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A unified approach towards *Trypanosoma brucei* functional genomics using Gibson assembly

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ABSTRACT

Trypanosoma brucei is the causative agent of human African trypanosomiasis and nagana in cattle. Recent advances in high throughput phenotypic and interaction screens have identified a wealth of novel candidate proteins for diverse functions such as drug resistance, life cycle progression, and cytoskeletal biogenesis. Characterization of these proteins will allow a more mechanistic understanding of the biology of this important pathogen and could identify novel drug targets. However, methods for rapidly validating and prioritizing these potential targets are still being developed. While gene tagging via homologous recombination and RNA interference are available in *T. brucei*, a general strategy for creating the most effective constructs for these approaches is lacking. Here, we adapt Gibson assembly, a one-step isothermal process that rapidly assembles multiple DNA segments in a single reaction, to create endogenous tagging, overexpression, and long hairpin RNAi constructs that are compatible with well-established *T. brucei* vectors. The generality of the Gibson approach has several advantages over current methodologies and substantially increases the speed and ease with which these constructs can be assembled.

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1. Introduction

Trypanosoma brucei is a protist parasite that causes enormous harm to both humans and livestock in Sub-Saharan Africa [1]. The parasite has been the focus of intense research efforts, originally focusing on morphological analysis and observational studies to understand the parasite's life cycle and how it evades the host immune response [2,3]. The advent of molecular biology has opened up a host of powerful tools for studying trypanosomes, including gene tagging and inducible expression using the tetracycline suppressor system [4,5]. Unlike several closely-related kinetoplastid parasites, *T. brucei* has retained the machinery necessary for RNA interference, which allows straightforward access to loss of function experiments [6,7]. These tools have been used to develop high-throughput approaches for gene analysis using inducible whole-genome RNAi and next generation sequencing (RIT-seq) [8]. This method has been used in a host of screens to identify genes involved in the parasite's transition from the mammalian-infectious bloodstream form to the insect-resident (procyclic) form and genes that are essential for resistance to several trypanocidal agents [9,10]. Protein-protein interaction approaches such as *in vivo* biotinylation using a mutant of the bacterial biotin ligase BirA (BioID) and high-throughput GFP tagging have begun to uncover components of many enigmatic cytoskeletal structures that are essential for cell polarity and motility [11–14]. Advances in genomic and proteomic methods have also identified key components of different cellular pathways that merit further study [15–19].

The initial characterization of *T. brucei* proteins relies on gene tagging for localization and tetracycline-inducible RNAi to establish function. There are numerous approaches for gene tagging, including constitutive overexpression, tetracycline-inducible overexpression, and tagging of the endogenous locus (Fig. 1**A, B**) [5,11,20–24]. Epitope tags such as the HA and Ty1 tags are commonly used, along with GFP for imaging of live cells [11,25]. Tetracycline-inducible RNAi plasmids originally employed flanking T7 promoters and tetracycline suppressors to produce double-stranded RNA suitable for triggering mRNA degradation, although single-stranded long hairpin RNAs (lhRNAs) are now favored due to lower levels of background expression and improved basepairing [26–30]. While these approaches are effective, there are significant

Abbreviations: lhRNAi, long hairpin RNAi; BioID, proximity-dependent biotin identification.

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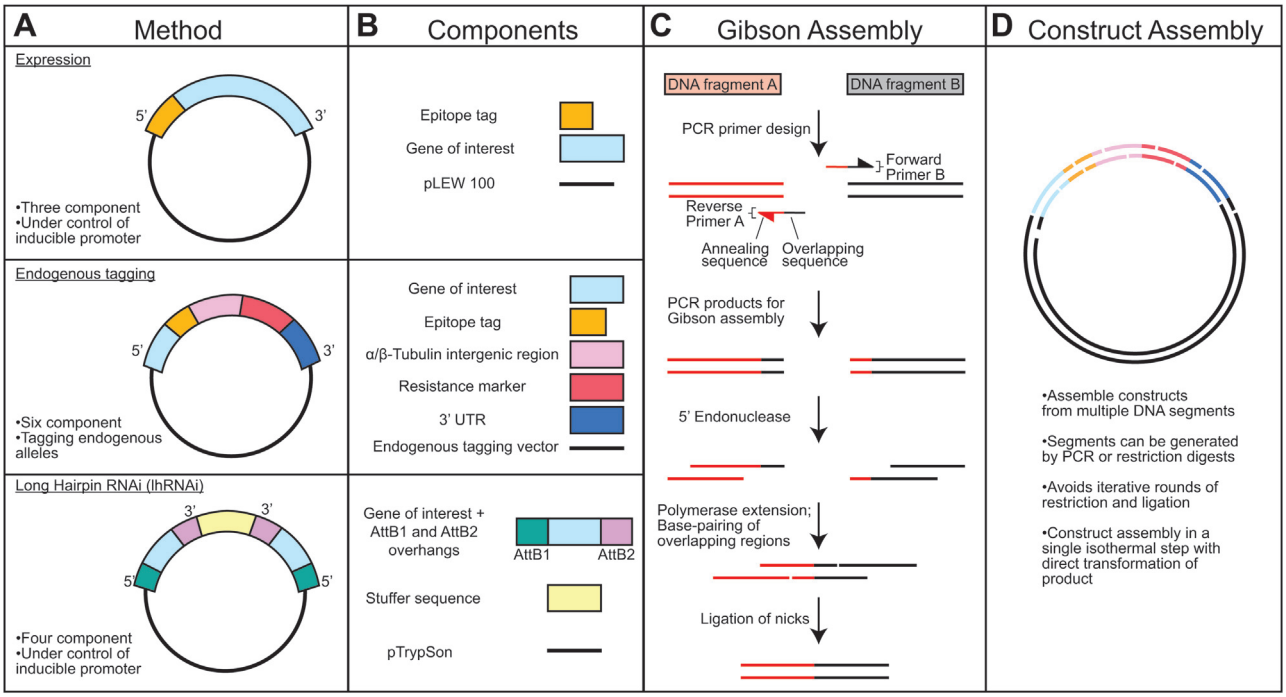

