



## Short technical report

## An improved and secreted luciferase reporter for schistosomes

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**Abstract**

Schistosomes are multicellular parasites of humans exhibiting interesting biological adaptations to their parasitic lifestyle. Concerted and in depth analyses of these adaptations and their cell and molecular biology requires further development of molecular genetic tools in schistosomes. In the current study, we demonstrate that a *Gaussia* luciferase reporter leads to significantly higher levels of luciferase activity in schistosomes compared to other tested luciferases. In addition, *Gaussia* luciferase can be secreted into culture media enabling non-invasive analysis of reporter activity. The secretion of *Gaussia* luciferase should allow a variety of new experimental paradigms for schistosome studies. Comparison of biolistic and electroporation transfection methods using luciferase RNA reporters and the luciferase activity produced indicates that electroporation of sporocysts and schistosomula is the most efficient transfection method for the four stages analyzed. These data should facilitate additional studies in schistosomes and provide a framework for further development of DNA transfection and gene expression analysis.

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Schistosomiasis is a major public health problem in 76 countries where it afflicts more than 200 million people [1]. Schistosomes exhibit a complicated life cycle including two free-living stages in water and parasitic stages in the intermediate snail host and definitive vertebrate host [2]. A large number of expressed mRNAs, genes and proteins have recently been identified or predicted in schistosomes from cDNA, genomic, and proteomic analyses [3–5]. However, in most cases, few functional details are available regarding these genes or proteins due to the lack of comprehensive molecular tools for schistosomes to elucidate their roles *in vivo* [6–8]. Some progress has been made toward the development and use of RNA interference methods, transfection methods, expression and integration of DNA constructs, and gene reporters [9–28]. There is a great need for further development and comparison of reporter genes and transfection protocols for different schistosome stages to provide researchers with a resource for choosing the most effective and appropriate tools for a particular experimental paradigm. In the present study, we evaluated the use of *Gaussia* luciferase

as a reporter for use in schistosomes. In addition, we evaluated a variety of electroporation and biolistic transfection protocols to compare the effectiveness of these transfection protocols in different schistosome stages.

*Gaussia* luciferase is a new reporter luciferase from the marine copepod *Gaussia princeps* [29,30]. This 185 amino acid (19.9 kDa) monomeric luciferase does not require any cofactors (e.g., ATP) and catalyzes the oxidation of the substrate coelenterazine in a reaction that emits light (480 nm) similar to *Renilla* luciferase. The reporter can be used in a Dual-Luciferase Assay with *Photinus* (Firefly) luciferase, enabling the use of a control luciferase reporter in experimental paradigms [34]. This new luciferase has several major advantages over previous luciferases (Firefly or *Renilla*): (1) human codon-optimized *Gaussia* luciferase generates at least 100-fold higher bioluminescent signal intensity in mammalian cells, compared to Firefly and *Renilla* luciferases; (2) *Gaussia* luciferase possesses a natural secretory signal consisting of 16 amino acids, is secreted into the media, and thus expression can be evaluated without lysis of the cells or organisms; (3) *Gaussia* luciferase coding region is 555 nt, the smallest luciferase reporter.

To evaluate *Gaussia* luciferase as a potential reporter for schistosomes, we transfected Firefly, *Renilla* and *Gaussia* luciferase RNA into different schistosome stages and compared the level of luciferase activity observed in schistosome lysates.

