# OPTIMIZING THE CULTURE OF *PLASMODIUM FALCIPARUM* IN HOLLOW FIBER BIOREACTORS

P Preechapornkul[1], K Chotivanich[1], M Imwong[1], AM Dondorp[1,2], SJ Lee[1,2], NPJ Day[1,2], NJ White[1,2] and S Pukrittayakamee[1]

[1]Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand;
[2]Centre for Clinical Vaccinology and Tropical Medicine, Churchill Hospital, Oxford, United Kingdom

**Abstract.** The hollow fiber bioreactor (HFBR) is a cell culturing system allowing continuous perfusion of medium. It was designed to grow microorganisms in a dynamically altering medium mimicking change in the *in vivo* intravascular and extravascular compartments. The cell compartment (extra capillary space) and medium compartment (intra capillary space) are connected through pores of semi-permeable fiber membranes. These membranes allow exchange of gas and nutrients. We have adapted this system for the *ex vivo* culture of *Plasmodium falciparum* at high parasite densities. A Thai *P. falciparum* isolate (TM036) cultured in RPMI, supplemented with 0.5% Albumax II, could be maintained continuously in the system by daily changes of a small volumes of medium. Under optimized conditions the HFBR cultures attained 8% parasitemia in 40% hematocrit, thereby providing a total parasite biomass of $6.0 \times 10^9$ parasitized erythrocytes. The main problem encountered was clogging of micropores in the hollow fiber system by cellular debris over time. Although 'reverse flushing' partly prevented this, a larger pore size might be needed to overcome this problem. The system opens new possibilities for the study of *in vitro* drug sensitivity under conditions mimicking *in vivo* pharmacokinetics, and the selection of anti-malarial drug resistance and associated parasite biological and genomic changes.

**Key words:** *Plasmodium falciparum, ex vivo* culture, hollow fiber bioreactor

## INTRODUCTION

The *in vitro* method for the continuous cultivation for *P. falciparum*, originally described in 1976, has made a significant contribution to malaria research (Trager and Jensen, 1976). This culture technique has become an essential component of malaria research leading to advances in our understanding of parasite biology (Udomsangpetch *et al*, 2007), phenotypic (Serirom *et al*, 2003) or genotypic characterizations (Kim *et al*, 2001), immunological studies (Vande Waa *et al*, 1984; Fievet *et al*, 2002), and drug susceptibility testing (Peel *et al*, 1993; Hatabu *et al*, 2005). The principle of this technique is to maintain parasitized erythrocytes in a culture bottle containing a thin layer of RPMI media to allow adequate gas exchange in a low oxygen tension environment. The technique

Correspondence: Professor S Pukrittayakamee, Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchwithi Road, Ratchathewi, Bangkok 10400, Thailand.
Tel/Fax: 66 (0) 2644 4541
E-mail: sasithon@tropmedres.ac

only permits culture at low parasite densities. Multiple large culture flasks are needed when larger numbers of parasites need to be studied.

Hollow-fiber membrane devices are widely used for a variety of purposes, including the study of monoclonal antibody production (Kreutz *et al*, 1997; Gramer and Britton, 2002), recombinant protein production (Inoue *et al*, 1999), lymphocyte culture(Malone *et al*, 2001), adenovirus vector production (Pan and Whitley, 1999), cartilage formation (Potter *et al*, 1998), studies on cytokine production (Lamers *et al*, 1999) and bioartificial organs (Chen *et al*, 2005). Applying the technique for the culture of *P. falciparum* parasites was first described in 2003 and offers a simple method for culturing parasites at high hematocrits and at parasite densities up to 14 - 18% (Li *et al*, 2003). This closed-continuous perfusion system offers some important advantages over the traditional suspension cultures in culture flasks, including protection of cells from shear stress, allowing high cell densities and higher concentrations of parasite products; and a reduction in the number of flasks, tedious manipulation, and medium requirement (Piret and Cooney, 1990). This system still requires large amounts of culture medium, although less than conventional *in vitro* methods.

