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A Gateway® compatible vector for gene silencing in bloodstream form *Trypanosoma brucei*

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Abstract

RNA interference is the most rapid method for generation of conditional knockdown mutants in *Trypanosoma brucei*. The dual T7 promoter (pZJM) and the stem-loop vectors have been widely used to generate stable inducible RNAi cell lines with the latter providing tighter regulatory control. However, the steps for cloning stem-loop constructs are cumbersome requiring either multiple cloning steps or multi-fragment ligation reactions. We report the development of a vector (pTrypRNAiGate) derived from pLEW100 that utilizes the Gateway® recombination system to facilitate easy production of hairpin RNA constructs. This approach allows the final stem-loop RNAi construct to be generated from a single cloning step of the PCR-derived gene fragment followed by an *in vitro* recombination reaction. The new vector facilitates high-throughput applications for gene silencing and provides a tool for functional genomics in *T. brucei*.

RNA interference (RNAi) is the technique used for down regulating the expression of gene targets in African trypanosomes [1]. Double-stranded RNA (dsRNA) is an effective trigger of gene silencing and operates by sequence specific mRNA degradation. In *T. brucei*, a useful method of silencing an endogenous gene is to transform the trypanosomes with a gene construct encoding hairpin RNA (hpRNA) using a stem-loop vector [2]. A major limitation of the use of stem-loop vectors to mediate RNAi for high-throughput applications, such as functional genomics and drug target identification, is the number of cloning steps needed to produce hpRNA constructs. The pQuadra system eliminates the multiple cloning steps but requires a challenging four-piece ligation to generate the final vector [3]. An alternative system is the inducible dual T7 promoter vector (pZJM) that allows rapid cloning of genes into the vector but which has been observed to provide leaky control of dsRNA expression, and thus has been of more limited value [4] [5].

In plants, various high-throughput cloning vectors have been reported that utilize the Gateway® cloning system to generate stem-loop vectors for RNAi induced gene knockdown [6]. Recently, in *T. brucei* a dual T7 promoter RNAi vector (p2T7-177) [7] was modified to be Gateway® compatible [8], and utilized for gene knockdown in *T. brucei* procyclic forms. However this approach does not solve the problem of leaky control of dsRNA expression from the dual T7 promoter system. Here we describe a vector, pTrypRNAiGate, which uses Gateway® recombination to facilitate rapid generation of hpRNA (stem-loop) constructs in a two-step process from PCR products via an intermediate vector (Fig 1A). We show that

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this vector is effective in silencing endogenous genes encoding *S*-Adenosylmethionine decarboxylase (AdoMetDC)(Tb927.6.4410/Tb927.6.4460), spermidine synthase (SpdSyn) (Tb09.v1.0380) and ornithine decarboxylase (ODC)(Tb11.01.5300) in bloodstream form (BSF) *T. brucei* 90-13 cells.

We generated the high throughput vector using a similar approach to that reported in plants for the pHellsgate vector. We inserted into the pLEW100 derived stem-loop vector (a gift from George Cross, Rockefeller University) [4] [9], a 3891 bp sequence containing two recombination Gateway® cassettes consisting of *ccdB*, a lethal gene that targets DNA gyrase and the chloramphenicol-resistance gene flanked by two *attR* recognition sequences necessary for LR recombination, *attR1-ccdB-attR2* (www.Invitrogen.com) in an inverted repeat configuration with a stuffer region in between. The 3891 bp sequence was synthesized (Genscript) and cloned into pUC57 vector between HindIII and XbaI sites. This sequence was then excised from the pUC57 vector with HindIII and MluI restriction sites and cloned into the stem-loop vector. The new vector was named pTrypRNAiGate (Fig 1B), which serves as the RNAi destination vector in a typical Invitrogen Gateway® cloning system (www.Invitrogen.com). This vector is maintained and propagated in competent DB3.1 or *ccdB* survival cells (Invitrogen cat # A10460) which are resistant to the toxic effects of the *ccdB* gene. The system incorporates a negative selection marker (CcdB) that selects against vectors that have not undergone a recombination reaction, resulting in high frequency recovery of recombined plasmids.

