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Short communication

Effective gene delivery to *Trypanosoma cruzi* epimastigotes through nucleofection

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ABSTRACT

New opportunities have raised to study the gene function approaches of *Trypanosoma cruzi* after its genome sequencing in 2005. Functional genomic approaches in *Trypanosoma cruzi* are challenging due to the reduced tools available for genetic manipulation, as well as to the reduced efficiency of the transient transfection conducted through conventional methods. The Amaxa nucleofector device was systematically tested in the present study in order to improve the electroporation conditions in the epimastigote forms of *T. cruzi*. The transfection efficiency was quantified using the green fluorescent protein (GFP) as reporter gene followed by cell survival assessment. The herein used nucleofection parameters have increased the survival rates (>90%) and the transfection efficiency by approximately 35%. The small amount of epimastigotes and DNA required for the nucleofection can turn the method adopted here into an attractive tool for high throughput screening (HTS) applications, and for gene editing in parasites where genetic manipulation tools remain relatively scarce.

Eight (8) to 10 million people in 21 endemic countries are infected with *Trypanosoma cruzi*, and 30% of these infected patients develop Chagas disease symptoms. This neglected tropical disease lacks an efficient treatment with reduced side effects [1]. The first version of the complete genome of *T. cruzi* was published almost a decade ago [2], and showed an estimate of 12,000 genes per haploid genome. Approximately 50% of these genes have unknown function and those genes with predicted function can perform additional activities. Although the genome sequence and transcriptomic data allow identifying the *T. cruzi* genes and their expression profile, new approaches are needed in order to determine gene function. Unlike what happens with *Trypanosoma brucei* [3], the RNAi machinery in *T. cruzi* is not functional [4], and it has deeply limited the high throughput functional genomics through RNA silencing. The RNAi approach has allowed large scale analysis in *T. brucei*, for example: [5] silenced all genes in chromosome I and searched for growth defects. Additionally, since the first report using *T. brucei* random RNAi library [6], elegant studies have been elucidating different aspects of *T. brucei* biology, such as drug resistance and *quorum* sensing [7,8]. Regarding *T. cruzi*, gene overexpression remains the main strategy to characterize genes, followed by the conventional gene knockout, which is too laborious. However, recent tests of the CRISPR-CAS9 system have evidenced that such system is a very promising tool in this organism [9,10], and that can be improved under better transfection conditions [11].

The transfection of different forms from *T. cruzi* with DNA vectors has become a widely used and powerful tool to investigate both gene regulation and gene function, in order to cause phenotypic changes. However, *T. cruzi* is considered a "hard-to-transfect" parasite, which shows relatively low transfection efficiency through conventional electroporation. However, recent publications have shown the potential of the nucleofector technology to improve the transfection efficiency in *T. brucei* [12,13] and, more recently, in *T. cruzi* trypomastigotes [14]. Such results have encouraged the systematic assessment of this transfection methodology in the epimastigote forms of *T. cruzi*. Accordingly, the main aim of the present report is to evaluate the transfection efficiency in *T. cruzi* using the nucleofector technology under different conditions.

Previous transfection efficiency between 4% and 8% was reported in the epimastigote forms of *T. cruzi* when it was assessed 24 hours-post transfection, using 30 and 60 μg of DNA, respectively, through conventional electroporation protocols [15]. In order to increment parasite transfection efficiency, 2.0×10^7 G strain epimastigotes in exponential growth phase were transfected with 10 μg of pTREXn-GFP vector [16] in the electroporation buffer 1× Tb-BSF [13], using different programs in the nucleofector apparatus. Plasmid DNA was prepared using PureLink® HiPure Plasmid Midiprep Kit (ThermoFisher Scientific), DNA quantity and quality was evaluated using a NanoDrop®

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spectrophotometer and electrophoresis. The U-033 and X-014 programs generated the highest transfection efficiency (between 12 and 15%), which is similar to the transfection efficiency reported by Xu et al. [17] (Fig. 1A). Although the U-014 and Z-001 programs have been used in hard-to-transfect cells [18], they were not efficient enough, because they just produced transfection efficiency below 4% (Fig. 1A). However, all programs tested with the Amaxa device yielded high survival rates (92 to 97% - 24 hpt) as shown through propidium iodide staining (Fig. 1A). It should be noticed that the cell viability, shortly after transfection (5 h), was also high; thus presenting survival rates between 90% to 97% (data not shown). The X-001, T-007 and Z-001 programs were also tested in the present study, however, none of them has generated transient transfection efficiency above 5% (data not shown). The following condition was tested in order to compare the transient transfection efficiency obtained here with the conventional electroporation protocol, which was carried as follows: a total of 4.0×10^7 epimastigotes were washed once and resuspended in 400 μL of ice-cold Cytomix buffer containing 50 μg of pTREX-GFP plasmid in 2-mm electroporation cuvettes with two pulses (450 V, 500 μF) delivered by the GenePulser II (BioRad) [15]. As shown in fig. 1A, the transfection efficiency was lower than that observed in the nucleofector device, which reached <5% of transfection efficiency and reduced cell viability (68% - 24 hpt). In order to test if the aforementioned difference in performance could due to the buffer composition, the epimastigotes were transfected in Tb-BSF buffer using the GenePulser II protocol, which did not increase the transfection efficiency, nor cell viability (Fig. 1A). It is worth highlighting that the results for transfection rate (% of fluorescent cells) were supported by the mean fluorescence intensity (Fig. 1B). The percentage of fluorescent cells herein obtained with *T. cruzi* epimastigotes are in the same range reported for the procyclic forms of *T. brucei* using the Amaxa (10% and 20% using the programs U-033 and X-014) (Burkard et al. 2007). It is important pinpointing that the transfection of non-replicative forms (trypomastigote) is less efficient than that of the epimastigotes [14].

