p*Tc*GW platform guideline

Gateway® cloning system: general overview - Gateway technology (Life Technologies) is a cloning system based on the recombinational properties of lambda phage in Es*cherichia coli*, where it alternates between lytic and lysogenic cycle. This recombination occurs between the attachment (*att*) sites present in the insert, donor vector and destination vector. p*Tc*GW plasmids are destination vectors, possessing the Gateway cassette RfA, including *att*R1 and *att*R2 sites, chloramphenicol resistance and *ccd*B gene. The *att*B1 and *att*B2 sites are added to the insert by polymerase chain reaction (PCR) amplification, then in the presence of BP Clonase mix, these sites recombine with *att*P1 and *att*P2 sites (BP reaction), found in the donor vector (e.g., pDONR 221). This will generate the *att*L1 and *att*L2 sites flanking the insert that at this stage is cloned in the donor vector (entry clone). The *att*L sites, in the presence of LR Clonase mix, recombine with *att*R1 and *att*R2 sites in the p*Tc*GW plasmids, generating *att*B sites. In these two steps of recombination, the *ccd*B gene provides a negative selection. Plasmids (donor or destination vectors) where the recombination did not occur will express the product of *ccd*B gene, which is toxic to the cell and in this way *E. coli* bearing those plasmids will not survive. The entry clone can be transferred to any destination vector. Once the insert is cloned into the p*Tc*GW plasmid flanked by *att*B sites it is ready to be transfected in *Trypanosoma cruzi*.

Plasmid propagation - Due to the presence of RfA cassette into p*Tc*GW plasmids, the propagation should be done in a *ccd*B resistant strain of *E. coli* [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G *fhu*A::*IS2*)]. p*Tc*GW plasmids confer resistance to ampicillin and chloramphenicol.

Primers design - The coding sequences to be inserted into p*Tc*GW plasmids generally are obtained by PCR amplification, where the *att*B sites required for recombination are inserted in both ends of the amplicon through the primers. For N-terminal fusions the start codon is present before the tag and the stop codon after *att*B2 Gateway site. However, we recommend the use of the original stop codon from the inserted gene to minimise the addition of exogenous sequences. For C-terminal fusions, the start codon of the inserted gene is required and a stop codon is present at the end of the tag and should be absent in the inserted gene sequence. The *att*B sites can be added in a single or double step, according to the templates of Table I.

Insert recombination - After the insertion of the *att*B sites in the amplicon, the recombination between *att*B sites present in the insert with *att*P sites present in pDONR vectors (BP reaction) is carried out using 1 µL of the enzyme BP Clonase II (Life Technologies) incubated overnight at 25°C. The transformation should be done in *E. coli* DH5α, TOP10 or DH10B strains. The screening of clones may be performed by picking at least five colonies and direct subject them to PCR amplification with the same primers used for the insert amplification. Once cloned into p*Tc*GW plasmids, the insert may be verified by Sanger sequencing with M13 forward and reverse primers. The recombination between *att*L (entry clone) and *att*R sites (p*Tc*GW plasmids) is carried out with 1 µL of LR Clonase II (Life Technologies) and incubation as described. Both BP and LR reactions are performed with circular instead linearised plasmids. For screening of the clones, an additional step for confirmation of the clone size is recommended. This may be done by running the bacterial lysate on an agarose gel. In this confirmation, a careful inspection of the clone size is important. Colonies with single or additional band, less than the expected clone size, should be discarded. The efficiency of recombination decreases as the size of insert increases, in this way, for big inserts optimisations in the recombination protocol may be required. Once the clone candidates are obtained we confirm it by DNA sequencing.

