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***Plasmodium falciparum*: Development of a Transgenic Line for Screening Antimalarials Using Firefly Luciferase as the Reporter**

Long Cui¹, Jun Miao¹, Jiaxu Wang¹, Qianjun Li², and Liwang Cui^{1,*}

¹Department of Entomology, The Pennsylvania State University, University Park, Pennsylvania 16802, USA

²Drug Discovery Division, Southern Research Institute, 2000 9th Ave. South, Birmingham, AL 35205, USA

Abstract

High-throughput screening (HTS) of small-molecule libraries against pharmacological targets is a key strategy of contemporary drug discovery. This study reports a simple, robust, and cell-based luminescent method for assaying antimalarial drugs. Using transfection technology, we generated a stable *Plasmodium falciparum* line with high levels of firefly luciferase expression. A luciferase assay based on this parasite line was optimized in a 96-well plate format and used to compare with the standard [³H] hypoxanthine radioisotope method. The 50% inhibitory concentrations (IC₅₀s) of chloroquine, artesunate, artemether, dihydroartemisinin and curcumin obtained by these two methods were not significantly different ($P > 0.05$, ANOVA). In addition, this assay could be performed conveniently with a luminescence plate reader using unsynchronized stages within as early as 12 h. Furthermore, the luciferase assay is robust with a Z' score of 0.77 to 0.92, which suggests the feasibility for further miniaturization and automation.

Keywords

Plasmodium falciparum; transfection; firefly luciferase; drug assay

1. Introduction

Despite intensive international efforts, malaria still affects ~5% of the world's population and brings a death toll of 0.5-2.5 million each year (Snow et al., 2005). Particularly, the emergence and spread of resistance to affordable antimalarials such as chloroquine (CQ) and the antifolates (pyrimethamine and sulfadoxine) have caused a sharp rise in malaria-caused mortality in Africa (White, 1999). This situation is further exacerbated in Southeast Asia, where the prevalence of multidrug resistant (MDR) parasites has become a great challenge for malaria management in endemic regions. To avert this disaster, artemisinin-based combination therapy (ACT) is recommended for treating MDR parasites (Ashley and White, 2005). ACT has proven to be very effective in treating *falciparum* malaria without evident clinical resistance, whereas both laboratory studies and suspected clinical cases of artemisinin resistance in *Plasmodium falciparum* suggest that development of resistance to artemisinin-related drugs is likely to occur (Inselburg, 1985; Luxemburger et al., 1998; Sahr et al., 2001; Menard et al., 2005; Afonso et

* **Corresponding author:** Liwang Cui Department of Entomology The Pennsylvania State University University Park, PA, 16802 Phone: 814-863-7663 Fax: 814-865-3048 Email: luc2@psu.edu

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al., 2006). Accordingly, there is a pressing need for discovering and developing novel chemotherapeutic agents against malaria parasites.

Recent advances in new chemical synthesis technologies and the identification of potential pharmacological targets through genomic studies demand the development of high-throughput screening (HTS) assays, which are at the central stage of early drug discovery. Most HTS assays developed so far are based on fluorescence or luminescence detection methods (Fan and Wood, 2007). While both types of detection methods are robust, fluorescent assays can have higher background because of the strong light emission. In contrast, luminescent assays are virtually absent of any background, thus resulting in better signal-to-noise ratios, which often outperform fluorescent assays in multiwell plate formats. Additional properties such as wide dynamic range and superior sensitivity in signal detection of luminescent assays also make miniaturization of the assays possible. Of the two types in vitro HTS assays (cell-free and cell-based), cell-based assays offer additional advantages since they measure the effect of the drugs in a proper biological context (Roda et al., 2003). For cell-based assays, a host cell is engineered to express a reporter gene, which can be one of the three commonly used luciferases, the firefly luciferase, *Renilla* luciferase, and aequorin (Deo and Daunert, 2001; Fan and Wood, 2007). Luciferase assays have been used widely for HTS, from screening drug candidates for viral infections (e.g., HIV and West Nile virus) to those targeting the G-protein-coupled receptors (Chiba-Mizutani et al., 2007; Puig-Basagoiti et al., 2005; Thomsen et al., 2005). While most of these studies were conducted with mammalian cell lines, they have also been used in drug screening in protozoan parasites such as different *Leishmania* species (Sereno et al., 2007). This underlines the potential feasibility of the cell-based HTS method for antimalarial drugs.

