

SHORT REPORT

CYQUANT CELL PROLIFERATION ASSAY AS A FLUORESCENCE-BASED METHOD FOR *IN VITRO* SCREENING OF ANTIMALARIAL ACTIVITY

Nongluk Sriwilaijaroen¹, Jane Xu Kelly², Michael Riscoe² and Prapon Wilairat¹

¹Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand;

²Medical Research Service, Department of Veteran's Affairs Medical Center and Oregon Health and Science University, Portland, Oregon, USA

Abstract. The appearance of drug resistant parasites and the absence of an effective vaccine have resulted in the need for new effective antimalarial drugs. Consequently, a convenient method for *in vitro* screening of large numbers of antimalarial drug candidates has become apparent. The CyQUANT cell proliferation assay is a highly sensitive fluorescence-based method for quantitation of cell number by measuring the strong fluorescence produced when green GR dye binds to nucleic acids. We have applied the CyQUANT assay method to evaluate the growth of *Plasmodium falciparum* D6 strain in culture. The GR-nucleic acid fluorescence linearly correlated with percent parasitemia at both 0.75 or 1 percent hematocrit with the same correlation coefficient of $r^2 = 0.99$. The sensitivity of *P. falciparum* D6 strain to chloroquine and to 3,6-bis-omega-diethylaminoamyloxyxanthone, a novel antimalarial, determined by the CyQUANT assay were comparable to those obtained by the traditional [³H]-ethanolamine assay: IC₅₀ value of chloroquine was 54 nM and 51 nM by the CyQUANT and [³H]-ethanolamine assay, respectively; IC₅₀ value for 3,6-bis-omega-diethylaminoamyloxyxanthone was 254 nM and 223 nM by the CyQUANT and [³H]-ethanolamine assay, respectively. This procedure requires no radioisotope, uses simple equipment, and is an easy and convenient procedure, with no washing and harvesting steps. Moreover, all procedures can be set up continuously and thus, the CyQUANT assay is suitable in automatic high through-put drug screening of antimalarial drugs.

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-

Correspondence: Dr Prapon Wilairat, Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand.

Tel: +66 (0) 2354-7140; Fax: +66 (00) 2354-7174

E-mail: scpwl@mahidol.ac.th

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 μ l 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 μ l 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 multi-well 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 $r^2 = 0.99$, 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 a 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-diethylaminoamyloxyxanthone (C5). Chloroquine (Sigma, MO, USA) and C5 (Interlab Corporation, OR, USA) were dissolved in water and then filter sterilized through 0.2 μ m HT Tuffryn® membrane (Pall Corporation, CA, USA) to pro-

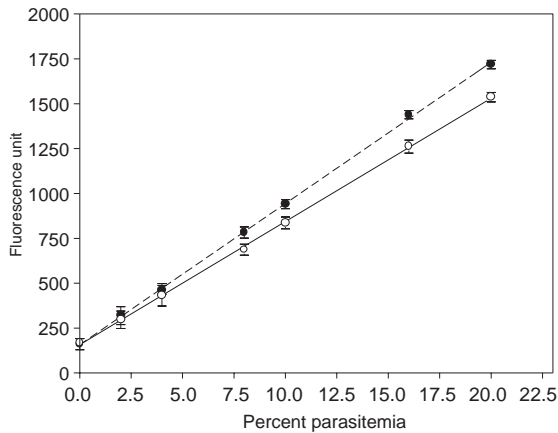
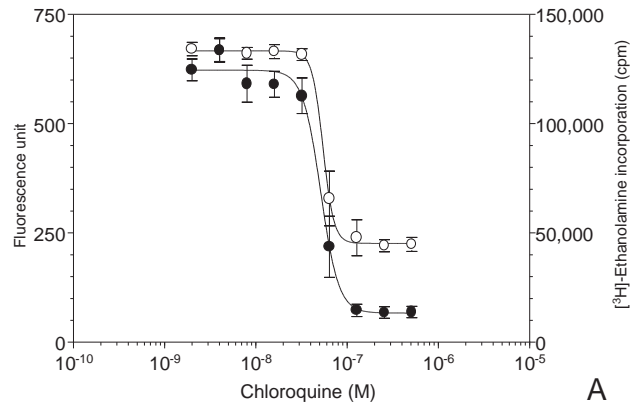


