

pTcGW plasmid vectors 1.1 version: a versatile tool for *Trypanosoma cruzi* gene characterisation

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The functional characterisation of thousands of Trypanosoma cruzi genes remains a challenge. Reverse genetics approaches compatible with high-throughput cloning strategies can provide the tool needed to tackle this challenge. We previously published the pTcGW platform, composed by plasmid vectors carrying different options of N-terminal fusion tags based on Gateway® technology. Here, we present an improved 1.1 version of pTcGW vectors, which is characterised by a fully flexible structure allowing an easy customisation of each element of the vectors in a single cloning step. Additionally, both N and C-terminal fusions are available with new tag options for protein complexes purification. Three of the newly created vectors were successfully used to determine the cellular localisation of four T. cruzi proteins. The 1.1 version of pTcGW platform can be used in a variety of assays, such as protein overexpression, identification of protein-protein interaction and protein localisation. This powerful and versatile tool allows adding valuable functional information to T. cruzi genes and is freely available for scientific community.

Key words: *Trypanosoma cruzi* - plasmid vectors - pTcGW platform - reverse genetics - cloning - gene characterisation

The sequencing of *Trypanosoma cruzi* genome revealed a high percentage of genes assigned with unknown function or with a role attributed only based on sequence similarity (El-Sayed et al. 2005, Weatherly et al. 2009). In order to improve this scenario and to add functional information to *T. cruzi* genes, our group previously constructed the pTcGW platform, which is composed by plasmid vectors containing several N-terminal fusion tags (Batista et al. 2010). In the current work we improved the previous 1.0 version of the pTcGW platform and created the pTcGW 1.1 version by implementing three changes.

First, in order to make our platform fully flexible, we aimed that each restriction site was present only once in the vectors. For this, we replaced the *XhoI* restriction site 5'-flanking the resistance marker by side by side *AscI* and *AgeI* sites through two cloning steps. The obtained plasmid (pTc6HisP-NH 1.1) containing N-terminal hexahistidine fusion tag comprises the backbone NH-vector of pTcGW platform 1.1 version (modification steps described in Supplementary Fig. 1). Second, to create vectors containing C-terminal fusion tags, the cassette comprising the 18S ribosomal promoter (RP), the first intergenic region (IR) (*TcUIR*), start codon and the N-terminal 6His tag were excised from pTc6HisP-NH 1.1 and replaced by a new cassette containing only the RP and *TcUIR*. Next, we

replaced the phleomycin resistance marker by neomycin and added a green fluorescent protein (GFP) fusion tag creating the pTcGFPN-CO 1.1, backbone for the CO-vectors (Supplementary Fig. 2). Third, for protein complex purification, we added either protein C (ProtC) or ProtC-TEV-ProtA (PTP) tags [PTP amplified from Schimanski et al. (2005)] in both N and C-terminal vectors.

Finally, in order to broaden the applicability of these tools towards different assays we replaced the tag and/or resistance marker through single cloning steps, generating five different amino 1.1 and four carboxy 1.1 plasmids (Fig. 1). Our destination vectors allow performing a range of functional assays to characterise *T. cruzi* genes, such as protein overexpression, identification of interactors and protein localisation. The sequences of the plasmids are available on GenBank (accessions described on Supplementary data 3). The sequences of primers here employed and all material and methods can be found in Supplementary data 4. We also provide technical details for users in the pTcGW Platform Guideline. Briefly, the guide contains an explanation of how the Gateway® cloning system works, instructions for primers design, recombination reactions, plasmids propagation, *T. cruzi* transfection and selection.