Asexual erythrocytic stages of the malaria parasite are the prime targets of antimalarial drugs. With the development of a continuous in vitro culture system for *P. falciparum* erythrocytic stages by Trager and Jensen (1976), in vitro methods measuring the incorporation of radioactive substrates such as [³H]-labelled hypoxanthine and ethanolamine have been developed as the standard malaria drug assays (Desjardins et al., 1979; Elabbadi et al., 1992). This technical advance also made cell-based in vitro drug screening possible. Several methods using colorimetric enzyme-linked immunosorbent assay (ELISA) that recognizes parasite antigens such as histidine-rich protein 2 and lactate dehydrogenase have been developed and found to be as sensitive as the radioisotope method (Druilhe et al., 2001; Noedl et al., 2002; 2005). These methods are particularly useful in drug resistance monitoring with clinical parasite samples in endemic areas, where radioactive assay is not possible (Brockman et al., 2004; Noedl et al., 2004; Attlmayr et al., 2005). Yet, although all aforementioned methods are reliable and accurate, they are expensive, with multiple processing steps, and unsuitable for HTS purpose. In recent years, the demand for higher-throughput, non-radioactive assays have resulted in the development of fluorescence-based techniques (Smeijsters et al., 1996; Corbett et al., 2004; Bennett et al., 2004; Smilkstein et al., 2004; Baniecki et al., 2007; Johnson et al., 2007). These methods rely on the binding of fluorescent dyes (e.g., PicoGreen, SYBR Green I, YOYO-1 and DAPI) to parasite DNA, which are often found to be as sensitive as the standard radioisotope method and colorimetric method (Bacon et al., 2007). The simplicity, robustness, and accuracy of these assays make them possible for HTS and the DAPI-based method has been used in 384-well microtiter plates for drug screening (Baniecki et al., 2007). The advance in malaria parasite transfection technology also allows the generation of transgenic parasite lines expressing different reporters (Crabb, 2002). For example, *P. falciparum* parasite expressing green fluorescent protein (GFP) offers a flow cytometry-based assay, which proves to be as sensitive as standard radioactive assay (Kadekoppala et al., 2000; Sanchez et al., 2007). In comparison, luciferase reporters have only been used to determine the activities of 5' and 3' regulatory elements in malaria genes in transient transfection assays (Frank et al., 2006; Militello et al., 2004; Oguariri et al., 2006), to study protein transport in the parasite

(Burghaus and Lingelbach, 2001), and to study murine malaria pathology by in vivo imaging (Franke-Fayard et al., 2006). Despite the advantages of a bioluminescent assay, it has not been developed for HTS for malaria medicine. To fully explore the potential of transgenic parasites in HTS, we created *P. falciparum* lines stably expressing a firefly luciferase reporter, which may serve as a convenient, radiolabel-free system for future antimalarial drug screening.

2. Material and Methods

2.1. Parasites culture

The malaria parasite *P. falciparum* was cultivated by the method of Trager and Jensen (1976) using human O⁺ erythrocytes in complete medium (RPMI 1640 supplemented with 25 mM Hepes, pH 7.5, 25 mM sodium bicarbonate, 50 mg/L hypoxanthine, 0.5% Albumax II, 40 µg/ml gentamicin sulfate). Cultures were maintained at 37°C in a gas mixture of 5% CO₂, 3% O₂ and 92% N₂ at a 5% hematocrit.

