

OPTIMIZATION OF A NEW CELL-BASED FLUORESCENCE ASSAY FOR U.S. ARMY GLOBAL MALARIA SURVEILLANCE EFFORTS IN SUPPORT OF THE WARFIGHTER

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ABSTRACT

High-throughput *in vitro* anti-malarial drug screens have traditionally incorporated the use of radioactive substrates to measure the effect test compounds have on parasitic growth. Several alternative growth inhibition screening assays using fluorescent nucleic acid intercalating dyes have been recently published. In this study we evaluated the malaria SYBR Green I-based fluorescence (MSF) assay, described by Smilkstein et al., for its use in laboratory research and in support of the U.S. Army malaria drug resistance program and the Global Emerging Infection Surveillance and Response System (GEIS) objectives. We expanded upon Smilkstein's initial characterization and validation of the MSF assay to fit our program-specific drug screening needs by testing various culture conditions commonly used for resistance screening. *Plasmodium falciparum* strains D6 and W2 were treated with a panel of known anti-malarial drugs and their respective IC₅₀s were determined using the MSF assay. The results were then compared to our IC₅₀ data generated using our standard [³H]hypoxanthine incorporation assay. Assay conditions that could potentially affect MSF assay readout, including assay length, starting parasite density and hematocrit levels, microtiter plate selection, and different culture medium components, were also examined. The IC₅₀ values from the MSF assay showed the expected pattern of drug resistance for both parasitic strains tested when compared to the values from the [³H]hypoxanthine incorporation assay. One possible limitation of the MSF assay for some drug resistance applications is due to a significant edge effect observed, which could influence IC₅₀ calculation. The MSF assay was easily amended for use with our robotic plate and handling equipment. Compared to our gold standard radioactive assay, the MSF assay is more cost-effective, simple, and less hazardous, while still allowing for accurate high-throughput, automated drug screening.

1. INTRODUCTION

The complete realization of the US Army's Objective Force vision, to "See first. Understand first. Act first. Finish decisively," will require more than lightweight, fully integrated individual combat system ensembles and

other platform-based capabilities for the Objective Force Warrior soldier. It necessitates the capability to see *all* of our enemies first, to understand *all* of them first, and to act first. To be properly inclusive, our enemies must therefore include the silent adversaries that are indigenous to the foreign lands our Warrior soldiers routinely operate in - infectious diseases. High-speed surveillance capabilities allows DoD soldier and civilian scientists to spot potential infectious threats before our Warrior soldiers are even on the ground. The use of cutting-edge scientific technology has further provided greater understanding of the pathobiology and molecular mechanisms of infectious agents. This situational awareness, in turn, has created opportunities to act first, including the creation of new prophylactic and treatment drugs for Warrior disease prevention and treatment.

U.S. troops are constantly exposed to many potential infectious threats while deployed, such as viruses, bacteria, and parasites. According to the Center for Disease Control and Prevention (CDC), one of these threats - malaria - "is one of the most severe public health problems worldwide" (CDC, 2006) and, therefore, as an extension should be considered one of the most severe U.S. military health problems to our deployed Warrior soldiers. Malaria has afflicted many of our soldiers during our military operations, including the Spanish-American War, World War II, the Vietnam War, the Korean War, Operation Restore Hope in Somalia, and in peace keeping operations in Liberia (Kitchen et al., 2006; Susi et al., 2005). In fact, the World Health Organization's (WHO) World Malaria Report indicates that 350 to 500 million clinical malaria infections occur every year, resulting in at least one million deaths per year (WHO, 2005). Furthermore, most of these infections occur in developing countries where many of our U.S. troops proudly serve. It is predicted that clinical infections and death will start to increase due to rapid spread of drug resistance parasites (Hastings and D'Alessandro, 2000; Hyde, 2002; May and Meyer, 2003; Price and Nosten, 2001; Wongsrichanalai et al., 2002).

There are four strains of the malaria parasite that infect humans. Of these, *Plasmodium falciparum* is the most deadly. *P. vivax*, although it rarely causes death, is a constant annoyance and the leading cause of morbidity due to the dormant phases of the parasite that reside in

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liver hepatocytes. Both *P. vivax* and *P. falciparum* have developed resistance to numerous antimalarial drugs, which has undermined the available options for prophylaxis and treatment (Mordmuller and Kremsner, 2006.). Unfortunately, the incident and specificity of malaria drug resistance is not homogenous throughout the world. Different geographical locations within the same country can yield malaria parasites with varying degrees of sensitivity to commercially available drugs (Mbaisi et al., 2004). These findings support the need for valid surveillance efforts to predict and determine the level of malaria drug resistance.

