Malaria remains a serious health problem throughout the tropical and sub-tropical areas of the world, with over 300 million cases and at least 1 million deaths a year (Guerin et al, 2002). An effective malaria vaccine has yet to be developed and parasites have developed resistance to nearly all available antimalarial drugs. Thus, there is an urgency to find new effective drugs and to explore the possibility of drug combinations.

An accurate and simple in vitro antimalarial sensitivity assay is an essential tool for estimating the effects of candidate drugs and drug combinations on parasite growth. The first in vitro antimalarial sensitivity assay was developed by Rieckmann et al (1968) based on the measurement of the ability of the parasite to mature from early ring forms to schizont forms in the presence of various amounts of drugs (schizont maturation assay). After the success of development of continuous in vitro cultivation of the erythrocytic cycle of *P. falciparum* (Trager and Jensen, 1976), the schizont maturation assay was simplified to the so-called micro schizont maturation assay (Rieckmann et al, 1978). At almost the same time, several drug sensitivity assays based on measurement of an increase in the percent parasitemia were developed using longer incubation times (Nguyen-Dinh and Payne, 1980; Nguyen-Dinh and Trager, 1980; Richards and Maples, 1979). However, the labor- and time-consuming process in determining percent parasitemia by microscopically counting parasite numbers, and in-
dividual variability in the data, led to the development of automatic readings (reviewed in Noedl et al, 2003). Several techniques using different indicator/equipment have been proposed, viz (i) Hoechst 33258-DNA staining (van Vianen et al, 1990) or acridine orange-DNA staining (Saito-Ito et al, 2001)/flow cytometer, (ii) Hoechst 33258-DNA staining (Smeijsters et al, 1996)/fluorometer, (iii) [3H]-hypoxanthine incorporation into nucleic acids (Desjardins et al, 1979) or [3H]-ethanolamine incorporation into phospholipids (Elabbadi et al, 1992)/liquid scintillation counter, (iv) parasite lactate dehydrogenase (LDH)/spectrophotometer (Makler et al, 1993) or double-site enzyme-linked LDH immunodetection assay (Druilhe et al, 2001), and (v) histidine-rich protein II/enzyme-linked immunosorbent assay plate reader (Noedl et al, 2002).

CyQUANT GR cyanine dye, has been recently reported to be a good indicator for quantitating the number of cells in culture (Jones et al, 2001). The green GR dye when bound to cellular nucleic acid, which is a reliable indicator of cell number, exhibits strong fluorescence easily detectable when excited at 485 nm and measured at 530 nm using a flow cytometer or fluorometer. Since the GR dye provides the greatest sensitivity and has the widest range of linearity in comparison with the other cyanine dyes, it has been developed as a commercial fluorescence-based assay kit, CyQUANT Cell Proliferation Assay Kit (Molecular Probes, OR, USA), and is claimed to be much more sensitive than other fluorescence-based assays, viz Hoechst 33258, Hoechst 33342, propidium iodide (Jones et al, 2001). CyQUANT assay has been used in studies on cell proliferation, cell growth inhibition and cell adhesion in diverse human cell types (Davenport et al, 2003; Dufes et al, 2003; Neff et al, 2002). Here, we demonstrate that this assay can be applied to assess the number of malaria parasites and to determine parasite viability after treatment with antimalarial drugs in 96-well cultures.

The sensitivity of CyQUANT GR dye in quantitating parasite number and the effect of erythrocyte number (hematocrit) on fluorescence response were performed as follows. Human red cells at 1.5% hematocrit (without parasite) were combined with red cells at 1.5% hematocrit and 20% parasitemia in a 96-well plate to generate a concentration of parasitemias ranging from 0 to 20% in a final volume of 100 µl per well. Parasitemias ranging from 0-20% in 2% hematocrit were also prepared in the same way. The plate was then stored frozen until ready to be used in the assay. 200x CyQUANT GR dye (Molecular Probes, OR, USA) and 20x lysis buffer (provided by the manufacturer) were freshly diluted before use as follows. 20x lysis buffer was diluted 1:10 in water to make 2x lysis buffer. The 2x lysis buffer was then used for diluting 200x GR dye (1:50) to give a 4x GR dye in 2x lysis buffer. During the preparation of GR dye in lysis buffer, the plate containing parasite suspensions was thawed at room temperature. One hundred µl of GR dye in lysis buffer were added to each well. The mixture was gently mixed by pipetting and the plate was then incubated in the dark at room temperature for 1 hour. Fluorescence was detected with a Cytofluor II fluorescence multiwell plate reader (Perseptive Biosystems, MA, USA), at excitation wavelength of 485 nm and emission wavelength of 530 nm. Correlation between GR fluorescence and percent parasitemia was assessed by plotting relative fluorescence unit against percent parasitemia.