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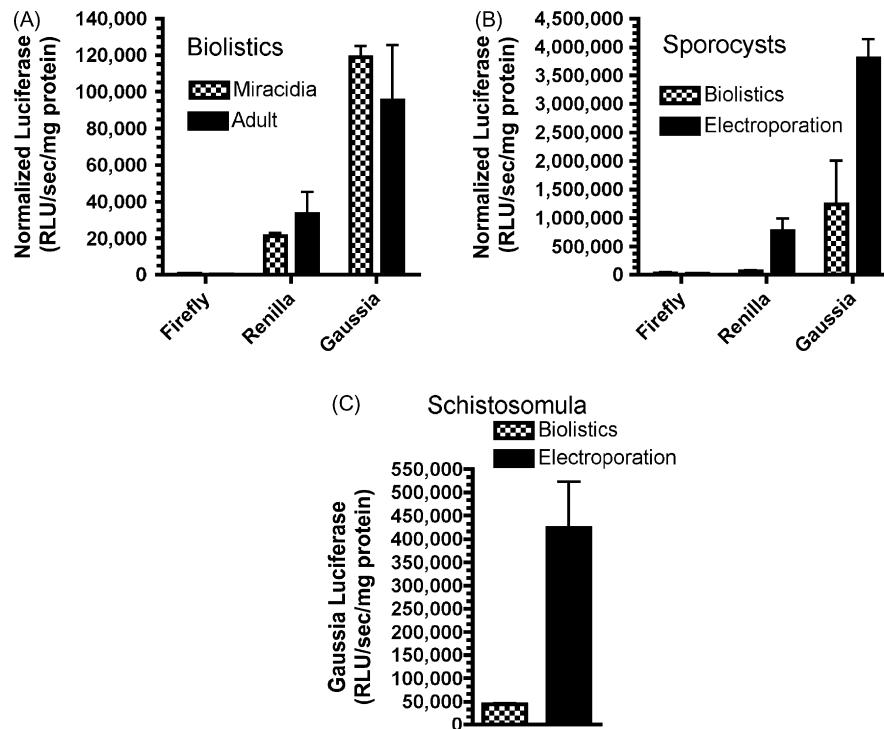


Fig. 1. Expression of luciferase reporters in *Schistosoma mansoni*. (A) Comparison of Firefly, *Renilla* and *Gaussia* luciferase activity in adults and miracidia following biolistic introduction of reporter RNAs. Firefly luciferase activity in miracidia (403 RLU/s/mg) and adults (166 RLU/s/mg) while much lower than other luciferases is reproducibly detected. (B) Comparison of Firefly, *Renilla* and *Gaussia* luciferase activity in sporocyst lysates following introduction of reporter RNAs using either biolistics or electroporation. Firefly luciferase activity in sporocysts, while much lower than other luciferases, is reproducibly detected using either biolistics (24,286 RLU/(s mg)) or electroporation (16,734 RLU/s/mg). (C) Comparison of *Gaussia* luciferase activity in schistosomula lysates following introduction of a reporter RNA using biolistics or electroporation. Biolistic and electroporation conditions used are provided in Table 1 and described in [10]. Luciferase activity in worm lysates in A–C was measured 3 h following RNA introduction using the Promega Dual-Luciferase Assay as previously described [10] with the activities normalized to protein concentrations in worm lysates using the Pierce BCA protein assay kit and Compat-Able protein assay preparation reagent set (Pierce, Rockford, IL). Luciferase activity represents 1/8 of total luciferase activity in parasite lysates. Error bars show standard deviation of a minimum of two independent results. Experiments illustrated are representative and were repeated a minimum of three times with similar results using independently prepared parasites and RNA transcripts. Firefly and *Renilla* DNA templates and their corresponding RNA transcripts were prepared using the primers and methods previously described [10]. *Gaussia* luciferase templates for RNA transcription were prepared using the plasmid pGlu-Basic (Targeting Systems, Santee, CA) and the primers 5'-TAATACGACTCACTATAGGACAAGCTTGGTACCGAGC-3' and 5'-T<sub>75</sub>GACAGTAAGAATTATTCTAGATGC-3'. RNAs were prepared and purified as previously described [10]. Miracidia, sporocysts, schistosomula and adult worms were harvested and cultured as previously described [10,12,35].

RNA reporters instead of DNA reporters were used to enable direct comparison of reporter activity and transfection efficiency independent of potential differences in promoter activity or RNA processing that could occur in different cells and stages. As illustrated in Fig. 1, *Gaussia* luciferase led to much higher levels of luciferase activity than either the Firefly or *Renilla* luciferase reporter in all schistosome stages examined (some data not shown). We next analyzed the time course of *Renilla* and *Gaussia* luciferase activity in sporocysts (Fig. 2A and B). *Gaussia* luciferase activity peaked rapidly within 3 h, began to fall ~12 h post-transfection, and was gone by 30 h. *Renilla* luciferase also peaked rapidly at 3 h, began to fall after ~12 h post-transfection, but significant levels of luciferase remained after 48 h. Thus, *Gaussia* luciferase protein appears to have a significantly shorter half-life than *Renilla*. We estimate that *Gaussia* protein half-life in worms is ~9 h. Similar data were observed in schistosomula (data not shown).