2.2. Development of a luciferase expression *P. falciparum* line

In order to develop a *P. falciparum* line suitable for bioluminescent drug assay, we attempted to generate transgenic parasites expressing the firefly luciferase. A plasmid construct Tg23-Luc was made in pDT.Tg23 (Wu et al., 1995), which carries a mutant *Toxoplasma gondii* dihydrofolate reductase (*dhfr*) gene conferring resistance to pyrimethamine (PY) as a selection marker with the *histidine-rich protein 3* (*hrp3*) promoter and the *histidine-rich protein 2* (*hrp2*) terminator (Fig. 1). To construct the luciferase expression cassette in pDT.Tg23, we cloned the luciferase coding region from the plasmid pGL-Basic (Promega, Madison, WI) in pSSPF2 (Sato et al., 2003) at the *Bgl* II and *Xho* I site. The luciferase expression cassette consisting of the *P. falciparum* heat shock protein 86 (*hsp86*) promoter and the 3' sequence of the *P. berghei* DHFR-TS gene (*PbDT-3'*) was moved into pDT.Tg23 at the *Spe* I and *Not* I site. The transgenic line was constructed in the common laboratory line 3D7. Transfection of the parasite was done using the erythrocyte loading method (Deitsch et al., 2001) and drug selection with PY was performed as described (Wu et al., 1995). Briefly, the transfected parasites were cultured under 20 µg/ml PY until resistant parasites emerged and reached 5% parasitemia, then the parasites were cultured in the absence of the drug for another two weeks. Afterwards, the parasites were cultured with 500 µg/ml PY for two or three weeks until the parasitemia reached 5%. After three drug on-off cycles, luciferase assay was performed to check whether the resistant parasite expressed luciferase. Parasite lines were obtained by limiting dilution (Rosario, 1981). Three clones with luciferase activity were selected for further characterization. Three predicted integration events in transgenic parasite lines were confirmed by PCR analysis using the primers shown in Figure 1A and Table 1. The primers allow the determination of integration events at the *hrp3* promoter, *hsp86* promoter, and *hrp2* 3' region, respectively (Wu et al., 1995). Genomic DNA was purified from selected clones as polymerase chain reaction (PCR) template (Cui et al. 2001).

Persistent drug pressure often leads to concatemerization of the plasmid, which is subsequently integrated in the parasite chromosome (O'Donnell et al., 2001). Such parasite clones carry multiple copies of the plasmid, thereby influencing the levels of the luciferase expression. The copy number of luciferase gene integrated into the genome of each parasite line was estimated by real-time PCR analysis using genomic DNA from each clone or transformed 3D7 parasites before cloning. The relative copy number of luciferase gene in each clone was determined by using $2^{-[Ct \text{ of transgenic line} - Ct \text{ of 3D7}]}$ with one pair of primers (CAACGTGAGAAATTAAG and TTCGAAATGTGGGAAG) to amplify a 323 bp fragment in the *hsp86* promoter region for clone 1 and 2, or a pair of primers (CATCTACTTTGCTTTTAAG and GTTCTGCATACATAAATAC) to amplify a 250 bp fragment in the *hrp3* promoter region for clone 3. The ratio was normalized with the single-

copy gene *seryl-tRNA synthetase* (Cui et al., 2007). Since the region used in homologous recombination in a single-crossover event is duplicated, the copy number of the luciferase gene was estimated as copy number of *hsp86* or *hrp3* promoter – 1.

2.3. Phenotype analysis of the transgenic line

To determine whether the transgenic line has any changes in growth phenotype, the transgenic line and 3D7 were synchronized at ring stage, seeded into a 24-well plate in triplicate at 0.5% parasitemia 5% hematocrit, and grown under the same conditions. Parasitemia was determined at 48 h and 96 h, and the developmental stages were compared between the two strains.

2.4. Luciferase assays

To determine luciferase expression in the transgenic *P. falciparum* lines, parasites at different parasitemias and hematocrits in a total of 100 μ l medium were seeded in each well of a 96-well plate. Luciferase assays were performed using the Luciferase Assay System (Promega) according to the manufacturer's instruction. Briefly, 80 μ l of supernatant was removed from the culture in each well and the plate was frozen at -80°C for at least 2 h. Five microliters of $5 \times$ lysis reagents from the Luciferase Assay System were added in each well after the lysates were completely thawed at room temperature. Each well was mixed with 100 μ l luciferase assay reagent and the light produced was measured immediately in a single-tube luminometer TD-20/20 (Turner BioSystems, Sunnyvale, CA). The luciferase assay for comparing the three original transgenic clones was done essentially the same except for that the parasites were grown in a 24-well plate. All assays were done in triplicates.

2.5. Assay optimization

To establish the best conditions for the luciferase assay, we optimized the following parameters: parasitemia, hematocrit, and parasite developmental stage. To determine the levels of luciferase expression at different developmental stages, the parasite clone was synchronized by two rounds of treatment with 5% sorbitol at the ring stage (Lambros and Vanderberg, 1979). Synchronized parasites at 2% parasitemia and 2% hematocrit were sampled every 12 h and parasites representing rings, early trophozoites, late trophozoites, and schizonts were determined by Giesma staining of thin smears of the parasites. Further, to determine whether assays performed at different stages would affect the outcomes, we compared the 50% inhibition concentration (IC_{50}) values of chloroquine (CQ) obtained at ring, trophozoite, schizont, and unsynchronized, mixed stages. IC_{50} s were calculated by a linear regression analysis method using KaleidaGraph, version 3.5 (Synergy Software, Reading, PA).

