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Short technical report

# A dual luciferase system for analysis of post-transcriptional regulation of gene expression in *Leishmania*

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ABSTRACT

Gene expression in kinetoplastid parasites is regulated via post-transcriptional mechanisms that modulate mRNA turnover, translation rate, and/or post-translational protein stability. To facilitate the analysis of post-transcriptional regulation, a dual luciferase system was developed in which firefly and *Renilla* luciferase reporters genetically fused to compatible drug resistance genes are integrated in place of one allele of the gene of interest and of an internal control gene, respectively, in a manner that preserves the cognate pre-mRNA processing signals. The sensitivity and reproducibility of the assay coupled with the ability to rapidly assemble reporter integration constructs render the dual luciferase system suitable for analysis of multiple candidates derived from global expression analysis platforms. To demonstrate the utility of the system, regulation of three genes in response to purine starvation was examined in *Leishmania donovani* promastigotes. This dual luciferase system should be directly applicable to the analysis of post-transcriptional regulation in other kinetoplastids.

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Transcription in kinetoplastid parasites is polycistronic, resulting in the production of long multigene pre-mRNAs that require coupled trans-splicing and polyadenylation reactions for processing into mature single-gene mRNAs [1]. Consequently, regulation of gene expression in these organisms occurs primarily post-transcriptionally through mechanisms that control mRNA abundance, translation rate, and post-translational protein stability [2]. Numerous studies have shown that control of mRNA and translational levels is mediated predominantly by elements encoded within the 5′ and 3′ untranslated regions (**UTRs**) of mRNAs [3–5], though regulatory elements have been also been found in coding sequences (CDS) [6,7]. The application of systems-level approaches (e.g., RNA-seq and whole proteome profiling) to the study of global gene regulation in these parasites is becoming more common [8–14]. While these approaches typically yield a multiplicity of candidates with altered mRNA or protein abundance in response to a particular growth condition or developmental program [9,10], understanding the contributions of translational and post-translational mechanisms to the regulation of individual candidates usually requires additional downstream analysis.

A variety of heterologous reporter systems (e.g., chloramphenicol acetyltransferase, β-galactosidase, β-glucuronidase, firefly (**Fluc**) and *Renilla* (**Rluc**) luciferases) have been employed to examine translational regulation and to identify *cis*-regulatory elements controlling mRNA abundance and translation in kinetoplastids [4,15–17]. The studies presented herein describe the development of a Fluc/Rluc dual luciferase reporter system that allows post-transcriptional regulation to be readily assessed for multiple candidates derived from systems-level gene expression studies of kinetoplastid parasites. A key feature of this system is the utilization of versions of the *Fluc* and *Rluc* genes fused in-frame with each of five differing drug resistance genes (referred to as **Luc-DRG** fusions; see Supplementary Materials and Methods). This enables direct selection for replacement of the CDS from one allele of the gene of interest with a luciferase reporter in a manner that preserves the cognate pre-mRNA processing signals; hence, the contribution of the 5′ and 3′ UTRs to regulation should be reflected in luciferase expression from the *Luc-DRG* allele. Integration of the reporter gene should maintain physiological levels of reporter message, and circumvents potential reproducibility issues due to cell-to-cell variation in copy number and non-physiological expression in episome-based reporter systems. In our preferred configuration of this system, *Rluc* fused to a puromycin resistance gene (***Rluc*-PAC**) is integrated in place of one allele of a control gene, the expression of which does not change under the conditions of the experiment

*Abbreviations:* Fluc, firefly luciferase; *Fluc-BSD*, firefly luciferase fusion to blasticidin S deaminase; Rluc, *Renilla* luciferase; *Rluc-PAC*, *Renilla* luciferase fusion to puromycin acetyltransferase; *UMPS*, uridine monophosphate synthase; UTR, untranslated region.

