

Short Communication

Whole-Cell SYBR Green I Assay for Antimalarial Activity Assessment

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Abstract

Malaria parasite growth assessment assays using nucleic acid dye SYBR Green I are frequently compromised by high background readings due to haemoglobin and detergents in lysis buffer. Here, we have assessed and further validated an intact-cell SYBR Green I-based fluorescence assay (Cell-MSF) for antimalarial activity assessment even of highly fluorescent compounds. Our modifications include:

- The absence of detergents and cell debris renders a high dynamic range and $z' > 0.7$
- Tested against fluorescent and non-fluorescent compounds
- Without cell lysis, one centrifugation and uses transparent 96 well plates

Graphical abstract



Method name: Cell-MSF - whole-cell SYBR Green I-based fluorescence assay

BACKGROUND

Antimalarial drug inhibitory concentrations determined through the measurement of fluorescent DNA binding dyes are based on the fact that the host, red blood cells, lacks DNA. The extensively used malaria SYBR Green I (SG) fluorescence assay (MSF) [1] has some technical problems due to the low signal-to-noise ratio, resulting from high background signals [2-5] in lysis

buffers, and haemoglobin (that drastically quenches fluorescence signal of DNA-SG adducts) has been implicated in this poor signal-to-noise ratio [5]. In addition, the absorption spectrum of haemoglobin overlaps almost exactly with the excitation/emission wavelengths for SG [1,6].

In our hands (also reported by others [1,3,5,7], either detergents or haemoglobin fluorescence quenching (or both),

were compromising the signal, rendering dispersed readings and low reproducibility results for the MSF assay.

To overcome this problem, we have assessed and further validated a simple whole-cell SG-based fluorescence assay (Cell-MSF) for use in antimalarial drug screening based on the work described by Bennett *et al.*, 2004 [3].

METHOD

Plasmodium falciparum strains 3D7, chloroquine (CQ) and mefloquine (MEF) susceptible, and Dd2, CQ and MEF resistant strains were cultured in standard culture conditions as previously described [8]. Cultures were synchronized by double sorbitol treatment [9] prior to the assays. Staging and parasitemia (%) were determined by light microscopy of Giemsa-stained thin blood smears. To determine the correspondent IC50s, 3D7 and Dd2 were cultivated for 48 h in the presence of a two-fold serial dilutions of CQ(500-0.5nM for 3D7 and 9000-8.8nM for Dd2), MEF (500-0.5nM for 3D7 and 9000-8.8nM for Dd2), dihydroartemisinin (DHA) (1000-1nM for both 3D7 and Dd2), acridine orange (5000-39µM for 3D7)and a control culture without drug.

Basic procedure

1 -100µl of *P. Falciparum* ring-stage (>85%) 3D7 and Dd2 cell culture is distributed in a 96-well transparent flat bottom plate

2 - After incubation (48h), 100µl of a solution of SG (0.001% v/v in PBS) is added to each well

3 - Plates are incubated for 60 min under standard culture conditions

4 - Centrifuged at 2750g for 2 min, supernatant (SN) is discarded

5 - Cells re-suspended in 100µl of PBS

6 - Fluorescence is collected in a fluorimeter plate reader with: Excitation 485nm and Emission 535 nm

Assessment of the linearity of fluorescence signal and Z' value of the assay

3D7 ring-stage parasitized red blood cells (iRBC) were serially diluted (1:2) with four suspensions of non-parasitizedRBCscontaining1, 2, 3 and 4% haematocrit (HTC). 100µl of mixture was distributed in triplicate in a 96-well transparent flat bottom plate and treated as above and fluorescence was collected. Parasitemia was plotted against the relative fluorescence units (RFU) values and analyzed by linear regression, the slope and R² values were determined to establish goodness of fit (Figure1).

Correspondent slope and R² values were as follows: 1% HTC slopes =424600±19800, R²=0.978; 2%HTC, slope=424600±28600,R²=0.950; 3%HTC, slope =627300±20280, R²=0.961; 4% HTC, slope =548100±47490, R²=0.898; all the four curves had a p<0.0001. The quality and robustness of the assay was verified using the statistical measure of Z' [10]. Z' was calculated as follows: $Z' = 1 - (3 \times \text{SDiRBC} + 3 \times \text{SDRBC}) / (\text{MiRBC} - \text{MRBC})$, where MiRBC and SDiRBC are the mean and standard deviation of RFU values from iRBC, respectively and MRBC and

SDRBC are the mean and standard deviation of RFU values from the RBC, respectively, the denominator is the absolute value of the difference of MiRBC and MRBC. Assays that display a Z' value of ≥0.5 are generally acceptable [11]. The haematocrit of 3% was chosen for further assays, based in the highest slope and R² for the correlation curve and the highest Z'-factor (Figure 1). Z' values ranged between 0.92 and 0.95 with a mean value of 0.94±0.01.