To assess the effect of parasitemia on assay sensitivity, the luciferase assay was performed in a 96-well plate using 2% hematocrit, ~ 30 h trophozoites and a parasitemia ranging from 0.2 to 8%. This was achieved by diluting ring-stage parasites at 10% parasitemia with fresh blood cells and luciferase assay was performed in late trophozoites after culturing the parasites for 12 h. To find out whether different parasitemias have significant impact on the assay outcomes, we compared the IC_{50} s of artemether and CQ obtained at 0.7, 1.5, 3 and 5 parasitemia. Briefly, ring stage (~ 18 h) parasites were diluted into the desired parasitemias using fresh blood cells and seeded into a 96-well plate. Luciferase assays were performed after 12 h of incubation, and IC_{50} s of each drug were determined.

Since background fluorescence from uninfected red blood cell may influence the sensitivity of the luciferase assay, the level of hematocrit was also optimized using a level from 0.2 to 5% using synchronized parasites at late trophozoite stage and 2% parasitemia. Given that HTS often uses dimethyl sulfoxide (DMSO) as the solvent for chemical compounds, we also evaluated the effect of different concentrations of DMSO (0.1-3%) on the sensitivity of the luciferase assay.

All the assay optimization experiments were done twice, each treatment was in triplicates and the means and standard deviations were calculated. The differences between the different stages, parasitemias, hematocrits, and DMSO concentrations were compared using ANOVA.

2.6. Standard drug assay

To determine the sensitivity of the luciferase assay and suitability for chemical screening, we have performed one experiment to compare this assay with the standard [^3H] hypoxanthine incorporation method (Desjardins et al., 1979) using four antimalarial drugs (CQ, artesunate, artemether, and dihydroartemisinin), and curcumin. Stock solutions of the test drugs were prepared at a concentration of 10 mM (CQ in water and the rest in DMSO), serially diluted in complete medium, and dispensed into triplicate wells to yield the designated final concentrations. For the standard luciferase assay, parasites were synchronized once with 5% sorbitol at ~8 h post invasion and luciferase assays were performed using ~30 h trophozoites. Given that our synchronization had a window of 8 h ring development, we estimated that at the time of the assay, the majority (~95%) of the parasites was mid-late trophozoites and ~5% was early schizonts. In each well, 100 μl of late ring-stage (~18 h) parasites were seeded at 2% parasitemia and 2% hematocrit, and collected after 12 h of incubation for luciferase assay. Drug assays with the [^3H] hypoxanthine incorporation method were performed with 3D7 as previously described (Cui et al., 2007). Briefly, ring-stage parasites at 1% parasitemia and 5% hematocrit in a complete low-hypoxanthine medium (5 mg/liter hypoxanthine) were seeded in triplicate in 96-well plates. Drugs or DMSO control was added to 200 μl of culture and incubated at 37°C for 24 h. Subsequently, 100 μl supernatant was removed and 100 μl of complete low-hypoxanthine medium containing 1 μCi of [^3H] hypoxanthine (Amersham, Piscataway, NJ) was added. After further culture for 20 h, parasites were harvested onto glass-fiber filters, and radioactivity of the filters was counted using a TriLux microbeta counter (Perkin-Elmer, Wellesley, MA). IC_{50}s were determined for the two methods and compared using ANOVA.

To determine whether these assay conditions are suitable for drugs with different modes of action, we also compared the assay sensitivity using fast- (artemether and CQ) and slow-acting drugs (azithromycin and doxycycline). Briefly, these drugs were serially diluted into complete medium and dispensed into triplicate wells of a 96-well plate. Ring-stage parasites (18 h) were seeded into each well with 2% parasitemia and 2% hematocrit. The medium in each well was changed every 24 h with same concentrations of test drugs. Parasites were collected at 12, 24, 48 and 72 h for luciferase assay. IC_{50}s were determined and the differences of each drug between different assay time points were compared using ANOVA.