As seen in Fig 1, both curves, one generated from keeping hematocrit constant at a final concentration of 0.75% and the other at 1%, were linear up to 20% parasitemia with the same correlation coefficient of r² = 0.99, and did not reach a plateau, indicating that at 0.75 and 1% hematocrit the dye concentration was sufficient to detect parasite nucleic acid concentrations of up to parasitemia of 20% in 1% hematocrit. The background fluorescence in the absence of parasites was negligible.

To test whether the CyQUANT assay can be used for testing antimalarial agents, the effects on the growth of chloroquine-sensitive P. falciparum D6 strain were evaluated for a conventional antimalarial drug, chloroquine, and a novel antimalarial compound, 3,6-bis-omega-diethylaminoamylxoxanthone (C5). Chloroquine (Sigma, MO, USA) and C5 (Interlab Corporation, OR, USA) were dissolved in water and then filter sterilized through 0.2 µm HT Tuffryn® membrane (Pall Corporation, CA, USA) to pro-
vide stock solutions, which were then freshly diluted in medium without serum to give the appropriate concentrations before use. Assays were performed in triplicate wells in 96-well plate. Drug solution and parasite suspension were added to each well giving a final volume of 200 µl per well containing 1% parasitemia in 1.5% hematocrit. The plates were then incubated at 37°C and the parasite survival was determined using the CyQUANT method and a radioisotopic assay (Elabbadi et al., 1992).

For the CyQUANT assay, after the plate was incubated for 48 hours, parasites in each well were resuspended by pipetting and 100 µl aliquot of the cell suspension was then transferred to a new plate. The plate was frozen and thawed at room temperature. Fluorescence in the wells was measured as described above. IC_{50} value was determined by GraphPad Prism (CA, USA) software from a plot of relative fluorescence unit against log of drug concentration.

For the radioisotopic assay, after 24 hours of incubation, 20 µl of 50 µCi/ml [3H]-ethanolamine (American Radiolabeled Chemicals, MO, USA) in culture medium were added to each well. The cultures were further incubated for 24 hours and the contents of each well were then harvested onto glass filters using a semiautomated Tomtec harvester (CT, USA). After drying, the dried filters were soaked with liquid scintillation fluid (Wallac,
MD, USA) and then counted by a liquid scintillation counter (1205 Betaplate, Wallac, MD, USA). IC\textsubscript{50} value was determined by the GraphPad Prism software from a plot of [\textsuperscript{3}H]-ethanolamine incorporation against log of drug concentration.

Although the CyQUANT and [\textsuperscript{3}H]-ethanolamine-incorporation assay measure different indicators, the shapes of the dose-response curves obtained by these two different assay methods were very similar as shown in Fig 2. The IC\textsubscript{50} values of chloroquine (54 nM) and C5 (254 nM) determined by CyQUANT assay were nearly the same as those determined by [\textsuperscript{3}H]-ethanolamine assay, namely 51 nM and 223 nM, respectively. The good agreement of these results indicates that the CyQUANT assay can be used for evaluation of in vitro antimalarial activity.

Since the CyQUANT assay is a fluorescence-based assay, it obviates the need to handle radioisotopes, eliminates problems associated with radioactive waste disposal and does not require specialized expensive equipment, such as liquid scintillation counter. Unlike isotopic assays that are based on measurement of radioisotope incorporation into a metabolite during a particular stage of parasite growth in culture, the CyQUANT assay measures parasite number at the end of culture. Thus, the CyQUANT assay should be more suitable for estimating parasite growth under various metabolic conditions.

In comparison with other in vitro antimalarial assays (Makler et al, 1993; Smeijsters et al, 1996; Druilhe et al, 2001; Saito-Ito et al, 2001; Noedl et al, 2002), the CyQUANT assay is easier and more convenient to perform, requires fewer steps, and uses simpler equipment. After culturing the parasites in a 96-well plate in the presence of drugs, the plate can be stored frozen for a long period prior to the assay. To perform the assay, the plate was thawed and 100 ml of the solution was transferred to a new plate for fluorescence measurements. However, this transfer step can be eliminated in order to make the assay easier by increasing concentration of dye/lysis buffer, which is added to the plate directly on a fluorescence microplate reader, thereby allowing the whole assay procedure to be completed within 2 hours. Since the media components did not interfere with the assay, the protocol did not require any washing steps nor changing growth medium. Although the previously reported fluorometric assay (Smeijsters et al, 1996) is highly sensitive, it requires many steps of repeated pipetting and centrifugation to extract DNA from the parasites and, therefore is labor-intensive, time-consuming and prone to human error.

In summary, the CyQUANT assay reported here is an accurate, non-radioisotopic, simple, convenient, rapid and practical procedure for in vitro determination of malaria parasite sensitivity to antimalarial drugs, tested alone or in combination. All the CyQUANT assay steps (freezethawing, transferring, lysis-staining, incubation and fluorescence measurement) can be automated, making this assay practical in high through-put in vitro screening of potential antimalarial candidates.

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