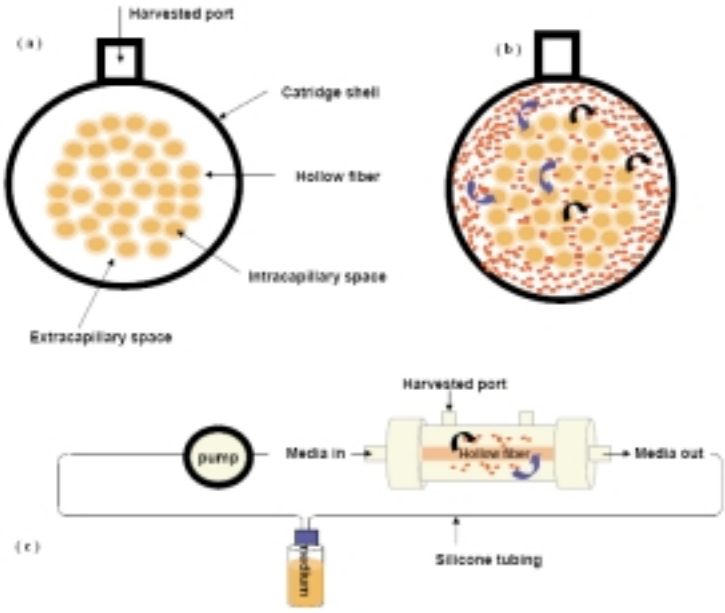


Fig 1–Schematic diagram of Hollow fibers bioreactor. a) Cross section of the HFBRS showing the different component. b) HFBR in use, with the erythrocyte suspension in the extracapillary space. The black arrows denote the outward flow of medium from the hollow fibers; the grey arrows represent the exchange of waste products back into the intracapillary space. c) Hollow fiber bioreactor system overview.

The aim of the current study was to optimize the hollow fiber bioreactor method for culturing *P. falciaparum* at high hematocrits and parasite densities, reducing the volumes of required culture medium, and supplementation with Albumax II instead of human serum, both of which confer important cost savings.

## MATERIALS AND METHODS

**Description of the hollow fiber bioreactor**

The system consists of three parts: a hollow-fiber cartridge, a pump, and the tubing system that connects the cartridge with a reservoir for medium (Fig 1). The cartridge contains 2 compartments: an ECS (Extra capillary space) and an ICS (Intra capillary space). Cells are trapped at the ECS side which is located between multiple hollow semi permeable fibers. The medium inside the ICS supplies nutrients, evacuates metabolites and enables gas ex-

change between the cells through the pores of the semi permeable fiber membranes.

These fibers are ultra filters with selective permeability depending on the molecular weight cutoff, which varies between 10-100 kDa corresponding to a micropore size between 0.1-0.2 µm. (Gramer *et al*, 1999). A microprocessor-controlled pump circulates medium inside the hollow fibers at a preset rate.

**Preparation of culture medium**

RPMI-1640 medium (Sigma, St Louis, MO) was complemented with $NaHCO_3$ 2 g/l, Hepes 5.958 g/l, gentamicin 1 ml/l, hypoxanthine 13.6 mg/l and D-glucose 4.5 g/l in de-ionizing tissue grade water. Culture medium was sterilized by filtering through a 0.45 µm pore filter unit (Nalgene, NY) and kept at 4ºC or at -20ºC in case storage for more than 2 weeks was required.

Culture medium was further supplemented with Albumax II (Gibco, New Zealand) on a daily basis. For this a 5% (m/v) stock solution was prepared from 10 g of Albumax II powder in 200 ml of RPMI 1640, stirred at 37ºC by a movable magnetic stirrer until dissolved completely, sterilized as described above, and stored at 4ºC until further use. This stock solution was added to 250 ml of culture medium to obtain a final concentration of 0.5% Albumax II. The pH of the completed medium was set between 7.2 and 7.4.

**Red blood cell preparation**

Human type O blood was collected in acid citrate dextrose [citric acid 7.3 g, trisodium citrate 22 g, glucose (dextrose) 24.5 g, distilled water 1,000 ml]. Buffy coat and plasma were removed after centrifugation at 2,500 rpm for 5 minutes. Packed cells were washed three times in RPMI-1640, centrifuged at 2,000 rpm for 5 minutes and resuspended at a 50% hematocrit in culture medium.

**Evaluating Albumax II as a replacement of human serum for culture medium supplementation**

In order to justify the substitution of the commercially available Albumax II, a lipid enriched bovine albumin solution, for human serum as supplementation of the culture medium, we compared the two in a conventional culture following the standard method as described by Trager and Jensen (Oduola *et al*, 1985). The parasites were cultured in 50 ml polystyrene tissue culture flasks (Becton Dickinson - Lab ware) containing 5% hematocrit in RPMI 1640 medium (Sigma, St Louis, MO) with $NaHCO_3$ 2 g/l, Hepes 5.958 g/l, gentamicin 1 ml/l, hypoxanthine 13.6 mg/l, D-glucose 4.5 g/l and supplemented with different types of serum. The growth rates of parasite culture in medium containing 0.5% Albumax II (Gibco, New Zealand) versus 10% human serum was compared. Growth rates were analyzed as previously described (Trager and Jensen, 1976) using the following equation:

$$\text{Exponential growth rates (R)} = \frac{\ln A_2 - \ln A_1}{T_2 - T_1}$$

Where $A_1$ and $A_2$ = the number of parasites per ml blood at time $T_2$ and $T_1$, respectively.