To determine whether this luciferase assay performed with a single-tube luminometer is suitable for a plate reader format that is required for HTS, we performed an experiment to assay three artemisinin-derivative drugs in 96-well plates using the parameters determined earlier. Briefly, 100 μl of late ring-stage (~18 h) parasites were seeded in 96-well plates at 2% parasitemia and 2% hematocrit, and incubated with different concentrations of each drug. For each drug concentration, the assay was performed in triplicates. At 12 h post treatment, 100 μl of Steady-Glo® Luciferase Assay System (Promega, WI) was added into each well, mixed and incubated at room temperature for 10 min. The luminescence values were obtained using a multi-well luminometer (Centro XS LB 960, Berthold Technologies, Oak Ridge, TN). IC_{50}s were calculated and compared with those determined using a single-tube reader by ANOVA.

2.6. Z' factor determination

To determine the suitability of the luciferase assay for HTS, we used the standard drug assay conditions (2% parasitemia, 2% hematocrit and 12 h treatment) and a drug concentration close

to the IC_{50} of each drug. The experiment was repeated once and each treatment was replicated at least three times. The Z' value was calculated from the drug assay data using the formula $Z' = 1 - 3X (StDev_+ + StDev_-) / (Mean_+ - Mean_-)$ (Zhang et al., 1999), where $StDev_+$ and $StDev_-$ are standard deviations from the positive (with drug) and negative control (without drug), respectively, and $Mean_+$ and $Mean_-$ are the mean values of drug treated samples and negative controls, respectively.

3. Results

3.1. Development of luciferase-expressing transgenic lines

We have transfected *P. falciparum* 3D7 clone with the plasmid Tg23-Luc using the erythrocyte loading method (Deitsch et al., 2001). Resistant parasites were obtained after three weeks of PY selection, following which three drug on-off cycles were performed to enrich parasites with stable chromosomal integration of the plasmid. Through parasite cloning by limiting dilution, we selected three parasite clones with stable plasmid integration and luciferase expression to characterize the integration events and the level of luciferase expression. Using a plasmid- and a chromosome-specific primer (Fig. 1A and Table 1), we determined by PCR that three possible integration events were present in uncloned parasites (data not shown), whereas the plasmid was integrated at the *hsp86* promoter site of chromosome 7 in clone 1 and 2 and at the *hrp3* promoter site of chromosome 13 in clone 3 (Fig. 1B). Since single-crossover integration often results in concatemerization of the plasmid, which may affect the level of luciferase expression, we determined the copy number of the plasmid and luciferase expression in each clone. Using real-time PCR analysis normalized with 3D7 (where the *hrp3* and *hsp86* promoters are of single copy) and internal reference gene *seryl-tRNA synthetase*, we found that three and two copies of plasmid (or luciferase gene) were inserted at the *hsp86* promoter region in clone 1 and 2, respectively, while one copy of the plasmid was inserted at the *hrp3* promoter region in clone 3 (Fig. 2A). These clones were indistinguishable in their growth phenotype (data not shown). However, by assaying luciferase production in a 24-well plate, we found that the luciferase expression levels of the three clones differed and appeared to correlate with the plasmid copy number (Fig. 2B). Since clone 1 had the highest luciferase level, it was selected for assay optimization and drug efficacy studies. Furthermore, since over expression of a foreign enzyme may affect the fitness of the parasite, we compared the growth phenotype of clone 1 with its parent strain 3D7. The growth curves of these two strains were highly similar (Fig. 2C), and there were no apparent differences in developmental rates between the two strains.

3.2. Optimization of the assay conditions

We have chosen clone 1 to develop and optimize a luciferase assay in a 96-well plate format. Because *P. falciparum* undergoes changes in both morphology and cell mass during its asexual erythrocytic cycle, we firstly tried to establish the developmental stage with the highest luciferase production. Using the same number of parasites, we determined that luciferase production increased steadily from rings to schizonts (Fig. 3A). To determine whether the assay done at different stages affect the assay outcome, we determined the IC_{50} of CQ using synchronized parasites at ring, early trophozoite, schizont stages, and unsynchronized parasites of mixed stages. The results showed that there were no significant differences in IC_{50} s determined at different developmental stages (Fig. 3B). In addition, the IC_{50} obtained with unsynchronized parasites was not significantly different from synchronized parasites (ANOVA, $P > 0.05$), suggesting that synchronization of the parasites is not necessary for future assays.