The basic structure of the platform characterised by the presence of a strong RP (18S), IR from the ubiquitin locus (*TcUIR*) and the Gateway® cloning system are maintained from version 1.0. The RP, recognised by RNA polymerase I, ensures an efficient transcription of genes under its control and had already been successfully used in *T. cruzi* vectors (Martinez-Calvillo et al. 1997, Vazquez & Levin 1999). IRs provide signals to drive the processing of pre-mRNAs transcribed in the long polycistronic units. In order to be functional *T. cruzi* mRNAs receive a 39-nucleotide splice-leader in the 5' region and a PolyA tail in the 3' region (Liang et al. 2003). Given the pivotal role of IRs, the functional elements of our

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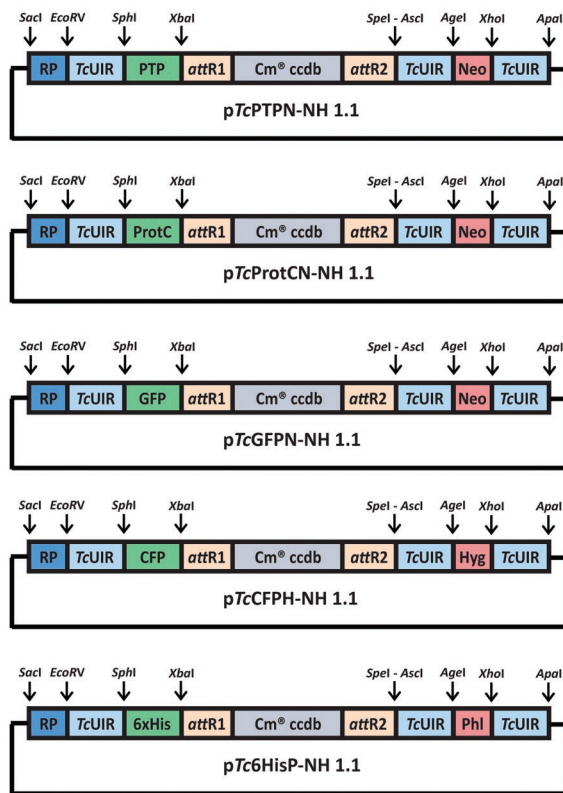
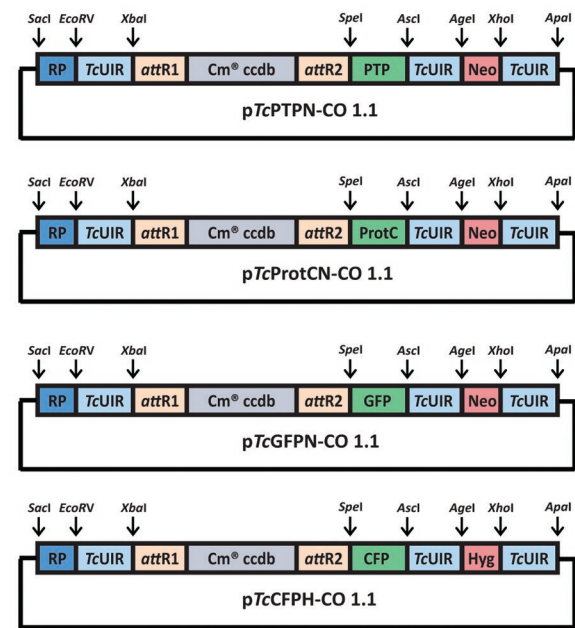
Amino vectors**Carboxy vectors**

Fig. 1: available pTcGW 1.1 vectors with amino and carboxy-terminal fusion tags. Arrows indicate the restriction sites flanking each element of the vectors. *att*: attachment; *attR1* and *attR2*: attachment sites required for Gateway[®] recombination; *ccdB*: gene for negative selection; Cm[®]: chloramphenicol selectable marker; CO: carboxy-terminal fusion; H: hygromycin; N: neomycin; NH: amino-terminal fusion; P: phleomycin; p: plasmid; RP: ribosomal promoter; Tag: the fusion tag used, such as green fluorescent protein (GFP), cyan fluorescent protein (CFP), ProtC-TEV-ProtA (PTP), protein C (ProtC) or hexahistidine (6xHis); *Tc*: *Trypanosoma cruzi*; *TcUIR*: *T. cruzi* ubiquitin intergenic region.

vectors are flanked by *TcUIR*, which had already been used in a *T. cruzi* vector (Wen et al. 2001) and is present three times in our system. Additionally, our destination vectors contain a Gateway[®] cassette composed by the attachment (*att*) sites *attR1* and *attR2* required for recombination, the chloramphenicol selectable marker and *ccdB* gene for negative selection.