A unique attribute of *Gaussia* luciferase is the presence of a native secretory signal. To examine the secretion of *Gaussia* luciferase in schistosomes, we transfected a *Gaussia* RNA

reporter into sporocysts and measured levels of luciferase activity in both parasite lysates and the culture media over time. As illustrated in Fig. 2E, secretion of *Gaussia* luciferase increased in a linear fashion up to ~9 h, was relatively stable in the media to ~22 h, decayed relatively rapidly after 22 h, and was gone by 30 h. Maximal *Gaussia* luciferase activity was ~1/3 greater in sporocyst media than in lysates. The time course of *Gaussia* secretion in schistosomula was similar to that observed in sporocysts (Fig. 2C and D).

During the course of these studies we also compared levels of luciferase expression using available biolistic or electroporation RNA transfection methods in different schistosome stages (see Table 1). Notably, RNA electroporation of miracidia and adults under a variety of conditions was never successful. Whether this represents the presence of multiple membranes in miracidia that limit efficient transfection or degradation of the RNA at the schistosome surface remains to be determined. In contrast, biolistic introduction of nucleic acids into miracidia and adults is readily achieved (Fig. 1A). However, we have not observed significant secretion of *Gaussia* luciferase following biolistic

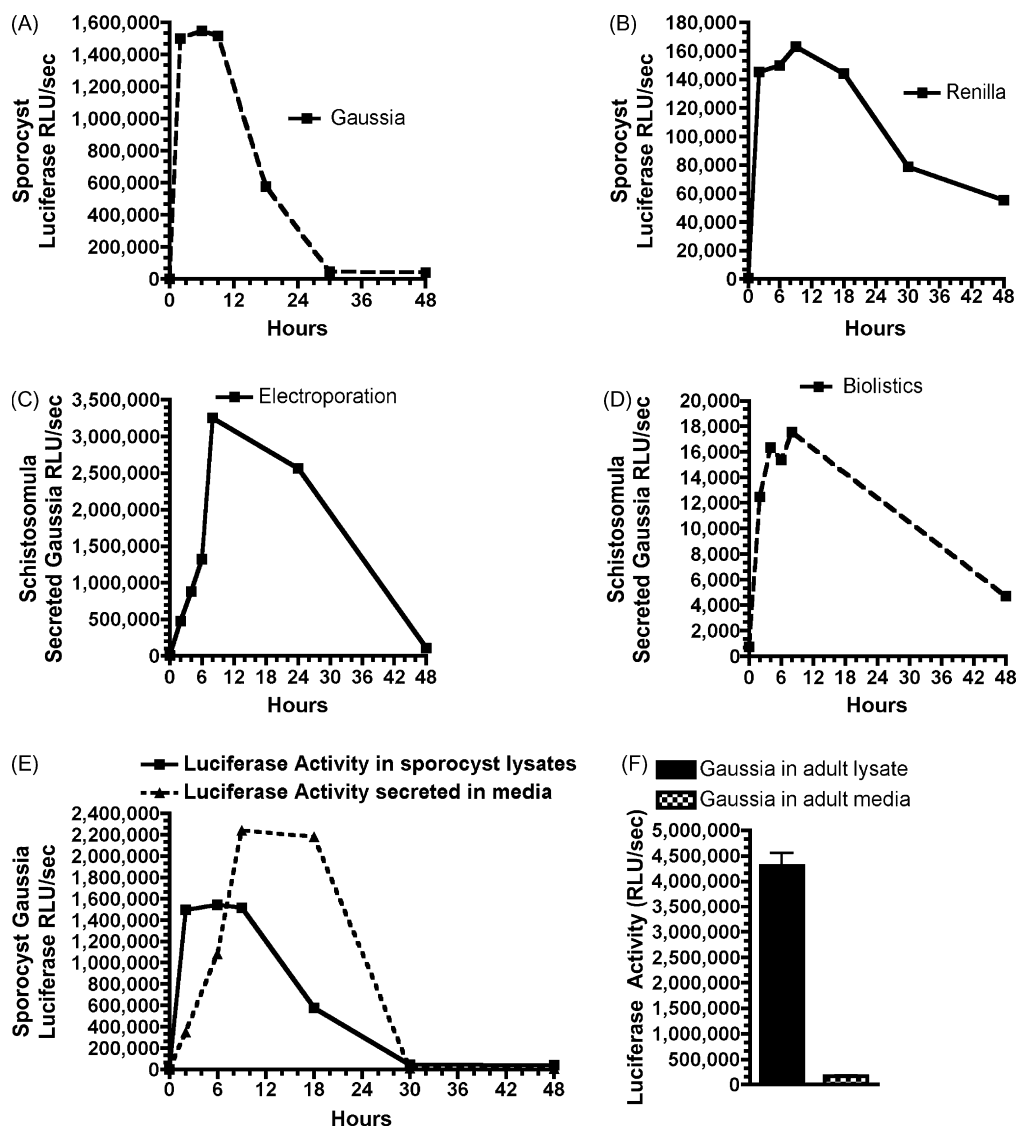


Fig. 2. Time course of *Renilla* and *Gaussia* luciferase activity. Time course of (A) *Gaussia* and (B) *Renilla* luciferase activity in sporocysts following electroporation of RNA reporters. Time course of secreted *Gaussia* luciferase activity following (C) electroporation or (D) biolistics of RNA reporter into schistosomula. (E) Comparison of *Gaussia* luciferase activity over time in sporocyst lysates and secreted into the culture media following introduction of reporter RNA by electroporation. (F) Comparison of *Gaussia* luciferase activity in adult lysates and culture media following biolistic introduction of RNA reporter. Experiments were carried out as in Fig. 1 except that 20  $\mu$ l of culture media were directly assayed for luciferase secreted into the media. Luciferase activity represents 1/8 of the total *Gaussia* activity in worm lysates or culture media.

transfection of RNA into adults (Fig. 2F). One possible interpretation of these results is that the prevailing biolistic conditions (1550 psi) might deliver the gold particles carrying the nucleic acid to a depth within the worms that cannot lead to secretion. However, we have observed gold particles in the tegument and GFP expression has been observed in the adult tegument using biolistics and an actin promoter [27]. Thus, it