T. cruzi transfection - For transfection in *T. cruzi* 10-15 µg s of the circular plasmid is recommended. Until now, the transfections have been carried out in *Dm*28c strain. Here we describe the protocol used in our lab for *T. cruzi* transfection. Epimastigote forms of *T. cruzi* grown at 28°C in liver infusion tryptose (LIT) medium, supplemented with 10% foetal calf serum, to a density of approximately 3×10^7 cells mL^{-1}. Parasites are harvested by centrifugation at 4,000 *g* for 5 min at room temperature, washed once in phosphate-buffered saline and resuspended in 0.4 mL of electroporation buffer pH 7.5 (140 mM NaCl, 25 mM HEPES, 0.74 mM Na_2HPO_4) to a density of 1×10^8 cells mL-1. Cells are then transferred to a 0.2 cm gap cuvette and 10-15 µg of DNA is added. The mixture is placed on ice for 10 min and then subjected to two pulses of 450 V and 500 µF. After electroporation, cells are maintained on ice until being transferred into 4-10 mL of LIT medium and then incubated at 28°C. After 24 h, the antibiotic (hygromycin or G418) is added to an initial concentration of 125 µg mL-1. Then, 72-96 h after electroporation, cultures are diluted 1:10 and antibiotic concentrations doubled. Stable resistant cells are obtained approximately 18 days after transfection.

In our tests, wild type *Dm*28c has been demonstrated to be resistant to high concentrations of phleomycin and we could not generate any transfectant. Therefore, before using this resistance marker the efficacy of phleomycin should be better evaluated. The set of p*Tc*GW plasmids 1.1 version available is described in Table II.

TABLE I

Fusion	Step	F/R	Primer sequence (5'-3')
Amino	Single	F	GGGG**ACAAGTTTGTACAAAAAAGCAGGCTTC**ATG…
		R	GGGG**ACCACTTTGTACAAGAAAGCTGGGTC***TCA*…
Carboxy	Single	F	GGGG**ACAAGTTTGTACAAAAAAGCAGGCTTC**ATG…
		R	GGGG**ACCACTTTGTACAAGAAAGCTGGGTC**…
Amino/carboxy	1	F	**GCAGGCTTC**ATG…
		R	**AGCTGGGTC***YCA*...
Amino/carboxy	2	F	GGGG**ACAAGTTTGTACAAAAAAGCAGGCTTC**ATG
Amino		R	GGGG**ACCACTTTGTACAAGAAAGCTGGGTC***TCA*
Carboxy		R	GGGG**ACCACTTTGTACAAGAAAGCTGGGTC**CCA

F/R column refers to forward or reverse primers. Bold nucleotides represent *att*B sites and italic ones indicate stop codon. Degenerated nucleotide Y represents a pyrimidine and generation of a stop codon depends on the primer used in the second polymerase chain reaction step for *att*B insertion. The ellipses indicate the gene specific nucleotides in the primers.

TABLE II

p*Tc*GW 1.1 plasmid vectors

Plasmid vector	Resistance marker	Fusion TAG	GenBank accession
p*Tc*6HisP-NH 1.1	P	6xHis	KR233510
p*Tc*CFPH-CO 1.1	H	CFP	KR233511
p*Tc*CFPH-NH 1.1	H	CFP	KR233512
p*Tc*GFPN-CO 1.1	N	GFP	KR233513
p*Tc*GFPN-NH 1.1	N	GFP	KR233514
p*Tc*ProtCN-CO 1.1	N	*P*rotC	KR233515
p*Tc*ProtCN-NH 1.1	N	*P*rotC	KR233516
p*Tc*PTPN-CO 1.1	N	PTP	KR233517
p*Tc*PTPN-NH 1.1	N	PTP	KR233518

H: hygromycin; N: neomycin; NH/CO: amino/carboxy-terminal fusion tag; P: phleomycin; p: plasmid; Tag: the fusion tag used, such as green fluorescent protein (GFP), cyan fluorescent protein **(**CFP), *P*rotC-TEV-*P*rotA (PTP), protein C (*P*rotC) or hexahistidine (6xHis); *Tc: Trypanosoma cruzi*.