To assess the suitability of this system, we used the vector to generate *T. brucei* bloodform cell line (90-13) and targeted inducible RNAi to previously characterized genes encoding AdoMetDC, SpdSyn and ODC and compared the phenotype with the previously generated RNAi lines using the standard stem-loop vector [10] [12]. The entry clones were created by producing a PCR product of about 400-500 bp length and then cloning it into pCR®8/GW/TOPO® vector (Invitrogen cat # K2520-02). This vector includes a TOPO cloning site for rapid and efficient cloning of Taq-amplified PCR products, and *attL1* and *attL2* sites for recombination-based transfer of the gene of interest into any Gateway® destination vector with *attR1* and *attR2* sites. The PCR products were generated using the forward primer 5'-(AAAGTACTGTTTGCGGCGAAGT)-3' and reverse primer 5'-(CCCAAACGAACAGTGCTCCTCA)-3' for AdoMetDC, forward primer 5'-(TTTACCACGAAATGTTGAGCCA)-3' and reverse primer 5'-(AAAGGTTGCGATTCAACCAAC)-3' for SpdSyn and forward primer 5'-(CCCTACGTTGCTTCAGCTTTCACACTTG)-3' and a reverse primer 5'-(CCCGGACAACATGGTCTGGTAGCCCGG)-3' for ODC with high fidelity platinum Taq DNA polymerase (Invitrogen). They were then incubated with pCR®8/GW/TOPO® vector (supplied ready to use) for 5 min at room temperature and transformed into Top10 competent cells and plated on spectinomycin (100 µg/ml) containing LB plates as described (www.invitrogen.com). Plasmid DNA were isolated from positive transformants and sequenced to verify the correct orientation of the genes between *attL1* – *attL2* sites. It was then used in the LR recombination reaction with the newly created pTrypRNAiGate destination vector.

LR recombination reactions were performed with 100 ng of entry clone and 100 ng of destination vector in a final volume of 10 µl. LR clonase enzyme mix (2 µl) was added and the volume made up to 10 µl with TE (Tris-EDTA buffer). Following overnight incubation at 25°C, 1 µl of proteinase K was added and incubated at 37°C for 10 min followed by transformation into Top10 competent cells and selection on ampicillin (100 µg/ml) plates. In a single step, the AdoMetDC, SpdSyn and ODC encoding genes were recombined into the final vector by replacement of the *ccdB* gene by a LR recombination reaction. Only recombined colonies grow under this negative selection with the *ccdB* gene. In a single step

recombination reaction, the gene of interest was inserted in opposite orientations. Transformants were screened by isolation of plasmid DNA and examined by both restriction analysis and sequencing for the genes of interest. The efficiency of the recombination reaction was between 85-90%. The correct RNAi constructs were then linearized and used to generate stable cell lines.

Bloodform cell line 90-13 (a gift from George Cross, Rockefeller University) was cultured in HMI-9 medium with 10% chicken serum at 37°C under 5% CO₂ [11]. Cells were grown with appropriate antibiotics as described [12]. Exponential growth phase *T. brucei* 90-13 BSF cells were transfected with the linearized stem-loop RNAi Gateway® vectors (10 µg) for AdoMetDC, SpdSyn and ODC and phleomycin (2.5 µg/ml) resistant cells containing the construct integrated into the rRNA locus were selected as previously described [13]. Three independent clonal lines were generated for each gene by limited dilutions. Five sets of dilutions were prepared so as to have approximately 100 cells/ml, 10 cells/ml, 2 cells/ml, 1 cell/ml and 0.5 cell/ml.