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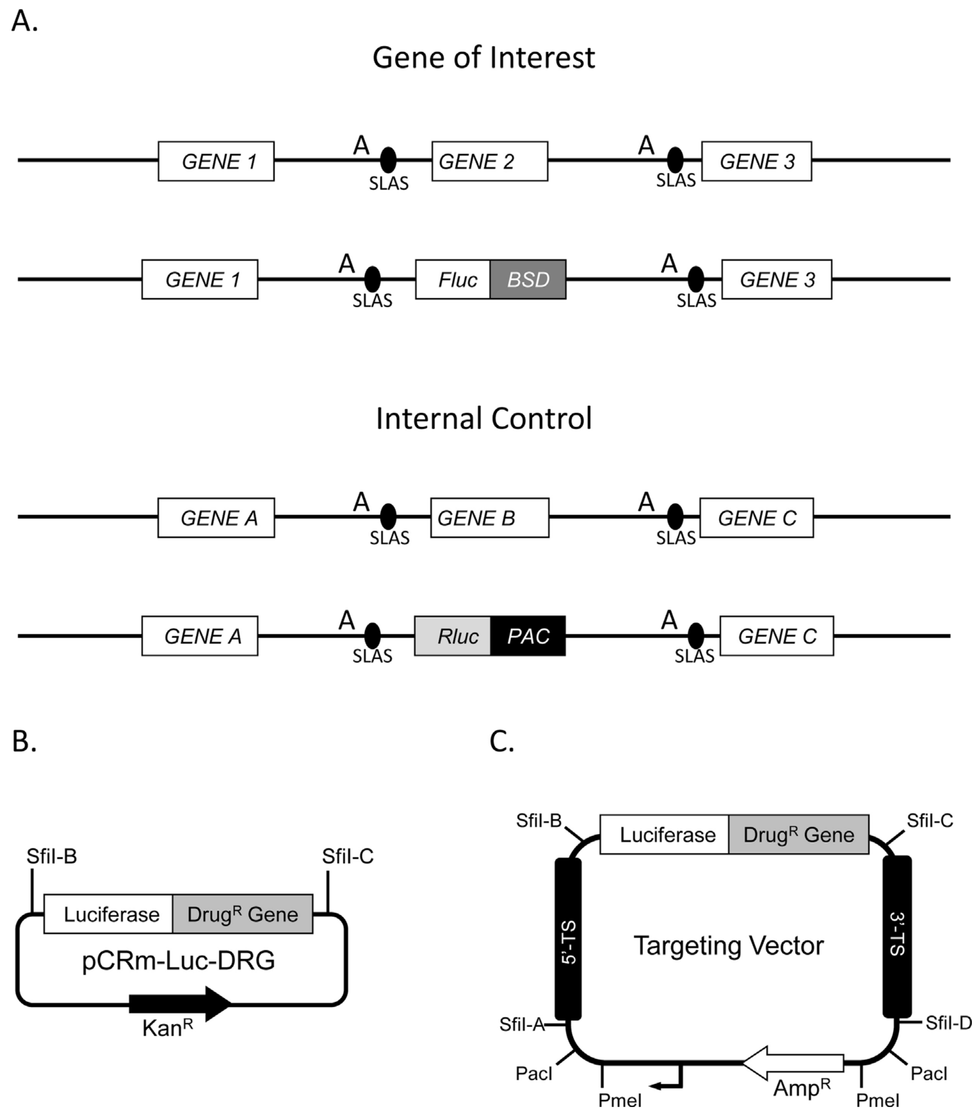


**Fig. 1.** Configuration and key components of the dual luciferase system. (A) *Rluc*-puromycin resistance gene (Rluc-PAC) and *Fluc*-blasticidin resistance gene (Fluc-BSD) fusions flanked by appropriate targeting sequences (TS) are sequentially transfected to replace the CDS of one allele of either a control gene or a gene of interest, respectively, via homologous recombination. The cognate spliced-leader acceptor site (SLAS; represented by black ovals) and polyadenylation site (represented by the "A" adjacent to the SLAS) are preserved and drive expression of the drug resistance gene, obviating the need for exogenous pre-mRNA processing signals that could disrupt regulation of the reporter. (B) The *Fluc* and *Rluc* genes were fused to genes encoding resistance to blasticidin, hygromycin, neomycin, phleomycin, and puromycin and inserted into pCRm to generate donor vectors. All of the luciferase-drug resistance gene (*Luc-DRG*) fusions retain robust luciferase activity and the ability to confer drug resistance upon chromosomal integration (data not shown). The *Luc-DRG* fusions are flanked by SfiI restriction sites designed to present non-identical 3′-overhangs upon SfiI digestion (designated SfiI-B and SfiI-C) that fit into our modular targeting vector construction strategy (18). (C) The targeting fragment, consisting of a *Luc-DRG* reporter flanked by the appropriate 5′ and 3′ TSs, can be released from the targeting vector by digestion with either PacI or PmeI. GenBank accession numbers for the pCRm-Luc-DRG donor vectors, as well as construction details for the *Luc-DRG* fusions, donor vectors, and targeting vectors are provided in the Supplementary Materials and Methods.

(Fig. 1A). The resultant cell line serves as the recipient for subsequent transfections of *Fluc*-blasticidin resistance gene fusions (***Fluc*-BSD**) targeting various genes of interest. The power of a dual luciferase system lies in the ability to sequentially measure Fluc and Rluc reporter luminescence from the same aliquot of a cell extract using a luminometer. In our system, the presence of an *Rluc* reporter integrated at a control locus in the same cell allows normalization of Fluc luminescence, adjusting for experimental variations such as cell number, pipetting errors, and efficiency of cell lysis.

The availability of a variety of *Luc-DRG* fusions provides the flexibility to find a compatible pair of *Fluc* and *Rluc* reporters for integration into cell lines that may already express one or more drug resistance markers. To enhance the efficiency with which the system can be implemented, the luciferase-drug resistance gene fusions were incorporated into donor vectors compatible with a previously described method from our laboratory for rapidly generating gene targeting constructs via multi-fragment ligation [18] (Fig. 1B). In this method, all of the targeting vector components (5′ and 3′ targeting sequences, a luciferase-drug resistance gene fusion, and a minimal plasmid backbone) are digested with SfiI, gel purified, and combined for directional ligation to form the completed targeting vector (Fig. 1C). Luciferase reporter targeting vectors can be assembled in three to four days and several constructs can be processed in parallel, greatly facilitating the analysis of multiple candidates.

As a first step in validating the system, it was important to examine the sensitivity and linear range of detection for integrated *Fluc* and *Rluc* reporter constructs. An *Leishmania donovani* promastigote line in which *Fluc-BSD* and *Rluc-PAC* reporters had replaced one allele each of the *LdNT2* and UMP synthase (***UMPS***) genes, respectively, was grown to mid logarithmic phase and 5-fold serial dilutions of the culture were independently processed for analysis

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