Suitability of the Cell-MSF assay for antimalarial activity assessment

3D7 and Dd2 strains were diluted to 3%HTC and 1% parasitemia and cultivated for 48 h in the presence and absence of two-fold serial dilutions of CQ, MEF, and DHA and processed as above. Correspondent IC50 values were estimated from dose response curves generated using Graph Pad Prism 5 (trial version) (Figure 2). To determine the Z' for the drug assays, wells containing culture media and wells containing maximum dose of each compound were compared using the formula above (Figure 2). Overall, drug assays produced satisfactory Z' values ranging from 0.71 to 0.80. These are similar to those previously described for other fluorescence based assays: SG (0.73-0.95) (12), YOYO-1 (0.75) (11) and 4',6-diamidino-2-phenylindole (DAPI) (0.80) (7).

The IC50s determined for CQ, MEF and DHA using the Cell-MSF assay are comparable to those in literature [4,13-15]. Additionally, absolute IC50 values for CQ and MEF in *P. falciparum* strains vary across the literature, but most data show identical relative drug susceptibilities between strains, obtained in a given growth inhibition format. For example, Dd2 parasites are >10-fold more resistant to CQ than susceptible strains (such as 3D7), assuming the same method for IC50 calculation [14]. This is reflected by our results where the IC50 for CQ in Dd2 is 20.7 times higher than in 3D7.

Assessment of fluorescent compounds interference with the Cell-MSF dynamic range

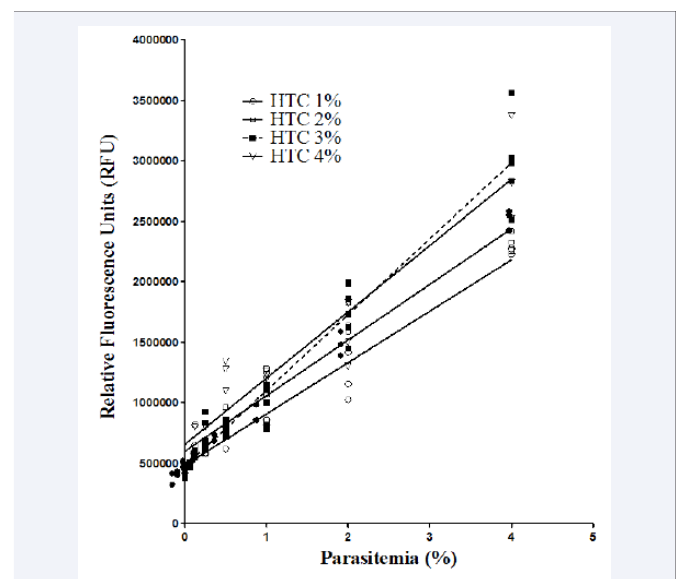


Figure 1 Cell-MSF assay: linear regression line and correspondent R² for RFU from ring-stage 3D7 parasites plotted over a range of parasitemia and haematocrit.