We next determined the effect of parasitemia on the assay sensitivity using a wide range of parasitemias (0.5-8%). We found that there was a significant linearity between parasitemia and light production ($R^2 = 0.93$, $P < 0.001$) (Fig. 4A). To determine the parasitemia range within

which the parasite's growth and luciferase production were optimal, we normalized the luminescence data against the parasite number at each parasitemia. Though we did not find significant differences among the parasitemias (ANOVA, $P > 0.05$), the parasitemias of 1.5-2% produced the best results (Fig. 4B). To further evaluate whether parasitemia affected the outcome of the assay, we determined IC_{50} s of CQ and artemether under four different parasitemias (Fig. 4C). The results showed that IC_{50} s of artemether at different parasitemias were not significantly different ($P > 0.05$). Whereas IC_{50} s of CQ were not significantly different among parasitemias of 0.7, 1.5 and 3%, the IC_{50} at 5% parasitemia was significantly higher than those obtained at lower parasitemias (ANOVA, $P < 0.05$). This suggests that the assay needs to be performed at lower parasitemia for drugs that act on parasite food vacuole. Thus, we have chosen 2% parasitemia for subsequent assays.

As it is desirable to perform the assay with minimum steps of manipulation, the luciferase assays were performed using whole culture lysates. Accordingly, hematocrit may not only affect the parasite growth but also the sensitivity of the assay due to background fluorescence. Using the same number of parasites, we found that light production was gradually reduced with increasing hematocrits and the optimum hematocrit was at 2% (Fig. 5A). Consequently, all subsequent drug assays were performed using late trophozoites at 2-3% parasitemia and 2% hematocrit. We also determined the background level of untransfected 3D7 under the same culture conditions. Light production in the 3D7 controls was less than 0.1 unit and not significantly different from that of luciferase assay reagent alone; therefore, background light production was not taken into account in subsequent experiments. Since many compound libraries for drug screening use DMSO as the solvent, the assay has to be tolerant to the effect of DMSO at 0.1-1%. We have determined that DMSO at a concentration from 0.1 to 3% did not affect the sensitivity of the assay (Fig. 5B).

3.3. Comparison of the luciferase assay with the 3H -hypoxanthine assay

To determine whether our luciferase assay was comparable to the standard radioactive assays (Desjardins et al., 1979; Elabbadi et al., 1992), we determined the sensitivity of the transgenic parasite to four antimalarial compounds using parasite clone 1 and the optimized conditions established earlier (Table 2). The results showed that the IC_{50} values obtained using these two methods were not significantly different ($P > 0.05$, ANOVA).

3.4. Validation of the luciferase assay for drugs with different modes of action

Drugs that have delayed effects on parasites require more extended time of treatment to achieve a clinically meaningful antimalarial effect (Dahl et al., 2006; Dahl and Rosenthal, 2007). To validate the luciferase assay for a broad spectrum of antimalarial drugs, we compared the IC_{50} s of the two fast- (artemether and CQ) and two slow-acting drugs (azithromycin and doxycycline) at different time points post drug treatment. For artemether, there were no significant differences between IC_{50} values obtained at the four time points of drug treatment, suggesting that the luciferase assay can be performed within 12 h of drug treatment for fast-acting drugs (Table 3). For CQ that acts in the food vacuole, IC_{50} s determined between 12 and 72 h ranged from 32.41 to 27.54 nM (Table 3). IC_{50} s determined at 48 and 72 h were not significantly different between each other, but significantly lower than that determined at 12 h, suggesting that luciferase assay needs to be performed at a later time point. For the two slow-acting drugs, results obtained at 72 h were significantly lower than those obtained at earlier time points, which were within the range determined earlier for *P. falciparum* field isolates (Pradines et al., 2000; Noedl et al., 2007). This suggests that the luciferase assay needs to be performed at 72 h in order to obtain more meaningful IC_{50} s for the slow-acting drugs.

3.5. Validation of luciferase assay for an automatic plate reader

To further demonstrate the potential of this luciferase assay for HTS applications, we evaluated it in a 96-well plate format using the Steady-Glo® Luciferase Assay System. This system offers stable luminescence with little light reduction in 4 h (data not shown). Using a luminescence plate reader, we determined the IC₅₀s for artemether, artesunate and dihydroartemisinin (Table 2). The results showed that the IC₅₀ values determined with the two types of luminometers were not significantly different ($P>0.05$, ANOVA).

3.6. Z' score test

Z' factor analysis has been applied to determine the robustness of HTS assays. Typically, a Z' value of >0.5 is considered to be quite favorable for HTS. To determine the Z' values of the selected compounds, clone 1 parasites at 2% parasitemia and 2% hematocrit were treated with the aforementioned drugs at the concentrations near their IC₅₀s (Table 4). Using untreated clone 1 as a negative control, we calculated the Z' values for the tested drugs to be 0.77-0.92 (Table 4).