Here, to demonstrate that the modifications in the plasmid preserved its functionality, the plasmids pTcPTPN-NH 1.1, pTcPTPN-CO 1.1 and pTcGFPN-CO 1.1 were used for protein overexpression and localisation assays.

The 117 kDa exportin Crm1 (TcCLB.511725.150) was cloned into the N-terminal PTP plasmid (pTcPTPN-NH 1.1) whereas the 67 kDa mRNA export factor Mex67 (TcCLB.506127.20) and the 95 kDa nucleoporin Nup95 (TcCLB.510181.50) were inserted in the C-terminal PTP plasmid (pTcPTPN-CO 1.1). Fig. 2A shows that the recombinant proteins were successfully expressed with the expected molecular weight. Furthermore, *T. cruzi* Mex67 and Nup95 demonstrated nuclear localisation, same as described for the *Trypanosoma brucei* orthologue proteins (Kramer et al. 2012, Dostalova et al. 2013), whereas the exportin Crm1, which shuttles between nucleus and cytoplasm, was observed to localise in both compartments (Fig. 2B).

To exemplify the usefulness of pTcGW vectors to characterise hypothetical proteins we used the plasmid pTcGFPN-CO 1.1 to clone the 58.1 kDa conserved hypothetical protein TcCLB.506825.40. The polyclonal serum raised using the recombinant protein recognised both the endogenous and the GFP-tagged protein in epimastigotes and metacyclic trypomastigotes forms of *T. cruzi* (Fig. 2C). Additionally, the intracellular localisation of the GFP-tagged TcCLB.506825.40 could be determined, which is concentrated in the posterior region of *Dm28c* epimastigotes (Fig. 2D).

A striking feature of pTcGW 1.1 version refers to the versatility of the system. All elements of the 1.1 vectors are flanked by exclusive restriction sites allowing an easy exchange of each element of the vector in a single cloning step, such as promoter, fusion tags, resistance markers and IRs. This feature empowers the system through the range of opened options. For example, one can easily adapt the vectors for use in other trypanosomatid species by simply changing IRs and promoter if required. Furthermore, by selecting different IRs containing developmentally regulated 3'UTR signals, the expression can be controlled in a life-cycle stage dependent manner (Coughlin et al. 2000, Clayton & Shapira 2007). Additionally, colocalisation studies can be performed by combining different tags with resistance markers.