**Cultivation of parasites in hollow fiber bioreactors (HFBR)**

The 15 ml HFBR with high molecular weight cutoff (MWCO; 20kd@50%) hydrophilic fiber (C2011, Fiber Cell system, MD) was used to culture the laboratory adapted *P. falciparum* strain TM036. A microprocessor-controlled pump was used to circulate the medium at 75 ml/minute. The bioreactor cartridge, pump, and the medium containing bottle were kept inside the incubator which was filled with 5% $CO_2$ and maintained at 37ºC.

Samples of cells and medium were collected daily after gentle cell resuspension using a syringe attached to the harvest port. One ml of the red cell suspension was removed to assess parasitemia and hematocrit. The parasitemia and stage distribution of the parasites were assessed microscopically on a thin blood film stained with Field's stain. Parasitemia was expressed as the number of erythrocytes containing asexual stages of *P. falciparum* per 1,000 erythrocytes under oil emersion at a 1,000x magnification. Metabolic activity was assesses by evaluating glycolyses. Since the total volume of the system remained constant over time (250 ml), the changes in lactate and glucose concentrations over time represent the lactate production and glucose consumption of the experimental total parasite biomass. Dividing the total glucose consumption and lactate production by the total parasite numbers gives the average consumption/production per parasite. Lactate and glucose were assessed using the YSI 2300 TSAT plus (YSI Incorporated, OH). Harvesting of the cultured parasites was achieved by tilting the bioreactor to one side and flushing the system with complete medium from the elevated end of the bioreactor allowing accumulation of the red cell suspension in the syringe connected to the lower harvest port.

The erythrocyte suspension was washed daily with 50 ml freshly prepared complete culture medium. The rest of the culture medium was replaced with 200 ml of freshly prepared complete culture medium in a frequency of once or twice a day depending on the total number of parasites in the system. The red blood cells were resuspended twice daily by gentle rotation of the cartridge. During the development of the method, we introduced 'reverse flushing', which consisted of applying negative pressure with a syringe to the harvest port and aspirating culture medium through the fenestrated hollow fiber, in order to clean and maintain patency of the micropores.

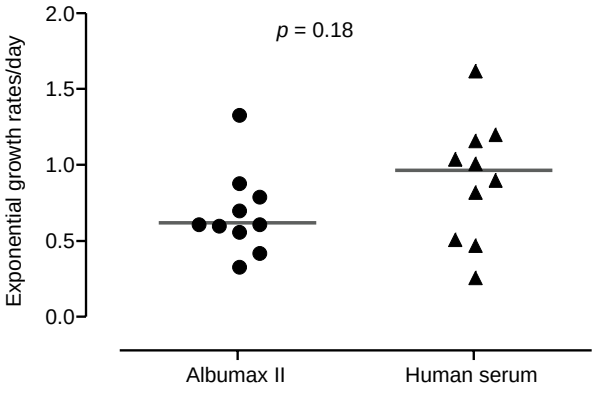


Fig 2–Growth rate of parasite strain TM036 in culture medium supplemented with Albumax II (●) versus human serum (▲).

**Statistical methods**

For the comparison between Albumax II and serum, the evaluation of the exponential parasite growth rates was assessed using Student's *t*-test. Once a continual decline in parasitemia was observed, the correlation between parasite biomass, lactate production and glucose consumption per parasite was assessed using Spearman's correlation coefficient. A continual decline was defined as 3 or more decreasing values over consecutive time points.

## RESULTS

**Evaluation of Albumax II**

Fig 2 shows the difference in the exponential growth rates of parasites cultured in RPMI-1640 medium supplemented with 0.5% Albumax II compared to 10% human serum. Mean ± SD growth rates were 0.69±0.28 in medium contain-

ing Albumax II compared to 0.91± 0.40 in medium containing human serum ($p$=0.18). Parasite strain TM036 could be cultured by using this Albumax II containing medium recipe, maintaining a total parasite biomass in the bioreactor of $1.6x10^8$ to $1.3x10^9$ parasite. The maximum parasitemia obtained with either growth medium was 10%. In contrast to medium supplemented with human serum, Albumax II supplemented medium required hypoxanthine and D-glucose as mandatory additional supplements to sustain parasite growth without transformation to gametocyte production (data not shown).