4. Discussion

The use of in vitro drug sensitivity assays is a commonly used strategy for monitoring antimalarial drug resistance and screening new drugs. We have generated a transgenic *P. falciparum* line expressing firefly luciferase, and optimized this assay using a 96-well microtiter plate format. This assay proved as sensitive as the standard radioisotope incorporation method with near absence of background luminescence (<0.1 unit), suggesting that future assays do not need empty wells or nonparasitized erythrocytes as controls. Besides, there were no significant differences between synchronized and unsynchronized parasites, suggesting that this assay could be simplified using unsynchronized parasites. While most current malaria drug assays rely on measuring the quantity of parasite DNA thus requiring 48-72 h of incubation time, the luciferase assay can be performed within 12 h for fast-acting drugs, suggesting that the reporter expression is altered even before parasite replication occurs. We speculate that altered reporter expression may be associated with changes in parasite physiological conditions from drug treatments. Intriguingly, even for drugs like CQ that acts on the food vacuole, the 12 h assay was also as sensitive as the 48 h [H^3] hypoxanthine incorporation assay. The reason for this observation is not clear, but recent work on the mechanism of CQ suggests that this drug may have additional cellular targets (Gligorijevic et al., 2008). Based on the results using a variety of drugs, we consider that a 24 h luciferase assay would probably suit most antimalarial drugs except for slow-acting drugs with delayed parasitocidal effects (Dahl et al., 2006; Dahl and Rosenthal, 2007).

While not designed for drug resistance monitoring purpose, the simplicity of the assay indicates the feasibility of this cell-based luminescent assay for HTS of antimalarials. HTS demands a minimum of a 384-well microtiter plate. We have shown that this assay performed well in a 96-well format using an automatic plate reader. Its robustness under these conditions suggests that the miniaturization of this assay to 384-well or even higher format should be possible. Besides, the Z' scores of >0.77 obtained in this assay for a number of drugs and compounds further supports this claim. Furthermore, use of the Steady-Glo® Luciferase Assay System allows easy processing of large batches of samples.

One caveat of this assay in comparison with the DNA stain-based fluorescence assay is that it requires a transgenic luciferase-expressing parasite line. Consequently, the drug used for selecting the transgenic line, PY, may potentially cause cross-resistance to certain candidate drugs. Yet, this problem can be easily solved by using another transgenic line with a mechanistically different marker as a complementary approach. So far, a number of selectable

markers have been successfully developed for malaria parasite transfection (Fidock and Wellems, 1997; Mamoun et al., 1999; de Koning-Ward et al., 2001; Gardiner et al., 2003). Furthermore, future technological development in parasite transgenesis may lead to the generation of transgenic lines with subsequent removal of the drug-resistant marker from the parasite genome (Carvalho and Menard, 2005). Taken together, the luciferase-expressing *P. falciparum* line may offer another drug assay method for malaria parasite, while its potential for HTS of antimalarials awaits further investigations.

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Abbreviations

ACT, artemisinin-based combination therapy; HTS, high-throughput screening; IC₅₀, 50% inhibitory concentration; DMSO, dimethyl sulfoxide; CQ, chloroquine; MDR, multidrug resistant.

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A. **Fig.1.**

A. Schematic representation of two single-crossover integration events at the *hsp86* promoter region (hatched box) in chromosome 7 and at the *hrp3* promoter region (stippled box) in chromosome 13. Shown here are plasmid Tg23-Luc with the *Tgdhfr* expression cassette and the luciferase expression cassette. The orientations of the promoters are shown as arrows. Chromosome 7-specific primer 1 and *luciferase* primer 2 were used to demonstrate the integration event in chromosome 7, while chromosome 13-specific primer 3 and *Tgdhfr* primer 4 were used to illustrate the integration event in chromosome 13. Another possible integration event with homologous recombination at the 3' region of *hrp2* (checked box) was not shown.

B. Integration events were confirmed by PCR using primer pairs 1×2 and 3×4, respectively. PCR were performed using DNA from three luciferase-expressing clones (1, 2, and 3), uncloned transfected parasite culture (mix), and control 3D7. M – molecular marker.

Fig. 2.