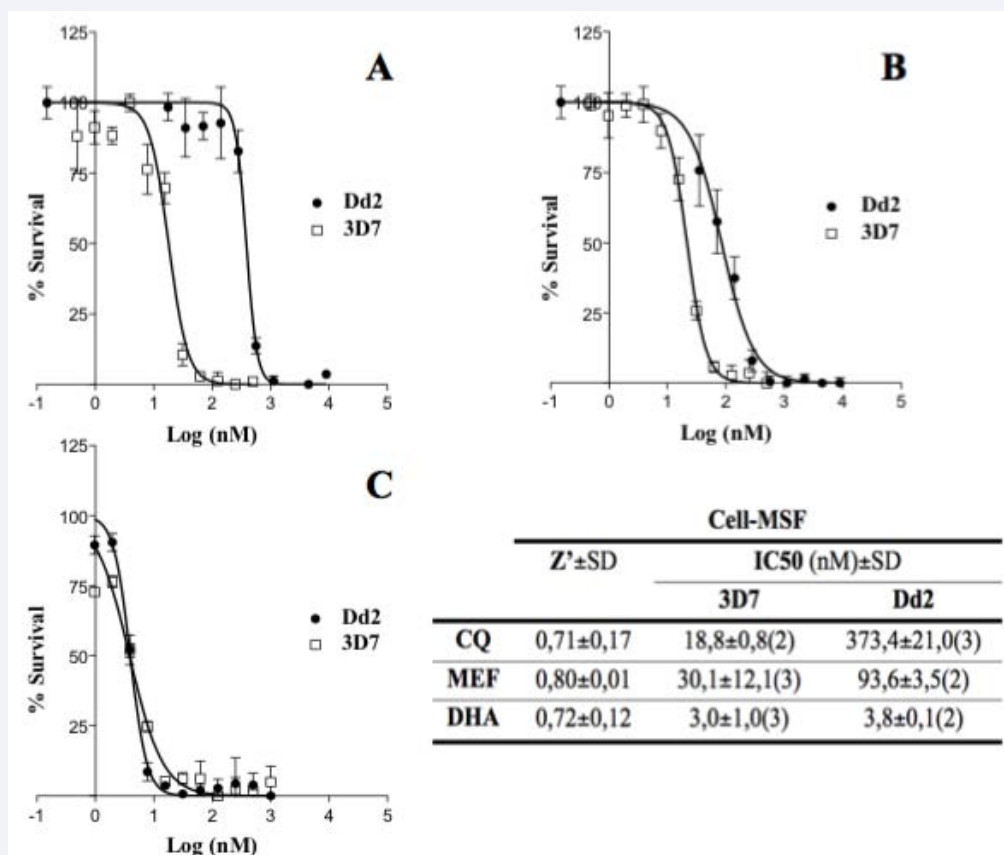


Figure 2 Cell-MSF assay quality parameters and inhibitory concentrations of CQ, MEF and DHA for *P. falciparum*. Representative dose response curves generated with the Cell-MSF assay. CQ (A), MEF (B) and DHA (C) effect on 3D7 and Dd2 strain survival. Inserted table: mean IC50 of experiments run on different days ± standard deviation (SD), number of independent experiments is in parentheses. The mean ± standard deviation (SD) for the Z'-factor corresponds to the optimization of the assay with 3D7 strain.

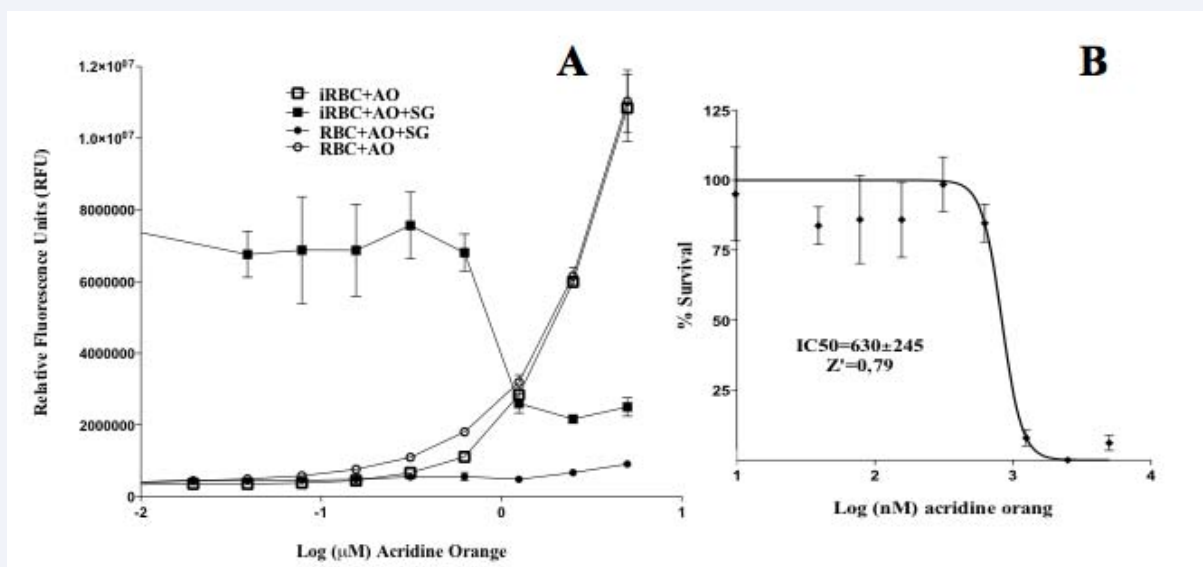


Figure 3 Assessment of acridine orange fluorescence interference with the Cell-MSF assay dynamic range. (A) Curves represent RFU before and after SG addition to cultures. iRBC+AO - iRBC before removing the culture media with AO (open squares); RBC+AO - non-parasitized RBC before removing the culture media with AO (open circles); iRBC+AO+SG - iRBC after incubation with SG (filled squares); RBC+AO+SG - non-parasitized RBC after incubation with SG (filled circles). (B) Representative dose response curve of AO effect on *P. falciparum* 3D7 strain growth, generated with the Cell-MSF.

The antimalarial activity of the highly fluorescent acridine orange (AO), with an emission peak of 550 nm, close to SG - 535 nm, was tested using the Cell-MSF assay. 3D7 parasite microculture (3% HTC, 1% parasitemia) was incubated for 48 h in the presence and absence of a two-fold serial dilutions of AO as described above for IC₅₀ determinations. After 48h of incubation, RFUs of AO were measured before adding SG. The resulting curves from iRBC and RBC nearly coincide (Figure 3), due to high fluorescence emitted by AO. In contrast, after incubation with SG and removing the SN, RFUs remained at a residual level in the RBCs and inversely correlated with AO doses in the iRBC, reflecting parasite growth inhibition by AO (Figure 3). We concluded that AO fluorescence does not influence the dynamic range of the Cell-MSF assay, since $Z' = 0.79$ was within the range of those determined for CQ, MEF and DHA (0.71-0.80). Furthermore, the resulting IC₅₀ for AO in 3D7 (630.6±245.1 nM) was similar to that described in the literature determined by HRPII assay (465.7±274.9 nM) [16].

Because a $Z' \geq 0.5$ is considered favorable for high-throughput drug screening due to the high degree of day-to-day reproducibility and large dynamic range that the score reflects [11], in this report we provide evidence that the Cell-MSF assay is simple, robust and amenable for *in vitro* screening of antimalarial compounds even those that emit high fluorescence with a wave length close to SG, such as AO.

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