seems more likely that the adult tegumental syncytium might either degrade the *Gaussia* reporter protein during secretion or its structure limits secretion. Comparison of levels of luciferase expression observed in either sporocysts or schistosomula following RNA transfection using biolistics or electroporation demonstrated that electroporation leads to much higher levels of luciferase activity (Fig. 1). Preliminary data suggest that this is also true for DNA transfection and that parameters in Table 1 can also be used for

DNA transfection (data not shown). Overall, current methods for delivery and analysis of protein normalized luciferase reporter expression are most efficient in sporocysts and schistosomula. From these data, we suggest that additional development and analysis of vectors and expression elements for DNA transfection might be most readily achieved through electroporation of either sporocysts or schistosomula.

To date, fluorescent or luciferase reporters have been tested and used in schistosomes [10,13,16,19,21–24,27,31]. Each of these reporters has its advantages. Fluorescent reporters provide a non-invasive and extremely useful method for evaluating the localization of expression. Furthermore, a variety of differently colored fluorescent reporters are currently available. A potential limitation of these reporters is the level of expression observed. Analysis of several different fluorescent reporters we

Table 1  
*Schistosoma mansoni* RNA transfection conditions

	Adults	Miracidia	Sporocysts	Schistosomula
Number of organisms	50	800–2000	800–2000	100–500
Post-transfection recovery media	RPMI media 1640 with 10% FCS and 200 µg/ml penicillin and streptomycin	MEJMSE-J with 5% FCS and 200 µg/ml penicillin and streptomycin	MEJMSE-J with 5% FCS and 200 µg/ml penicillin and streptomycin	RPMI Media 1640 with 10% FCS and 200 µg/ml penicillin and streptomycin
RNA amount	1 µg RNA per shot	1 µg RNA per shot	1 µg RNA per shot or 10 µg/ml in 200 µl of transfection buffer	1 µg per shot or 10 µg/ml in 200 µl of transfection buffer
Electroporation conditions	— <sup>a</sup>	— <sup>a</sup>	280 V, 0.5 ms, 1 pulse in MEJMSE-J	125 V, 20 ms, 1 pulse in RPMI media 1640 <sup>b</sup>
Biolistic conditions	1550 psi helium pressure, 15 in. Hg, 3 cm stage distance, 1.6 µm or 2.2 µm gold particles, double shots	450 psi helium pressure, 15 in. Hg, 3 cm stage distance, 0.6 µm gold particles	450 psi helium pressure, 15 in. Hg, 6 cm stage distance, 0.6 µm gold particles	450 psi helium pressure, 15 in. Hg, 3 cm stage distance, 0.6 µm gold particles

Biolistics were carried out using a Bio-Rad Biolistics PDS-1000/HE particle delivery system and electroporation using 4 mm cuvettes and a BTX ECM830 square wave electroporator as previously described [10,13].