Subsequently, there was the interest in investigating whether the transient transfection efficiency could be improved by rising the DNA amount. When the DNA amount was increased to 20 μg, there was transfection efficiency increase, which has reached the fluorescent cell percentage up to 35%, when it was analyzed 48 h after transfection (Fig. 1C). In addition, twofold increase was observed in the mean fluorescence intensity when the DNA amount was raised to 20 μg (Fig. 1D). Of note, neither the transfection efficiency nor the mean fluorescence intensity have increased when 40 μg of DNA was used (Fig. 1C and D).

Aiming to test if the buffer composition could be have an impact in the transfection efficiency, several homemade buffers, previously tested to transfect primary T cells using the Amaxa [19], were used in *T. cruzi*. The T cells, such as *T. cruzi*, are considered hard to transfect and the cell viability after their transfection is very low. As shown in the Table 1, any of the herein used homemade buffers could improve the transient transfection when the GFP expression was assessed 24 h after transfection. In addition, the transfections conducted using some of these buffers (Tc-EF2 and Tc-EF3 buffers) have seriously compromised the cell viability, and it has revealed that the electroporation buffer composition of plays an important role in the transfection process.

Since the success of the transfection depends on the efficient delivery of the DNA into the nucleus, and on the cellular machinery, to express its information, we decide to analyze the cell permeabilization using a fluorescent marker (FITC-Dextran – 150 kDa). All tested programs and transfection protocols were able to label >60% of the cells

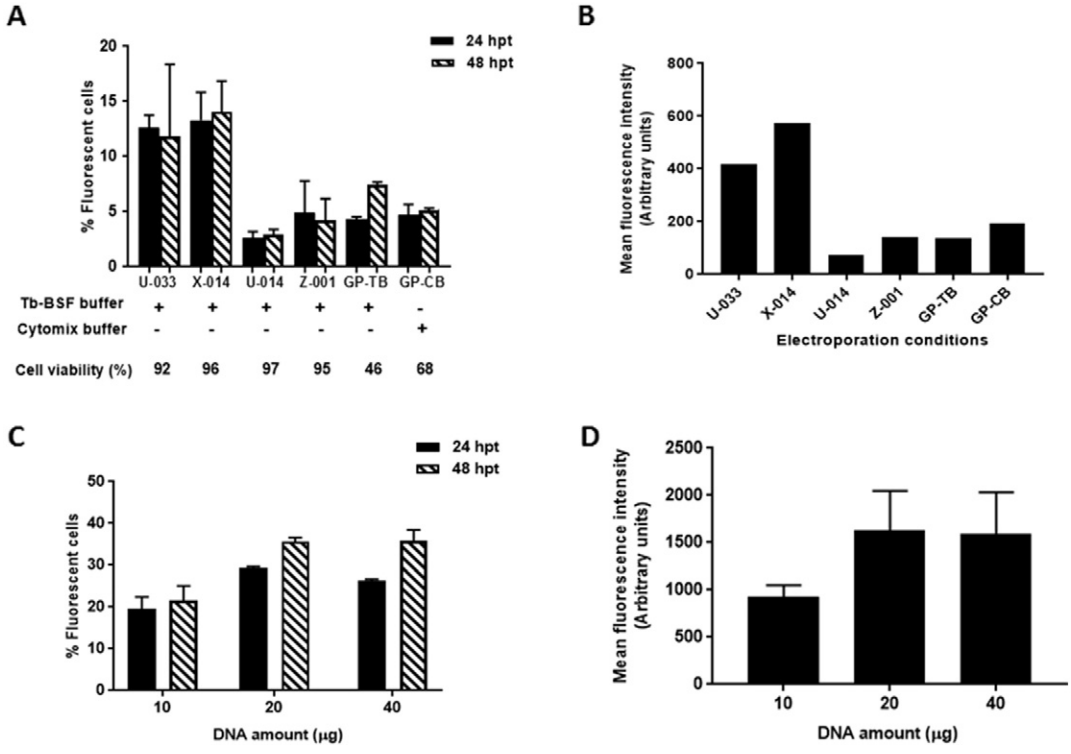

Fig. 1. Transient transfection efficiency in epimastigote forms from *Trypanosoma cruzi* using different electroporation conditions. (A) 2.0×10^7 epimastigote forms of G strain in exponential growth phase were washed once and resuspended in 100 μL of 1× Tb-BSF buffer and mixed with 10 μg of pTREX-GFP vector. The parasites were transferred to 2 mm gap cuvettes and electroporated using U-033, U-014, X-014 and Z-001 programs in the Amaxa Nucleofector 2b (Lonza Cologne AG, Germany). In parallel, 4.0×10^7 parasites were electroporated in GenePulser II equipment (BioRad), using the Tb-BSF buffer (GenePulser-TbBSF buffer, GP-TB), or in Cytomix buffer (GenePulser-Cytomix buffer, GP-CB). After transfection, the parasites were transferred to 5 mL of LIT medium supplemented with 10% FCS and incubated at 28 °C. The flow cytometry analyses were carried out in order to assess the green fluorescent protein expression at 24 and 48 h after transfection (hpt). (B) The mean fluorescence intensity was evaluated 24 hpt in each one of the transfection conditions used in A (a representative experiment is shown). (C) Epimastigote forms were transfected with 10, 20 or 40 μg of the pTREX-GFP vector using the X-014 program according to the transfection conditions set in panel A. The GFP expression was measured by flow cytometry 24 and 48 hpt. (D) The mean fluorescence intensity was measured at 24 hpt of the same transfections shown in C. These values were normalized according to those for mock-transfected parasites and were presented through the means (±SDM) of three independent experiments.