The dsRNA to AdoMetDC, SpdSyn and ODC was induced by tetracycline (1 µg/ml), which was added fresh every 24 h. Proliferating cultures were maintained through periodic dilutions and monitored by counting motile parasites. Results shown are average values of three independent clonal lines for each gene. Induction of AdoMetDC RNAi and ODC RNAi lead to cell growth arrest within 48 h followed by cell death in 10 days and 6 days respectively, for all three of the tested clonal lines (Fig 2). Induction of SpdSyn RNAi led to slowed growth in 1/3 clonal lines that were tested, but no significant effects on growth were observed for the other two lines. Addition of exogenous spermidine (0.1 mM) to tetracycline induced AdoMetDC and SpdSyn RNAi cells and putrescine (0.5mM) to the induced ODC RNAi cells restored normal growth relative to the uninduced controls (Fig 2). The results for AdoMetDC and ODC are similar to previously reported results using the standard stem-loop RNAi construct [10] [12] and the pZJM vector [4]. For SpdSyn it was previously reported that induction of double stranded RNA from the stem-loop RNAi construct led to growth arrest but not cell death [10]. This effect is similar to what we observed for one of the clonal lines using the Gateway® construct, but not all three, suggesting that clonal variation occurs when using RNAi for gene knockdown experiments. At least in this study clonal variation is more pronounced for the gene (SpdSyn) that shows a less pronounced growth effect, than for those like AdoMetDC and ODC where strong phenotypes are observed that lead to cell death.

Expression of AdoMetDC, SpdSyn and ODC mRNA was monitored by both northern blot and quantitative real-time PCR. For northern blots total RNA was prepared using TRIzol Reagent (Invitrogen), separated on 1% denaturing agarose gels (10 µg/lane), and transferred to a positively charged nylon membrane (BrightStar-Plus; Ambion). Northern blot analysis for AdoMetDC and ODC mRNAs was performed as previously described [12] with radio-labeled probes and tubulin as a loading control. SpdSyn RNAi analysis was carried out with the non radio-labeled probe using Psoralen-Biotin probe labeling kit (Ambion). After RNAi induction, the level of mRNA of all three genes was reduced or undetectable by 24 h (Fig 2, insets) similar to previously described results [10] [12].

Quantitative real-time PCR was performed using SYBR Green Mastermix (Bio-Rad). Total RNA was extracted from parasites using TRIzol Reagent (Invitrogen), treated with DNase I (Worthington) and reverse-transcribed using High capacity cDNA reverse transcription kit (Applied Biosystems). A 100 bp fragment of AdoMetDC, SpdSyn and ODC was amplified using SYBR Green Mastermix on a CFX96 Real-time system (BioRad) and compared to levels of a constitutively expressed control, telomerase reverse transcriptase, TERT [14]. Oligonucleotides were forward primer 5'-(GAGCGTGTGACTTCCGAAGG)-3' reverse

primer 5'-(AGGAACTGTCACGGAGTTTGC)-3' for TERT, forward primer 5'-(GCTGGTGTCAACAATGC)-3' reverse primer 5'-TACGGCAAGTGTGAAAGCTG)-3' for ODC, forward primer 5'-(GTTCCAGCACCTGTGCGATT)-3' reverse primer 5'-(TGGCTCAACATTTTCGTGGT)-3' for SpdSyn and forward primer 5'-(AGCGCTTGGAGGTGATAATG)-3', reverse primer 5'-(CTCACGGGAAACAATGTGTG)-3' for AdoMetDC. Real-time quantitative PCR results for the three clonal lines showed a reduction in mRNA levels of 72-74% for AdoMetDC, 61-71% for ODC and 30-70% for SpdSyn 48 h after addition of tetracycline. (Fig 3). The greater variability observed between clonal lines for SpdSyn and the on average poorer knockdown is consistent with the observation that only one cell line showed a growth defect. The cell line with the best knockdown efficiency (70%) was also the line that showed a growth defect, while the line that showed poor knockdown efficiency (30%) did not show a growth effect. In previous reports of RNAi knockdown of these genes qPCR was not used to quantitate the level of RNA knockdown, though for AdoMetDC quantitation was performed by evaluation of the Northern blots using a Typhoon scanner and knockdown levels of 70-80% were observed [12], similar to the effects observed for the Gateway AdoMetDC clone.

