended to choose recipient *E. coli* strains that tolerate these motives such as SURE (Stop Unwanted Rearrangement Events) (Agilent Technologies, USA).
4. Subcloning in pZErO-2 or pUC vectors is not mandatory but is recommended to ensure that the PCR fragment coding for *T. cruzi* antigen is properly digested at both 5′and 3′ ends for an efficient directional cloning.
5. The 5′restriction sites of *Sal*I or *Xba*I allow the in-frame cloning of the sequence of interest with an N-terminal secretory signal peptide. The 3′ restriction sites of *Msp*CI or *Kpn*I allow the in-frame cloning of the sequence of interest with a C-terminal 6xHis-tag. A map of the pLEXSY_I-ble3 vector can be downloaded from https://www.jenabioscience.com/images/PDF/EGE-244.pdf. Detailed consideration for choosing the restriction sites for cloning can be found at https://www.jenabioscience.com/images/ae3a4f50f1/EGE-1410.pdf.
6. The addition of a short spacer sequence between the gene of interest and the poly-histidine codons provides spatial freedom to the His-tag to interact with the affinity resin or the anti-His antibody. Fusion of *T. cruzi* coding sequence to affinity tags other than poly-histidine can be achieved using standard molecular biology techniques (i.e., restriction or restriction-free cloning). Due to the presence of a thrombin-like proteolytic activity in *Leishmania*, do not use such protease motif between the target gene and the affinity tag as this will lead to the loss of the tag.
7. An alternative restriction-/ligase-independent cloning strategy can be used to circumvent any incompatibility between gene sequence and restriction sites at the vector MCS or unsuccessful cloning due to plasmid instability (please, *see* **Note 3** above).
8. If a synthetic gene coding for the *T. cruzi* antigen will be expressed in LEXSY system, the DNA sequence can be

analyzed based on the codon usage table of L. tarentolae prior to cloning. For this, go to the Codon Usage Table of different organisms available at http://www.kazusa.or.jp/codon and select the data for *L. tarentolae*. Copy and paste the entire codon usage table from the database together with the *T. cruzi* DNA sequence into the online program Optimizer (http://genomes.urv.es/OPTIMIZER/) and submit the data for analysis. Select the retrieved sequence to synthesize your gene. Moreover, OPTIMIZER program can also be used to remove or add recognition sites for restriction endonucleases within the optimized coding sequence, especially when the target sequence has the same recognition sites present in the multiple cloning site [35].

9. Growth at 30 °C instead 37 °C favors the stability of pLEXSY vectors in *E. coli*.
10. If the plate from the self-ligation control has a similar or higher number of colonies than the plate corresponding to cells transformed with the insert-vector ligation reaction, then repeat **steps 1–7**.
11. Even after transformation and selection, *E. coli* can easily loss pLEXSY_I-ble3 plasmids retaining the antibiotic resistance. To avoid this, do not allow recombinant colonies to stay too long in a Petri dish or liquid media before extraction of plasmid DNA. Once the identity of the target gene is confirmed, grow larger cultures, and store bacterial pellets from 50 mL culture aliquots at −20 °C for further plasmid purifications. Otherwise, keep a small aliquot of the plasmid miniprep at −20 °C for transforming competent *E. coli* SURE2 cells and to prepare fresh recombinant vector whenever it is necessary.
12. Liver infusion tryptose (LIT) medium supplemented with hemin and antibiotics can also be used as growth medium for T7-TR *L. tarentolae*. Passage of parasites from BHI to LIT should be done stepwise by adding 10% of the new medium every time a subculture is made.
13. Once digestion is complete, two main DNA bands are visualized on the agarose gel. The upper band corresponds to the *T. cruzi* coding sequence flanked by the necessary elements for the expression and homologous recombination in the *L. tarentolae* genome. The lower band corresponds to the cassette that allows the amplification and selection of pLEXSY_I-ble3 in *E. coli* (see Fig. 2).
14. Addition of 1% fetal bovine serum to the BHI medium during the first week of cultivation improves *L. tarentolae* recovery from a frozen stock.
15. At this point and whenever an antigen-specific serum is available, Western blot analysis of a sample from the culture supernatant could be performed to detect the presence of the

recombinant protein. Also, anti-6xHis antibodies can be used if the antigen harbors this tag.

16. As imidazole absorbs at 280 nm, set the reference blank with elution buffer when measuring the protein content in the elution fractions.

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