Fig. 1. Overview of the Gibson assembly method. [**A**] Three commonly used plasmids for functional genomics in *T. brucei*. Expression- A conventional expression vector for inducible control of a gene of interest. Endogenous tagging- A vector that introduces a tag and a selection marker to an endogenous locus. Long hairpin RNAi (lhRNAi)- A plasmid that provides inducible expression of a long hairpin RNAi for depletion of a protein of interest. [**B**] The specific components comprising each of the constructs described in **A**. [**C**] A schematic of the Gibson assembly process. [**D**] An overview of how multiple DNA segments can be assembled into a completed construct.

shortcomings that can decrease throughput when a large number of genes need to be assessed. Most available tagging and overexpression plasmids lack multiple restriction sites for cloning, which can require blunting or other workarounds. Endogenous tagging methods either require multiple assembly steps to clone portions of the gene of interest to direct the tag or use smaller targeting segments such as overhangs in primers, which can make PCR difficult and decrease homologous recombination efficiency, especially if the second allele is being targeted [21,31]. For the production of RNAi hairpins, intermediate steps are frequently necessary to assemble the hairpin prior to insertion into the final tetracycline-inducible plasmid [28,29]. A rapid, general strategy for making these constructs would allow more genes to be studied in a more cost-effective manner.

Gibson assembly is a single-step isothermal reaction that rapidly assembles segments of DNA with overlapping termini (Fig. 1**C**) [32]. The method employs a mixture of three enzymes: a 5′ exonuclease that exposes overhanging sequence for specific annealing of the complimentary DNA segments, a DNA polymerase that fills in the overhangs, and a DNA ligase that links the segments. The method requires 15–20 base pairs of homologous sequence, so specificity can easily be encoded within non-annealing overhangs in PCR primers. Gibson-compatible segments can also be generated by restriction digest, which is especially useful for including plasmid backbones in the assembly reaction. Gibson assembly is remarkably robust and has been used to assemble whole genomes from small DNA segments, showing the generality of the method [33]. In this work, we show how the Gibson approach can be used to create many of the constructs used for gene analysis in *T. brucei*, including tetracycline-inducible expression, endogenous replacement, and RNAi (Fig. 1**D**). The Gibson approach removes many obstacles such as restriction enzyme incompatibilities, the need for intermediate ligation steps, and the limited size of targeting segments, allowing the rapid assembly of the best-suited constructs for probing gene function.

2. Materials and methods

2.1. Molecular biology

Enzymes used in this study were from New England Biolabs (Ipswich, MA) and chemicals from Thermo Fisher Scientific. PCR was performed with Q5 High Fidelity Polymerase in Q5 buffer (NEB). Plasmids were prepared for transfection with GeneJET Plasmid Midiprep Kit (LifeTechnologies). PCR primers used in this work are included in **Supplemental** Fig. 1, while the sequences of all the constructs are in **Supplemental** Fig. 2.

2.2. Gibson assembly

Gibson assembly, also known as isothermal chew-back-anneal assembly, was conducted as described [32]. Briefly, typically 10 μg of vector backbone was digested to generate linearized vector for assembly reactions, followed by treatment with 10 units of calf intestinal alkaline phosphatase (NEB) for 1 h at 37 °C. PCR was conducted using Q5 polymerase (NEB) with the manufacturer's standard conditions to generate fragments bearing 20 bp overhangs of desired homology to the flanking region. Both vector fragments and PCR products were purified by gel purification (Zymoclean Gel DNA Recovery Kit, Zymogen) prior to assembly. Fragments were combined in either commercially available (New England Biolabs Gibson Assembly Master Mix) or homemade Gibson Assembly Master Mix. The homemade Master Mix was prepared by combining 699 μL water, 320 μL 5 x isothermal reaction buffer (500 mM Tris-Cl, pH 7.5, 250 mg/mL PEG-8000, 50 mM $MgCl_2$, 50 mM DTT, 1 mM each of four dNTPs, 5 mM beta-NAD), 0.64 μL T5 Exonuclease (Epicentre, 10 U/μL), 20 μL Phusion DNA polymerase (NEB, 2 U/μL) and 160 μL Taq DNA ligase (NEB, 40 U/μL). This solution was divided into 15 μL aliquots and stored at −20 °C. Vector to fragment ratios were variable; typically, 100 ng of linearized vector was added to the mixture with a 2-fold excess of each PCR fragment. PCR frag-

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