Fig 1—Correlation between CyQUANT GR fluorescence and percent parasitemia. The frozen and thawed cell suspension was mixed with GR dye, in a total volume of 200 μ l containing 4x GR dye/1x lysis buffer and 0.75% hematocrit (open circle) or 1% hematocrit (solid circle). Fluorescence was measured at excitation wavelength of 485 nm and emission wavelength of 530 nm. Both linear curves have $r^2 = 0.99$. Each point is the mean \pm SD of triplicate.

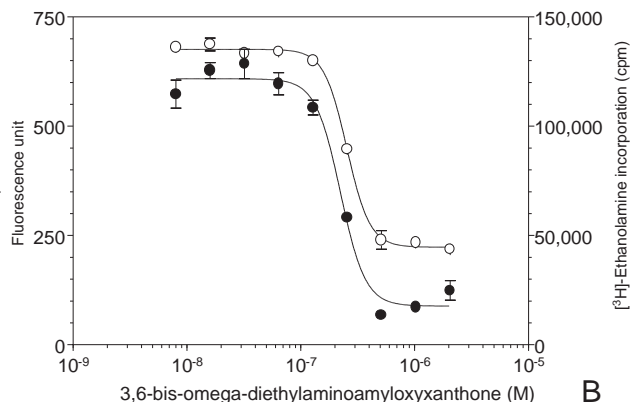
vide stock solutions, which were then freshly diluted in medium without serum to give the appropriate concentrations before use. Assays were performed in triplicate wells in 96-well plate. Drug solution and parasite suspension were added to each well giving a final volume of 200 μ l per well containing 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 μ l 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 were 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 μ l of 50 μ Ci/ml [3 H]-ethanolamine (American Radiolabeled Chemicals, MO, USA) in culture medium were added to each well. The



A



B

Fig 2—Assessment of sensitivity of *P. falciparum* D6 strain against chloroquine and 3,6-bis-omega-diethylaminoamyloxyxanthone using the CyQUANT and [3 H]-ethanolamine assay. Parasites were incubated with chloroquine (panel A) or 3,6-bis-omega-diethylaminoamyloxyxanthone (panel B) at 37°C in 96-well plate. In the CyQUANT assay (open circle), after a total incubation of 48 hours, 100 μ l aliquot of cell suspension was lysed and incubated with GR dye at room temperature for 1 hour in the dark. Fluorescence was then determined as described in the legend of Fig 1. In the radioisotopic assay (solid circle), after incubation of 24 hours, 1 μ Ci of [3 H]-ethanolamine was added to each well and the cell suspension was incubated for a further 24 hours. Radioactive isotope incorporation into parasites was detected in a liquid scintillation counter. Each point is mean \pm SD of triplicate.

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). IC₅₀ value was determined by the GraphPad Prism software from a plot of [³H]-ethanolamine incorporation against log of drug concentration.

Although the CyQUANT and [³H]-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 IC₅₀ values of chloroquine (54 nM) and C5 (254 nM) determined by CyQUANT assay were nearly the same as those determined by [³H]-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 (freezing-thawing, 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.

ACKNOWLEDGEMENTS

NS was supported by the Thailand Research Fund (TRF) Royal Golden Jubilee PhD Program (PHD/0010/2543) and PW is a TRF Senior Research Scholar. MR and JK are supported by a grant from the Medical Research Program of the US Department of Veterans Affairs.

