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Short technical report

Identification of essential and non-essential protein kinases by a fusion PCR method for efficient production of transgenic *Trypanosoma brucei*

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ABSTRACT

Manipulation of gene expression has been used to elucidate gene function, explore fundamental biological processes and to identify potential drug targets in *Trypanosoma brucei*. We show in bloodstream forms that CDC2-related kinase CRK12 (Tb11.01.4130) is essential since transcriptional inactivation in conditional null mutants is lethal but 19 other protein kinases are not essential since null mutants are viable. We did so using efficient methods for the generation of null and conditional null cell lines of *T. brucei* by approaches that generate transfection constructs with large targeting sequences and which use reliable transfection and selection conditions. These methods, which are described in detail in the supplementary material, employ multiple oligonucleotides and PCR reactions and several transfections but are cost effective and can simultaneously generate 24 transfectants thus shifting the rate limiting experimental steps from the production of cell lines to their analysis.

Manipulation of gene expression is a powerful tool for elucidating the functions of genes in the context of a biological system and for assessing the potential of the gene products as drug targets. The functions of numerous genes have been examined in *Trypanosoma brucei* in order to explore fundamental biological processes and to search for drug targets. In addition, this diploid protozoan extracellular pathogen has served as an experimental surrogate for the related intracellular kinetoplastid pathogens *T. cruzi* and *Leishmania* because of their highly homologous and syntenic genomes and because experimental genetic manipulation is more developed in *T. brucei* [1].

The robust homologous recombination system that is naturally present in *T. brucei* has been exploited to make gene knockouts (KOs) and other genome modifications. For gene KOs, cells are transfected by electroporation with a linear DNA construct that contains an antibiotic resistance gene, *i.e.* selectable marker, with flanking sequences that target recombination which results in replacement of the endogenous gene with the selectable marker. The creation of a null mutant, *i.e.* a cell in which both alleles eliminated, is a definitive way to identify non-essential genes. Conditional RNA interference (RNAi) [1], by which expression of a target gene is repressed *via* mRNA destruction in a tetracycline(tet)-dependant manner, has been commonly used in *T. brucei* to assess gene function and essentiality. RNAi cell line production entails the cloning of single RNAi construct and single transfection step into the appropriate cell line. The ability to efficiently generate RNAi cell lines has led to its systematic use to assess many genes on one chromosome [2] and for genome-wide analysis of genes using deep sequencing of a population of RNAi cell lines [3]. An additional advantage to the RNAi approach is that mRNAs from nearly identical paralogs can be simultaneously targeted with a single construct. However, RNAi has the disadvantages that it may not sufficiently reduce the mRNA levels to generate a definitive phenotype and can have off-target effects, *i.e.* degradation of mRNA from genes that were not intentionally targeted. Little is known about off-target RNAi effects in *T. brucei*, however, and genetic approaches can be used to verify that RNAi is acting on-target [4]. We prepared null cell lines by knocking out both endogenous alleles in cells and a "conditional null" cell line by knocking out both endogenous alleles in cells that have a tet-regulatable copy of the target gene's coding DNA sequence (CDS) to determine the essentiality of several protein kinases. Expression knockdown is very robust in conditional nulls cell lines but these cell lines have not been widely used because their preparation requires multiple transfections and cloning steps. However, we improved the efficiency of their production by minimizing the need to prepare plasmid constructs and improving transfection and selection conditions.

We employed an efficient PCR approach to make gene KO DNA constructs instead of a sequential PCR/cloning approach (*e.g.* [5–9]) since the latter is time consuming and has pitfalls that reflect the limitations of restriction enzyme digestion, DNA fragment purification, and ligation efficiency. A PCR approach has been used to directly produce the DNA constructs for transfection by a single

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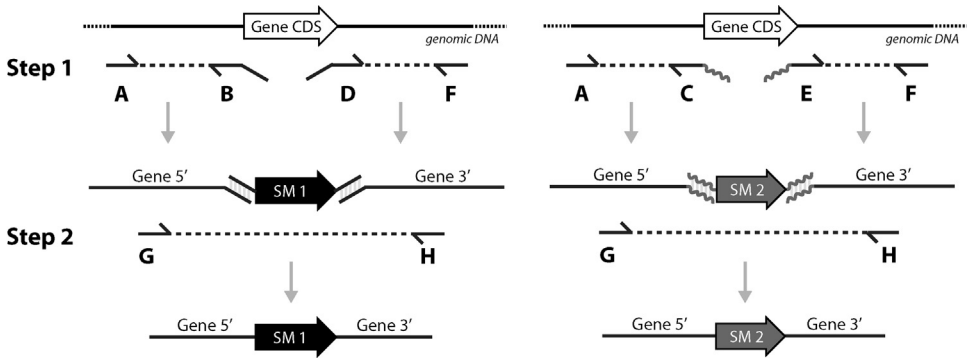

Fig. 1. Schema for generation of transfection constructs. Fusion PCR method that generates gene knockout (KO) constructs. Step 1: gene-specific 5′ and 3′ targeting sequences of both alleles are amplified by separate PCRs using six different oligonucleotides (oligos). Step 2: The 5′ and 3′ targeting fragments of each allele are fused by PCR with the coding DNA sequence (CDS) of their respective selectable marker (SM) that is amplified from a plasmid stock. Complementary sequences are designed into the termini of the oligos as illustrated and nested oligos G and H provide for specific amplification of the fusion PCR product. Approximately 25–50 ng each of 5′ and 3′ targeting sequences and a drug resistance CDS PCR products were combined and fused by PCR amplification. The fusion PCR products were verified and purified on an agarose gel and recovered with a Qiagen gel extraction kit. Fusion PCR protocols and oligo sequences are detailed in supplementary material.