When the complete culture medium with Albumax II was kept longer than 3 days, a red color change was observed, corresponding to a more acid pH of the medium, coinciding with a decrease in quality of parasite morphology.

**Optimizing *P. falciparum* cultivation in the HFBR**

Using the method described by Li *et al* (2003) with 500 ml of culture medium, we were able to culture *P.falciparum* strain TM036 up to 8 % parasitemia in 40 % Hct, corresponding to approximately $6.02x10^9$ parasites, with preservation of normal parasite morphology. This is a considerably higher total parasite biomass than achievable with conventional culture in culture flasks: 39 times higher when compared to the biomass in a 5 ml culture with 5% parasitemia, 5% hematocrit in a T25 flask, 10 times more than 20 ml conventional culture in a T50 flask and 5 times more than 40 ml culture in a T75 conventional flask.

The original method described by Li *et al* (2003) was modified subsequently by halving the amount of complete culture medium used, from 500 ml per day to 250 ml per day by reducing the volume of the medium reservoir. With the reduced volume of culture medium (250 ml per day), the culture could be sustained without subculturing for 3 days during which the parasitemia in the HFBR increased from 1.6% to 4% parasitemia at 40% hematocrit. However, after 3 days parasitemia decreased to 1.1% on Day 4 and further decreased until no parasites were observed on the thin film by Day 7 (Fig 3a) coinciding with a change in color of the medium resulting from increased red cell hemolysis.

In the subsequent experiment, reverse flushing for maintaining micropore patency was introduced (see methods) and the culture was sub-cultured when parasitemia reached 1.4% on the $4^{th}$ day. These measures resulted in healthy parasite growth up to a parasitemia of 3% at a 40% hematocrit on Day 6 corresponding to a total parasite biomass of $2.3 \times 10^9$ parasites. Repeated harvesting of the culture on Days 5, 6 and 9 yielded a total number of $2.9 \times 10^9$ parasites. To harvest a similar number from conventional parasite cultures in T25 flasks, 19 flasks would be needed. Fresh red blood cells were repeatedly administered to the culture in order to maintain a 40% hematocrit (Fig 3b).

Timing of harvesting, dilution with fresh red blood cells and 'reverse flushing' were further optimized as shown in Fig 3c. With this regimen, a parasitemia of 7% at a 40% hematocrit could be reached on Day 4 of the experiment, corresponding to a total parasite biomass of $5.3 \times 10^9$ parasites. The continuous culture could be maintained successfully for up to 35 days. Repeated harvesting up to 15 times yielded a total of $2.4 \times 10^{10}$ parasites, equivalent to the number of parasites in 152 T25 flasks using conventional culture techniques.

To monitor viability of the parasites

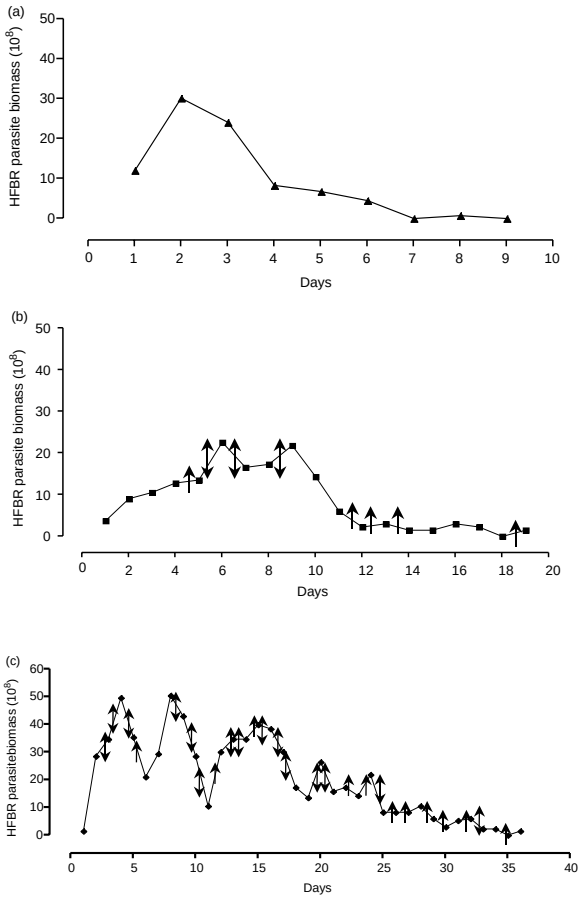


Fig 3–a) Parasite biomass in the HFBR over-time without subculturing; b) parasitized biomass in the HFBR over time with sub culturing. Two-headed arrows denote harvest and addition of red blood cells while single headed arrows represent addition of fresh red blood cells only; c) The parasitized biomass in the HFBR over-time. Introducing " reverse flushing" and intensified subculturing

in culture, glucose consumption and lactate production was monitored over time (Fig 4a-c).