Several organizations to include the DoD, WHO, Wellcome Trust, and CDC have implemented various drug resistance efforts. Within the DoD, Global Emerging Infections Surveillance and Response System (GEIS) maintains the largest network of malaria drug resistance surveillance program, including surveillance in Africa, Southeast Asia, South America, and the Western Pacific islands. These malaria drug screening laboratories use both molecular screening techniques to determine drug resistance genotypes and an *in vitro* cultivation and drug sensitivity assay to determine drug resistance levels against a battery of common antimalarial drugs (Aubouy et al., 2003; Labbe et al., 2003; Plowe, 2003; Mbaisi et al., 2004;). In response to the call for a standard assay platform to measure malaria drug resistance across all GEIS surveillance sites, our Antimalarial Drug Discovery Program has assessed and further optimized a new cell-based fluorescence assay for its use in monitoring drug resistance and identifying novel antimalarial agents.

Traditionally, high-throughput *in vitro* antimalarial drug screens have incorporated the use of radioactive substrates to measure parasitic growth in the presence or absence of known antimalarial drugs (Desjardins et al., 1979; Trager and Jensen, 1976). Although these isotopic assays are widely used, accurate, and reliable, they are very expensive, involve multiple processing steps, and require special handling and waste disposal procedures. Thus, the main utility of radioactive assays lies within a fixed research facility, and not in the field. Several fluorescence malaria drug surveillance assays have been recently published that use fluorescent nucleic acid intercalating dyes to measure *in vitro* malaria growth inhibition (Smilkstein et al., 2004; Bennett et al., 2004; Corbett et al., 2004; Kosaisavee et al., 2006). As mature erythrocytes lack RNA and DNA, binding of the dye is specific for malarial DNA in any erythrocytic stage of parasite development. The dyes also display preferential binding to double-stranded DNA versus single-stranded DNA or RNA. In these studies, malaria culture strains were treated with known antimalarial drugs and their respective IC₅₀ (50% inhibitory concentration) values were determined and compared to those obtained from traditional radioactive assays.

In this study, we have assessed and further validated the malaria SYBR Green I-based fluorescence (MSF) assay in our own laboratory. *P. falciparum* strains D6 and W2 were treated with a panel of known anti-malarial drugs and their respective IC₅₀s were determined using the MSF assay. The results were compared to our [³H]hypoxanthine incorporation assay. The effect of various assay conditions on MSF assay readout, including assay duration, parasitemia and hematocrit levels, microtiter plate selection, and culture medium components, were additionally examined.

2. MATERIALS AND METHODS

2.1 Reagents

SYBR Green I dye (10000x in DMSO) was purchased from Molecular Probes, Inc., Eugene, Oregon. [³H]hypoxanthine was obtained from American Radiolabeled Chemical Inc, Saint Louis, Missouri. Chlorquine diphosphate salt, quinine hemisulfate salt, and mefloquine hydrochloride were purchased from Sigma Chemical Co., Saint Louis, Missouri.

P. falciparum strains D6 (CDC/Sierra Leone) and W2 (CDC/Indochina III) were routinely maintained in continuous long term cultures in RPMI-1640 medium supplemented with 5% washed human A+ erythrocytes, 11 mM glucose, 25mM HEPES, 32 nM NaHCO₃, 29 μM hypoxanthine, and 10% heat inactivated A+ human plasma (modified from Trager and Jensen, 1979), herein called TCM. Human erythrocytes and plasma were obtained from Valley Biomedical Inc., Winchester, Virginia. The cultures were incubated at 37°C under an atmosphere of 5% CO₂ and 5% O₂, with a balance of N₂.