A. Relative copy numbers of firefly luciferase gene in the three selected clones and parasites before cloning (mix). The relative copy numbers were determined by real-time PCR quantification. **B.** Luciferase activity (as light unit) of the three clones and mixed parasites before cloning (mix). The parasites were grown in a 24-well plate, and luciferase assays were performed at late trophozoite to schizont stages. **C.** Growth curves of 3D7 and clone 1. Synchronized parasites of both lines were initiated at the same ring stage at 0.5% parasitemia, and parasitemia was determined at 48 and 96 h.

Fig. 3.
A. The effect of developmental stages (R – ring, ET – early trophozoite, LT – late trophozoite, Sc – schizonts) on the assay sensitivity; **B.** IC₅₀s of chloroquine obtained using parasites of different developmental stages and unsynchronized parasites of mixed stages (mix). T – trophozoite. Columns labeled with the same letter were not significantly different (ANOVA, $P > 0.05$).

Fig. 4.

A. Linear regression of luciferase production (light unit) of clone 1 at different parasitemias (0.5-8%). **B.** Values obtained in A normalized by using the same number of parasites found at 2% parasitemia to represent luciferase production in a unit number of parasites. **C.** IC_{50} s of CQ and artemether determined using different parasitemias. Columns of the same drug labeled with different letter indicate significant difference at $P=0.05$ level (ANOVA).

Fig. 5.

A. Luciferase assay performed using different hematocrit (0.5-6%). **B.** Luciferase assay performed at different DMSO concentrations (0.1-3%). Y axis indicates the light units from the luciferase assay.

Table 1

Primers for determining the chromosomal integration site of the plasmid

Integration site	Primers (5'→3')	Predicted PCR size (kb)
<i>hsp86-5'</i>	1: GGCGCCGGCCTTCTTTATGT 2: TTATATATAACTTCCACAAA	0.91
<i>hrp3-5'</i>	3: GCAATATTAATATACCCATAAACC 4: GGGGTCATCGCGACGACCAG	1.96
<i>hrp2-3'</i>	5: CAATACAAAATTATGTTTCATCATG 6: GCTACGTCCCGCACGGAC	0.76

Table 2

Comparison of the IC₅₀s (mean ± standard deviation) of the test compounds by the two methods.

Drug or compound	Methods [*]		
	Luciferase Assay [#]		[³ H]-Labeling Assay
	Single-well Assay	Plate Assay	
Artemether	11.84±0.24 nM	12.37±0.41 nM	12.92±2.25 nM
Artesunate	18.42±1.50 nM	20.03±0.07 nM	22.37±1.72 nM
Dihydroartemisinin	19.58±1.12 nM	18.90±1.06 nM	12.72±1.62 nM
Chloroquine	31.39±1.14 nM	ND	32.55±3.89 nM
Curcumin	29.16±0.67 μM	ND	24.69±0.47 μM

^{*} No significant difference between the three methods ($P>0.05$, Two-way ANOVA: IC₅₀ versus method)

[#] The single-well luciferase assay used the Luciferase Assay System (Promega), while the plate assay used the Steady-Glo® Luciferase Assay System (Promega) on an automatic luminescence plate reader. ND – not determined.

Table 3

Comparison of the IC₅₀s of the test drugs (nM) by performing the luciferase assays at different hours post drug treatment*

Test drugs	12	24	48	72
Artemether	11.84±0.24 ^a	11.21±0.57 ^a	10.33±0.80 ^a	10.87±0.93 ^a
Chloroquine	32.41±1.62 ^a	31.39±1.14 ^{ab}	28.55±3.12 ^b	27.54±2.03 ^{bc}
Azithromycin	6024±713 ^a	6077±224 ^a	5270±982 ^b	4626±410 ^c
Doxycycline	43630±819 ^a	34870±5526 ^b	10248±1575 ^c	3097±661 ^d

* The alphabetical letters following the numerical values indicate the results of ANOVA multiple comparison tests. The values with the same letter in each row were not statistically significant at $p = 0.05$ level.

Table 4

Z' factor analysis of luciferase-expressing clone 1

Test drugs	Concentrations	Z' factor
Artemether	10 nM	0.89
Artesunate	15 nM	0.79
Dihydroartemisinin	10 nM	0.77
Chloroquine	30 nM	0.90
Curcumin	20 μ M	0.91
Azithromycin	6 μ M	0.85
Doxycycline	25 μ M	0.92