In all three experiments, a biphasic pattern in glucose consumption and lactate production was observed, with increase in per parasite lactate production and glucose consumption occurring when

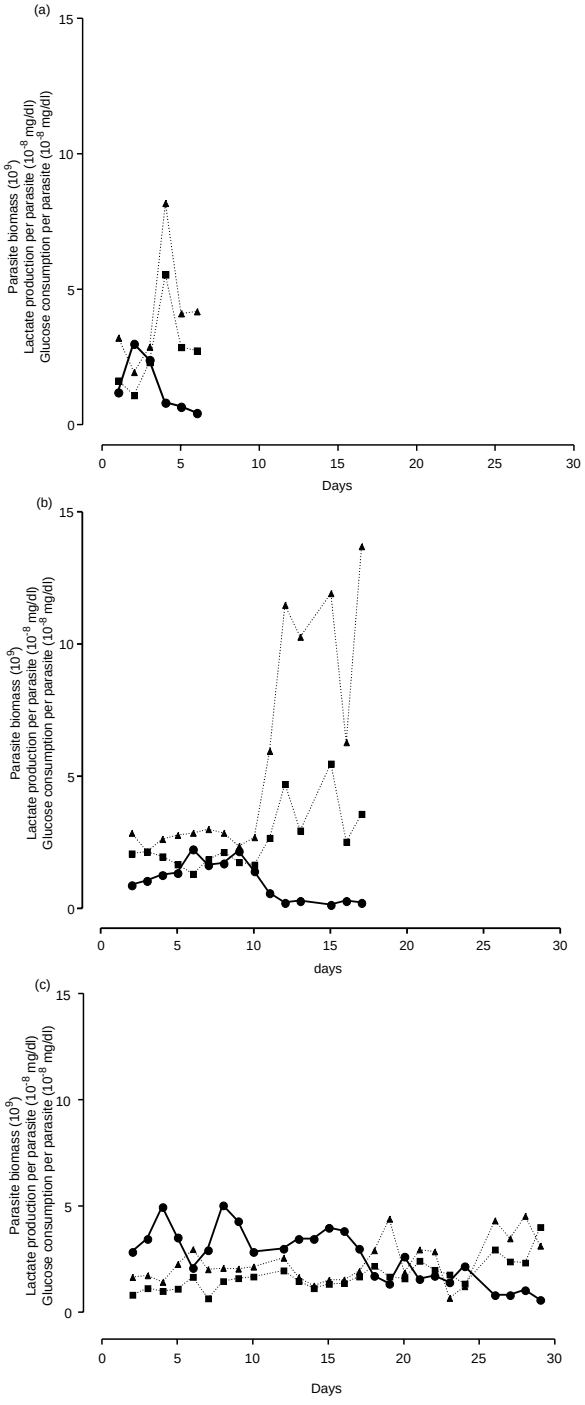


Fig 4a-c–The relationship between parasite biomass (circle), lactate production (square) and glucose consumption (triangle) in experiment 1 (Fig 4a), experiment 2 (Fig 4b), and experiment3 (Fig 4c).

parasitemia declined (Fig 4a-c). During parasite expansion, indicating a healthy parasite population, glucose consumption and lactate production per parasite showed little variation ($p>0.20$), except in experiment 1 which had only 2 time points. Once a continual decline in parasitemia was observed, however, glucose consumption and lactate production per parasite increased ($p< 0.004$).

## DISCUSSION

This investigation aimed to optimize the techniques used for culturing *P. falciparum* at high parasite densities at a physiological hematocrit of 40%, thereby approaching conditions similar to those of a patient with acute malaria. The availability of large volumes of human serum has previously limited attempts to recreate the conditions of a human infection. Albumax II showed to be a good alternative to serum as culture medium supplement although hypoxanthine and D-glucose were essential additions for the Albumax II containing medium. Albumax II has an additional advantage that variation in the composition of the complete culture medium will be reduced compared to that use of human serum, which will vary in antimalarial properties and nutrient content between individual donors. In areas of high malaria transmission, the presence of antibodies and other factors can vary considerably between donors (Schuster, 2002). Compared to human serum, however the use of Albumax requires more frequent changes of media.