2.3 Parasite drug assays

Prior to performing the drug assays, the parasites were cultured in their respective test culture condition for 3-4 days. The test culture conditions included: TCM (as described above); TCMA, TCM with AlbuMax (lipid-rich bovine serum albumin, 50 mg/ml; Gibco Inc, Grand Island, New York) substituted in place of the plasma; folic acid free and p-aminobenzoic acid free RPMI-1640 medium with the same additional constituents as TCM except for the absence of hypoxanthine (herein called FAF); and FAFA, FAF with AlbuMax substituted in place of the plasma. All drug assays were conducted at 37°C under a humidified atmosphere of 5% CO₂ and 5% O₂, with a balance of N₂ in sterile transparent tissue culture 96-well plates

Drug assay plates containing eleven two-fold serial dilutions of each antimalarial drug, originally suspended in DMSO or 70% ethanol at varying stock concentrations,

in culture medium were produced in advance and frozen at -80°C until use (stored no more than one week) or made freshly the day of the assay. No difference was seen in IC_{50} determination between previously frozen or fresh drug assay plates (data not shown). The Beckman Coulter Biomek 2000 automated laboratory workstation was used to produce all drug assay plates and to dispense the parasites (in late-ring/early trophozoite stages) and [^3H]hypoxanthine or SYBR Green I dye to the plates for each respective assay.

The [^3H]hypoxanthine incorporation assay was based on modifications of previously described methods (Desjardins et al., 1979; Chulay et al., 1983; Milhous et al., 1985). Briefly, *P. falciparum* strains were cultured in the pre-dosed 96-well microtiter drug assay plates in 200 μl volumes at a starting parasitemia of 0.4% and hematocrit of 1% for a total of 72 hours. [^3H]hypoxanthine in culture medium (25 μl) is added to the culture during the last 24 hours of the assay to allow for incorporation into the live parasites. After the 72 hour incubation was complete, the assay plates were frozen to lyse the cultures. The parasite DNA was recovered by harvesting the lysate onto glass-fiber filter plates using a Packard FilterMate Cell Harvester and the radioactivity (count per minute, CPM) was counted on a Packard TopCount microplate scintillation counter. The CPM per well (y-value) at each drug concentration (x value, transformed to the $\text{Log}[X]$) was plotted and analyzed using nonlinear regression analysis (sigmoidal dose response/four parameter equation) with in-house software to determine the IC_{50} value for each drug tested.

The malaria SYBR Green I-based fluorescence (MSF) assay, adapted from Smilkstein et al. (2004), used the same specimen processing and malaria culture techniques as the standard [^3H]hypoxanthine incorporation assay described above. However, 100 μl culture volumes at an optimized starting parasitemia 1% and hematocrit of 2% were used (data not shown). After culturing the plates for 72 hours, 100 μl of lysis buffer [20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% (wt/vol) saponin, and 0.08% (vol/vol) Triton X-100] containing SYBR Green I (1x final concentration) was added directly to the plates, gently mixed, and incubated for another hour at room temperature. The plates were examined for the relative fluorescence units (RFUs) per well using the Tecan Genios Plus. The drug concentrations (x value) were transformed using $X=\text{Log}[X]$ and plotted against the RFUs (y values). The data was then analyzed in GraphPad Prism (GraphPad Software, Inc., San Diego, California) by non-linear regression (sigmoidal dose-response/variable slope equation) to yield the IC_{50} , as described by Smilkstein et al (2004).

For both assays, microtiter plate wells containing noninfected erythrocytes in the absence of drugs serve as

negative controls, while parasitized erythrocytes in the presence of drugs serve as positive controls for each plate.

2.4 Assessment of SYBR Green 1 Fluorescence Linearity

Experimental conditions and Tecan Genios Plus settings were verified/adjusted by examining SYBR Green I fluorescence linearity of parasitemia values between 0 and 5 %, as determined by microscopic examination of Geimsa-stained parasites. To do this, triplicate wells of parasitized erythrocytes (in late ring stage) were serially diluted with noninfected erythrocytes at a constant 2% hematocrit in culture medium (100 μl final volume). SYBR Green containing lysis buffer was then added to the wells as described. Analysis of the counts was performed by MS Excel. The % parasitemia (x values) was plotted against the RFUs (y values, after background subtraction of noninfected erythrocytes) and analyzed by linear regression to determine the Goodness of Fit (r^2 value).

3. RESULTS

It has recently been reported that SYBR Green I, as well as similar fluorescent nucleic acid intercalating dyes, can be used for drug dose response analysis in *in vitro* malaria parasite screens (Smilkstein et al., 2004; Bennett et al., 2004). To ensure that the dye could quantify differences in parasitemia between wells, we first verified the fluorescence linearity of the SYBR Green I over a range of known parasitemia, as determined by microscopic examination of Geimsa-stained D6 parasites (Figure 1). As expected, the fluorescence intensity was linear over the range of parasitemia tested.