PCR reaction of drug resistance markers with long oligos (~100 nucleotides) that contain targeting sequences for gene-specific KOs (*e.g.* [10] and http://tryps.rockefeller.edu/trypsru2_genetics.html). We instead employed a "fusion PCR" approach to generate KO constructs with sizeable homologous targeting sequences (Fig. 1) since the use of long oligos for PCR adds significant cost and effectively limits the length of homologous targeting region. This targeting region limit can affect the frequency of homologous recombination since it has been shown that shorter targeting sequences result in lower recombination frequency in *T. brucei* [11] as has been seen for other organisms. Hence, we used long (~500 nt) targeting sequences to potentially provide for a greater recombination frequency, and thus a greater frequency of transfection. These larger targeting regions were used with a fusion PCR approach to enhance the efficiency of transfection construct production.

The fusion PCR method that we used (Fig. 1) was adapted from that used in other systems (*e.g.* [12,13]). Multiple PCR reactions were used to rapidly, inexpensively, and effectively generate DNA for direct use in transfections. Two separate PCR reactions were used to amplify 5′ and 3′ targeting sequences from *T. brucei* genomic DNA for each gene. The inner oligos each contain a sequence that is complementary to the outer sequences of a selectable marker that has been PCR amplified in bulk from plasmid DNA and gel purified. The 5′, 3′ and selectable marker PCR products were combined after removal of the PCR primers using a commercial spin column (Qiagen) and amplified by another PCR reaction which produces the fusion PCR product for transfection. Six unique oligos are needed to create the fusion product for KO of each allele (Fig. 1). For the 1st allele the two inner oligos (oligos B, D) contain sequences that complement those of the 1st selectable marker, the two outer oligos (oligos A, F) provide for amplification of ~500 nts of targeting sequence flanking the gene CDS, and two oligos (G, H) that are nested (*i.e.* internal to oligos A and F) for specificity and to create the fusion PCR product that will be used for transfection. Two additional oligos (oligos C, E) are needed which contain sequences that are complementary to the 2nd selectable marker to generate the PCR product that will be used in the fusion PCR to generate the construct to KO the 2nd allele. The original two outer oligos and those for the nested PCR can be the same (*i.e.* oligos A, F, G, H).

The fusion PCR transfection constructs for two alleles made in this manner will have the same targeting sequences. This can result in the construct for the 2nd allele KO recombining with the site of 1st allele KO. However the use of two drugs in combination will select against such cells making this approach suitable for high throughput studies. Nevertheless, recombination of the 2nd allele KO construct with the site of 1st allele KO can (and does) occur. This will not eliminate the 2nd endogenous allele but will insert at the site of the 2nd selectable marker and either retain or replace of the 1st selectable marker. This can be averted as detailed in the supplementary material by using oligo pairs that are internal to A, B and D, F to PCR amplify the targeting sequences for the 2nd selectable marker. This results in the need for two additional oligos and for awareness of possible consequences to adjacent genes (*e.g.* deletion of important regulatory or coding sequences). Thus, minimally eight, and maximally ten, oligos are needed to KO two alleles, which is more than the number used for the long oligo KO approach. However, the total cost of these eight to ten oligos is a fraction of the cost of the longer ones due to their smaller size. Currently the total reagents costs for the fusion PCR is 3–4 times less than for the single PCR method using long oligos and is due to the substantially greater cost of the long oligos. In addition, the larger flanking gene targeting regions of the fusion PCR products provide for higher recombination and transfection efficiencies. The oligos are designed to have similar annealing temperatures so that all of the PCR products can be generated under the same conditions. This PCR protocol has been scaled up to a 96-well format which enables simultaneous production of PCR products for the KO of 12–24 genes (see supplementary materials for details). More PCR products can be produced at a time (*e.g.* for 48–96 genes) but the subsequent steps of transfection, cell maintenance and confirmation of the desired transfections effectively limit production to 24 gene KOs at a time by one person.

Fusion PCR products were prepared and used to KO target genes in *T. brucei* to attempt to create null mutants, *i.e.* eliminate both endogenous alleles, for 20 predicted protein kinases. The creation of null mutants would definitively demonstrate that the eliminated gene is not essential for viability. A conditional null mutant was produced when null mutants could not be obtained. The protein kinases chosen are primarily members of the Sterile (STE) and Other protein kinase groups which had not been thoroughly studied genetically in *T. brucei*. Null mutants were generated by the higher-throughput fusion PCR transfection method using two different selectable markers to sequentially KO both endogenous copies of target genes (see supplementary materials for KO schematic). These constructs were designed to precisely delete the target gene from the start to the stop codon. One endogenous allele was eliminated by transfecting purified fusion PCR products by electroporation into bloodform (BF) *T. brucei* single marker 427 cells (SM427) [14]. The SM427 cell line expresses T7 polymerase and Tet repressor proteins in the β tubulin locus (maintained with *NEO* selectable marker) to control tet conditional expression