<sup>a</sup> Although a variety of electroporation conditions were evaluated, conditions were not identified that enabled RNA transfection in adults and miracida of *S. mansoni*.

<sup>b</sup> RNA transfection conditions for schistosomula were described by Correnti et al. [12].

have examined (data not shown) indicates only a small percentage of the parasites exhibit expression requiring considerable time and effort to identify and to evaluate expression of the reporter in parasites. In addition, while fluorescence can be quantitated, its measurement is more complex than luciferase measurement in a luminometer. Luciferase reporters are not typically used as imaging reporters. However, they can be used as imaging reporters and *Gaussia*'s output compared to *Renilla* is ~200-fold better. An advantage of luciferase reporters is their high sensitivity and ease with which quantitative data can be obtained. As shown here, electroporation of a *Gaussia* reporter RNA into sporocysts and schistosomula leads to the expression of high levels of luciferase activity. *Gaussia* luciferase is not only more robust than other luciferases examined, but has the advantage of being secreted in several stages enabling its level of activity to be measured non-invasively in the media. This offers a variety of advantages for experimental paradigms, particularly if the amount of secretion is high. In our experiments, we have easily measured luciferase activity in 20 µl of the media which corresponds to ~200,000 relative light units/s. The media can be concentrated to increase the sensitivity of the assay. As *Gaussia* is secreted from the parasites, this property might enable the identification of: (1) snails infected with sporocysts expressing recombinant DNA; (2) parasites expressing *Gaussia* luciferase following limiting dilution in culture; or (3) possibly the expression of luciferase from schistosomes *in vivo* in the definitive host. For example, as DNA transfection and integration technologies are further developed, it may be possible to monitor expression of *Gaussia* DNA constructs driven by different promoters and secretory signals in adults and during schistosome development in mice through analysis of secreted luciferase in samples of blood drawn from infected hosts. Secretion of *Gaussia* luciferase might also offer a new tool for evaluation of transcriptional regulation associated with signaling pathways. Thus, the influence of host ligands on transcription of schistosome genes could be examined using *Gaussia* reporter DNAs

driven by selected promoters in developing schistosomes in culture. Following DNA transfection, the role of various ligands in modulating transcription could be measured over time in culture media. Furthermore, the relatively short half-life of *Gaussia* protein might enable experimental approaches where the culture media is changed, the worms washed, and new ligands added to evaluate their role on transcriptional regulation of particular genes. Finally, *Gaussia* has recently been adapted for use in protein-fragment complementation assays (PCAs) to study the dynamics of protein–protein interactions *in vivo* and *in vitro* [32]. Using this strategy, a variety of potential applications might be devised to study protein interactions using PCA that could be implemented by schistosome researchers simply using RNA transfection [33].

In the current experiments, we have used a human codon-optimized form of the *Gaussia* coding region. It remains to be determined whether native *Gaussia* codons would lead to higher levels of *Gaussia* expression in schistosomes as human codon-optimized open reading frames may not be optimal for invertebrate expression. Substitution of a native, schistosome secretory signal might improve secretion and be necessary for adults. In addition, a polyclonal antibody against *Gaussia* luciferase is available commercially as an additional tool for *Gaussia* reporter studies (NanoLight Technology, Pinetop, AZ). Furthermore, a second secreted copepod luciferase (*Metridia*) was recently made commercially available (Ready-To-Glow<sup>TM</sup> Secreted Luciferase System, Clontech, Mountain View, CA). *Gaussia* can be used in co-transfection experiments with Firefly luciferase for transfection and data normalization within parasite lysates [34] (data not shown). Finally, promoterless vectors and non-secreted forms of *Gaussia* are also commercially available (Targeting Systems, Santee, CA).

In summary, we have demonstrated that *Gaussia* luciferase is a robust and secreted luciferase reporter that may enable a variety of experimental approaches in schistosomes. The data illustrate that: (1) *Gaussia* is the most robust luciferase reporter for all

stages examined; (2) biolistics can be used to introduce RNA into miracidia and adults where electroporation is not effective; (3) electroporation of luciferase reporters into sporocysts and schistosomula leads to the highest levels of luciferase activity. These data should facilitate additional studies and provide a framework for further development of DNA transfection technologies in schistosomes.

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