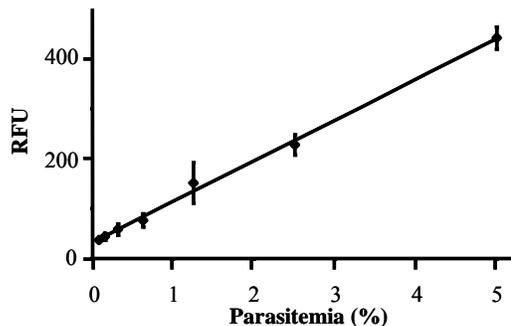


Figure 1. Assessment of SYBR Green I fluorescence linearity. RFU = relative fluorescence units.

We next assessed the malaria SYBR Green I-based fluorescence (MSF) assay (Smilkstein et al., 2004) for its use in monitoring drug resistance in *P. falciparum*. For these studies, two well-characterized *P. falciparum* strains, D6 and W2, were cultured in the presence or absence of known antimalarial drugs for 72 hours in various culture

conditions routinely used in the [³H]hypoxanthine incorporation assay by our malaria *in vitro* drug screening laboratory. The drugs tested included chloroquine, quinine, and mefloquine. Their respective IC₅₀s were determined using the MSF assay. Example test drug dose response curves are illustrated in Figure 2A and 2B. The results were then compared to our historical IC₅₀ data and/or side-by-side experiments generated using our standard [³H]hypoxanthine incorporation assay. Representative data from one experiment is shown Table I. Compared to the radioactive assay, the IC₅₀ values from the MSF assay showed the expected pattern of drug resistance for both parasitic strains tested.

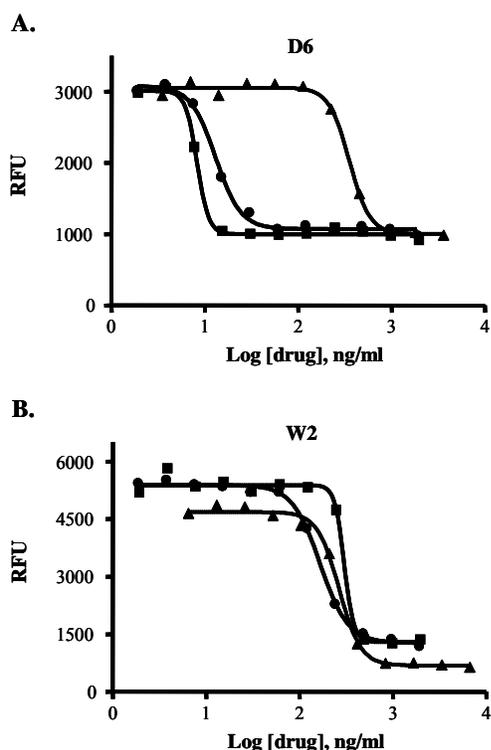


Figure 2. Example test drug dose response curves for D6 (A) and W2 (B) *P. falciparum* strains. ■ = Chloroquine. ● = Quinine. ▲ = Mefloquine. RFU = relative fluorescence units.

CONCLUSIONS

The drug resistance profile of D6 and W2 has been well established by our group and in the literature. D6 is slightly more resistant to mefloquine and susceptible to chloroquine and quinine; the W2 strain is resistant to chloroquine, and quinine. Importantly, the IC₅₀ values from the MSF assay showed the expected pattern of drug resistance for each parasitic strain tested when compared to the data generated using our standard [³H]hypoxanthine incorporation assay (Table I).

Furthermore, the different culture medium tested, whose main difference was the presence or absence of folic acid in combination with either plasma or Albumax, gave similar results to one another in the MSF assay. Taken together, these results support the findings of Smilkstein et al. (2004) and Bennett et al. (2004) and expand upon their initial characterizations of the MSF assay.