Protein levels were evaluated by western blotting as described previously [12] [13]. Protein (30 µg) was loaded for analysis onto SDS-polyacrylamide gel and transferred onto PVDF (Amersham) membrane. Membranes were probed with rabbit polyclonal antibodies against *T. brucei* AdoMetDC, SpdSyn and ODC. Dihydroorotate dehydrogenase (DHODH) was used as a loading control employing antibody dilutions and conditions as previously described [10], followed by visualization with ECL western blotting reagents (Amersham Biosciences/GE Healthcare). Western blot analysis indicated that the level of AdoMetDC, SpdSyn and ODC proteins were reduced by 24 h of induction. No change was observed in DHODH levels used as a loading control (Fig 2 insets). Three independent clonal lines were analysed for each gene and all showed similar results. Representative data for one cell line is shown in Fig. 2.

These results indicate that the pTrypRNAiGate vector has the potential to facilitate the generation of hpRNA constructs, rapidly and efficiently and thus provides a useful resource for *T. brucei* functional genomics. The data reported herein were collected in blood form parasites, however we have also found that the vector functions to generate knockdown in procyclic cells (data not shown). In conclusion, this vector facilitates the high-throughput cloning of gene libraries or a large number of defined genes, using an *in vitro* recombinase system. The system can be used for large scale determination and discovery of trypanosome gene functions in the same way as RNAi is being used to examine gene function in other organisms such *Caenorhabditis elegans*, plants and *Drosophila*.

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Fig 1A. A schematic flowchart of Gateway® cloning of hairpin RNAi constructs.

PCR product of the gene of interest from a High fidelity Taq Polymerase reaction is cloned into pCR®8/GW/TOPO® vector between the *attL1* and *attL2* sites to generate an entry clone. The entry clone is then recombined with the destination vector (pTrypRNAiGate) containing the *attR1* and *attR2* sites with the LR clonase enzyme. The final RNAi expression clone is obtained after transformation and selection with suitable markers.

Fig 1B. Map of Gateway® compatible vector (pTrypRNAiGate) for RNAi in *T. brucei*. pTrypRNAiGate is a modified pLEW100 vector containing two copies of the toxic *ccdB* gene and chloramphenicol gene in inverted direction with a stuffer in between. The “stuffer” DNA is a part of human protein Pex11 β . The two *ccdB* conversion cassettes with *attR* sites in opposite orientation and the stuffer sequence are inserted in the inducible expression site between the HindIII and MluI sites of pLEW100.

Fig 2. Effects of RNAi induction using pTrypRNAiGate vectors targeting AdoMetDC, SpdSyn and ODC.

Plots show growth curves of total cell numbers calculated as the product of cell density and the total dilution factor plotted against time (days) post-tet induction (1 µg/ml) for A) AdoMetDC and B) SpdSyn and C) ODC. Spermidine (Spd) (0.1 mM) or putrescine (Put) (0.5 mM) were utilized as rescue agents as indicated on the graphs. Three clonal lines for each gene were analyzed and data represent the average for the three experiments. Error bars for ODC and AdoMetDC represent the SEM calculated from average of three different clonal lines. For SpdSyn error bars represent the observed experimental range to illustrate the clonal variation that was observed. Northern analysis (Northern) of AdoMetDC (panel AD), SpdSyn (panel SS) and ODC (panel ODC) and tubulin (panel T) is displayed as an inset. Ten µg of total RNA was used in each lane. Western analysis (Western) is also displayed as an inset on each graph. Each lane contains 30 µg of total cell lysate. DHODH protein level (panel D) was used as a loading control.

Fig 3. Real-time quantitative PCR analysis.

RNAi reduces steady-state level of AdoMetDC, SpdSyn and ODC mRNAs. Total RNA isolated from cells after induction of AdoMetDC, SpdSyn and ODC RNAi for 24 h and 48 h was used. The abundance of mRNAs from induced cells is shown relative to that from uninduced cells. The mRNA levels were normalized to telomerase reverse transcriptase, TERT mRNA. Error bars represent the SEM calculated from average of three different clonal lines.