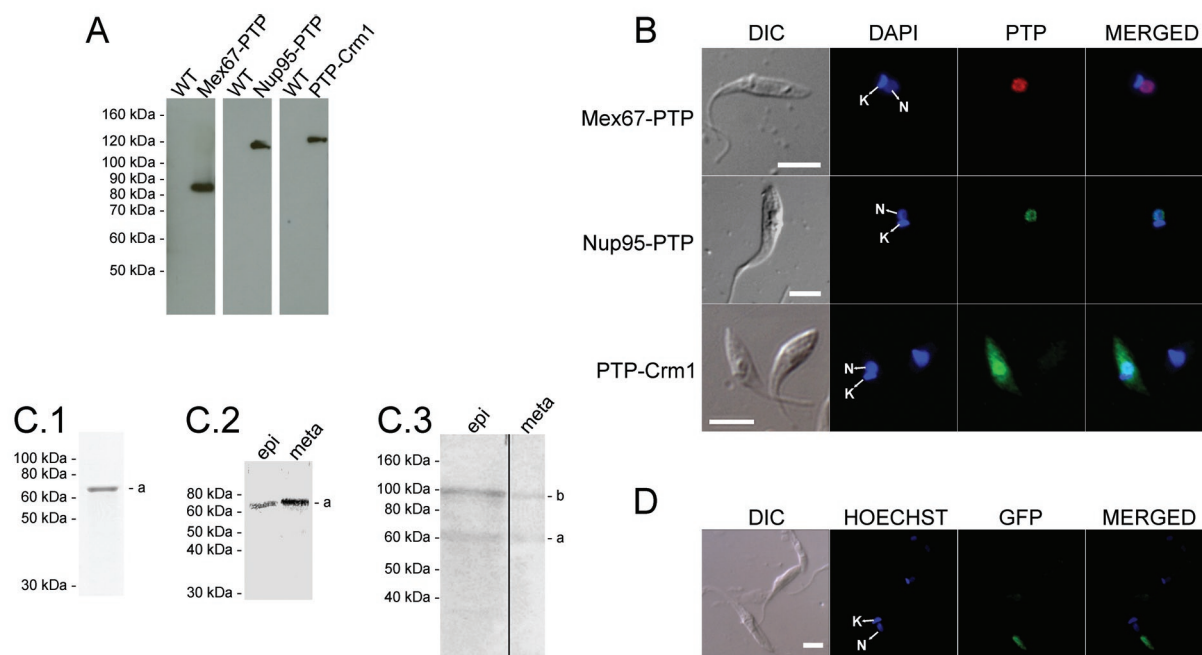


Fig. 2: cellular localisation and overexpression of tagged proteins in *Trypanosoma cruzi*. A: western blot (WB) using total extract of epimastigotes wild-type (WT) and expressing ProtC-TEV-ProtA (PTP)-tagged proteins; B: detection of mRNA export factor Mex67 and nucleoporin Nup95 tagged at C-terminal extremes and nuclear export factor Crm1 tagged at N-terminal extreme of PTP tag by indirect immunofluorescence microscopy; C: analysis of protein expressed by TcCLB.506825.40 gene; C1: polyacrylamide gel electrophoresis of the purified protein fused to a hexahistidine tag; C2: WB analysis using a specific anti-TcCLB.506825.40 antibody and total extract of WT epimastigotes (epi) and metacyclic trypomastigotes (meta); C3: WB analysis using a specific anti-TcCLB.506825.40 antibody and protein extracts of epi and meta expressing the conserved hypothetical protein (TcCLB.506825.40). The lower band (a) corresponds to the endogenous protein and the upper band (b) corresponds to the GFP-tagged protein expressed in the pTcGFPN-CO 1.1 vector; D: subcellular localisation of TcCLB.506825.40 protein fused with GFP in *T. cruzi* epi parasites; DIC: differential imaging contrast; K: kinetoplast; N: nucleus. Bar = 5 μ m (B), 10 μ m (D).

Another improvement in the 1.1 version consists in the possibility of choosing whether using N or C-terminal fusion tags. This represents a very important feature since signal peptides located at the N-terminal region of proteins might be hidden when fluorescent tags in this portion are used (Simpson et al. 2000).

In the previous version of the platform, to sequentially purify protein complexes we used a TAP tag, which is composed by a duplicated ProtA followed by Tobacco Etch Virus (TEV) protease site and calmodulin binding peptide. However, the second step of TAP tag purification was not efficient (Batista et al. 2010), issue previously reported in the literature and overcome by using a PTP tag (Shimanski et al. 2005). To offer an alternative to TAP tag, our vectors now have either a PTP or a ProtC tag. The PTP tag allows performing two steps purification whereas ProtC tag on its own can be used for single step protein complex purifications.

The vectors from 1.1 version of pTcGW platform are compatible with both high and low-throughput studies and had already been successfully used in different studies (Batista et al. 2013, Inoue et al. 2014, Kalb et al. 2014). Altogether the alterations presented at pTcGW 1.1 platform were designed to easily allow any further customisation and are available for scientific community.

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