Table I. Comparison of IC₅₀ values (ng/ml) determined using the MSF and [³H]hypoxanthine incorporation assays

TEST DRUG	D6				[³ H] HPX
	MSF				
	TCM	TCMA	FAF	FAFA	
Chloroquine	11.13	9.61	11.06	11.09	4.66
Quinine	42.99	55.99	54.81	53.20	11.06
Mefloquine	50.29	55.99	55.01	51.12	9.38

TEST DRUG	W2				[³ H] HPX
	MSF				
	TCM	TCMA	FAF	FAFA	
Chloroquine	293.50	276.20	263.80	349.90	143.77
Quinine	158.30	136.70	160.50	141.20	70.96
Mefloquine	13.12	11.64	13.99	6.64	2.33

For certain drugs, like chloroquine, a reproducible edge effect was seen with the MSF assay (data not shown). It resulted in significantly lower IC₅₀ values for those drugs affected and could potentially influence the determination of resistance during screening. The cause of the edge remains unclear, but was not due to volumetric differences or intrinsic fluorescence properties of the drugs (data not shown). To avoid this issue, the outer wells composing the edge of the microtiter plate were not used for testing drug resistance in our laboratory. Additionally, several assay conditions that could potentially affect MSF assay readout, including assay length, starting parasite density and hematocrit levels, microtiter plate selection, and plate freezing were also examined (data not shown). IC₅₀ values determined at 48h were similar for both types of assays. Initial parasitemia between 0.2% and 1% were examined, with the most reliable results occurring with parasitemia above 0.5%. No significant difference was seen in tests with hematocrit levels between 1 and 4%. Interestingly, the use of phenol red-free basal medium did not decrease fluorescence background in the MSF assay as has been demonstrated for other fluorescence techniques, including flow cytometry and confocal microscopy. Black microtiter plates commonly used to enhance fluorescence plate reader applications did not affect test outcome compared to standard transparent microtiter plates. Freezing the plates before or after the addition of dye did not affect IC₅₀ determination in the MSF assay. The use of the Biomek 2000 robotics platform reduced plate

manipulation time by 60% and significantly increased assay intra- and inter-plate reproducibility.

The advantages of the MSF assay are clear. Firstly, it is a one-plate assay, unlike the radioactive assays that require costly filter plates. Only standard, sterile transparent tissue culture microtiter plates already used in the [³H]hypoxanthine assay are necessary to perform the assay. Less culture reagents are required, as the culture volume has been scaled to one-half that of the radioactive assays to facilitate one-plate assay conditions. Secondly, the MSF assay is a one-step assay, meaning that dye/lysis buffer solution is added directly to the malaria cultures, briefly incubated, and then read on the fluorometer. The dye is stable enough for actual field use in tropical environmental conditions and is currently being developed for culture-free, patient whole blood assay conditions in Peru. Thirdly, the fluorometer model (Tecan Genios Plus, Tecan US, Research Triangle Park, North Carolina) we use to obtain our readouts in our own laboratory is the same model used in Peru by the Parasitology Program, U. S. Naval Medical Research Center, Lima (NMRCDC). The filter set needed for data acquisition is the standard set used for the detection of fluorescein (FITC; excitation at 485 nm and emission at 530nm) and is readily available worldwide. It is critical that we use the same acquisition platform in Kenya for the purposes of multi-site standardization. Fourthly, data analysis is easily standardized for multi-site and/or centralized capture and, hence, transfers worldwide. Our laboratory and NMRCDC directly export the readout data as MS Excel software files. These files are then analyzed by GraphPad Prism 4.0 software, where the data is transformed and IC₅₀ values determined as previously described (Smilkstein et al., 2004). Lastly, the cost per MSF assay is 100 times less than the [³H]hypoxanthine assay for reasons described above and the absence of expensive radioactive substrate use and disposal.

In conclusion, compared to our standard radioactive assay, the MSF assay is more cost-effective, simple, less hazardous, and field amendable, while still allowing for accurate high-throughput, automated drug resistance surveillance and testing at all of our global malaria surveillance sites. These efforts are important, because with several networks using the same assay system, the malaria drug resistance data can be compared and analyzed from various drug resistance programs (WHO, 2005). This in affect will increase the usefulness of the data generated through GEIS malaria drug resistance surveillance sites. The data from a standard assay can then be expressed in terms that are interpretable and useful for the physician to prescribe malaria prophylaxis or treatment options to the U.S. Warfighter (Labbe et al., 2003; Chen and Wilson, 2002; Kotwal et al., 2005; Susi et al., 2005; Tuck et al., 2003.). Thus, while the nature of war has not changed, even with our parasitic threats,

technological advances like the MSF assay support the Warrior soldier's undeniable military advantage by proactively surveying infectious threats and preventing and treating diseases.

The opinions expressed herein are those of the authors and do not reflect the views or opinions of the U.S. Army and the Department of Defense.

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