REFERENCES

- Davenport DR, Mailhot JM, Wataha JC, Billman MA, Sharawy MM, ShROUT MK. Effects of enamel matrix protein application on the viability, proliferation, and attachment of human periodontal ligament fibroblasts to diseased root surfaces *in vitro*. *J Clin Periodontol* 2003; 30: 125-31.
- Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob Agents Chemother* 1979; 16: 710-8.
- Druilhe P, Moreno A, Blanc C, Brasseur PH, Jacquier P. A colorimetric *in vitro* drug sensitivity assay for *Plasmodium falciparum* based on a highly sensitive double-site lactate dehydrogenase antigen-capture enzyme-linked immunosorbent assay. *Am J Trop Med Hyg* 2001; 64: 233-41.
- Dufes C, Alleaume C, Montoni A, Olivier JC, Muller JM. Effects of the vasoactive intestinal peptide (VIP) and related peptides on glioblastoma cell growth *in vitro*. *J Mol Neurosci* 2003; 21: 91-102.

- Elabbadi N, Ancelin ML, Vial HJ. Use of radioactive ethanolamine incorporation into phospholipids to assess *in vitro* antimalarial activity by the semiautomated microdilution technique. *Antimicrob Agents Chemother* 1992; 36: 50-5.
- Guerin PJ, Olliaro P, Nosten F, *et al.* Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. *Lancet Infect Dis* 2002; 2: 564-73.
- Jones LJ, Gray M, Yue ST, Haugland RP, Singer VL. Sensitive determination of cell number using the CyQUANT cell proliferation assay. *J Immunol Methods* 2001; 254: 85-98.
- Makler MT, Ries JM, Williams JA, *et al.* Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *Am J Trop Med Hyg* 1993; 48: 739-41.
- Neff T, Layman D, Jeansonne BG. *In vitro* cytotoxicity evaluation of endodontic sealers exposed to heat before assay. *J Endodont* 2002; 28: 811-4.
- Nguyen-Dinh P, Payne D. Pyrimethamine sensitivity in *Plasmodium falciparum*: determination *in vitro* by a modified 48-hour test. *Bull WHO* 1980; 58: 909-12.
- Nguyen-Dinh P, Trager W. *Plasmodium falciparum in vitro*: determination of chloroquine sensitivity of three new strains by a modified 48-hour test. *Am J Trop Med Hyg* 1980; 29: 339-42.
- Noedl H, Wernsdorfer WH, Miller RS, Wongsrichanalai C. Histidine-rich protein II: a novel approach to malaria drug sensitivity testing. *Antimicrob Agents Chemother* 2002; 46: 1658-64.
- Noedl H, Wongsrichanalai C, Wernsdorfer WH. Malaria drug-sensitivity testing: new assays, new perspectives. *Trends Parasitol* 2003; 19: 175-81.
- Richards WH, Maples BK. Studies on *Plasmodium falciparum* in continuous cultivation. I. The effect of chloroquine and pyrimethamine on parasite growth and viability. *Ann Trop Med Parasitol* 1979; 73: 99-108.
- Rieckmann KH, McNamara JV, Frischer H, Stockert TA, Carson PE, Powell RD. Effects of chloroquine, quinine, and cycloguanil upon the maturation of asexual erythrocytic forms of two strains of *Plasmodium falciparum in vitro*. *Am J Trop Med Hyg* 1968; 17:661-71.
- Rieckmann KH, Campbell GH, Sax LJ, Mrema JE. Drug sensitivity of *Plasmodium falciparum*. An *in-vitro* microtechnique. *Lancet* 1978; 1: 22-3.
- Saito-Ito A, Akai Y, He S, Kimura M, Kawabata M. A rapid, simple and sensitive flow cytometric system for detection of *Plasmodium falciparum*. *Parasitol Int* 2001; 50: 249-57.
- Smeijsters LJ, Zijlstra NM, Franssen FF, Overdulve JP. Simple, fast, and accurate fluorometric method to determine drug susceptibility of *Plasmodium falciparum* in 24-well suspension cultures. *Antimicrob Agents Chemother* 1996; 40: 835-8.
- Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976; 193: 673-5.
- van Vianen PH, Thaihong S, Reinders PP, *et al.* Automated flow cytometric analysis of drug susceptibility of malaria parasites. *Am J Trop Med Hyg* 1990; 43: 602-7.