Halving the volume of culture medium in the HFBR from 500 to 250 ml per day enabled continuous culturing of the parasites at a significantly reduced cost. Introducing the technique of 'reverse flushing' to preserve micropore patency, and optimized timing of dilution with fresh red blood cells and sub-culturing, allowed continuous culturing of the laboratory adapted parasite strain TM036 for up to more than a month. Parasite viability over time was well preserved, as shown by the relatively constant growth rates and associated constant glucose consumption and lactate production rates over time. Increased glucose consumption and lactate production was an early sign of reduced parasite viability in all our experiments.

The culture of parasites in the HFBR yielded a high parasite biomass of around $10^{10}$ parasites for prolonged periods of time and this can be obtained at less cost and labor compared to conventional methods. An important application of the method is the study of rare events such as the selection of genetic changes associated with drug resistance. High parasite numbers are also needed for the study of mRNA transcription, and studies on proteomics.

In the current study we observed that parasite growth was optimal below a total parasite biomass of $3.4 \times 10^9$ parasites, corresponding to a parasite density of approximately 1,000/µl in an adult. Since it is unlikely that the availability of unparasitized red blood cells necessary for invasion is the limiting factor, this suggested that there might be parasite mechanism of 'quorum sensing'. Although this phenomenon seems present during both *P. falciparum in vivo* and *in vitro* infection, the mechanisms involved have not been clarified. In the bioreactor system it could involve a negative growth regulatory factor secreted by parasitized erythrocytes accumulating inside the bioreactor with increasing numbers of parasites (Li *et al*, 2003). The bioreactor system provides a model which is suitable for further study of the mechanisms involved in *P.*

*falciparum* quorum sensing.

The HFBR method also enables more sophisticated assessment of antimalarial drug sensitivity, mimicking *in vivo* drug pharmacokinetics, especially of drugs with a short *in vivo* plasma half-life. The artemisinin derivatives have a short half-life of around an hour. Therefore conventional testing, where parasites are cultured in the presence of a constant concentration of the drug, does not represent the real life situation. Use of the HFBR system using our adapted method was less labor intense than the originally described methods, with only a daily frequency of cuture medium changes. However, if very high parasite/densities (*eg* >60% parasitemia, at high hematocrit like 50%) need to be achieved, medium need to be changed more frequently; that is twice or three times a day. In addition, the HFBR enables the frequent change of the culture medium with decreased manipulation and danger of contaminating the culture, since the culture medium reservoir can be easily replaced.

There is still room for improvement of the method, since it is currently not suitable for long - term culture for periods over a month. The main problem seems to be the clogging of micro pores in the hollow fiber by parasite waste products over time, despite flushing the pores by 'reverse flushing'. This compromises parasite growth in the long - term. The use of a bigger pore size might prevent this, but this will need further evaluation. Besides, an optimum training to limit problems of contamination is essential.

In conclusion, the *in vitro* culture of *P. falciparum* in the HFBR provides an important technique for studying parasite biology requiring a high parasite biomass as well as studies requiring frequent change of culture medium conditions such as *in vitro* drug sensitivity assays assessing short half life antimalarial drugs. The current study describes in detail an optimized method to further deploy this technique.

## ACKNOWLEDGEMENTS

We would like to thank the staff of malaria laboratory, Faculty of Tropical Medicine, Mahidol University for technical support. This work was a part of Mahidol Oxford Tropical Medicine Research Programme supported by the Wellcome Trust of Great Britain. P Preechapornkul was supported by the University Development Committee Scholarship Thailand.

## REFERENCES

Chen Z, Ding Y, Li G. Configuration of a new bioartificial liver support system and *in vitro* evaluation of its functions. *Ann Clin Lab Sci* 2005; 35: 7-14.

Fievet N, Tami G, Maubert B, *et al.* Cellular immune response to *Plasmodium falciparum* after pregnancy is related to previous placental infection and parity. *Malar J.* Epub 2002 :1:16. Epub. [Cited 2010 Jan 10]. Available from: URL: http://www.malariajournal.com/content/1/1/16

Gramer MJ, Britton TL. Antibody production by a hybridoma cell line at high cell density is limited by two independent mechanisms. *Biotechnol Bioeng* 2002; 79: 277-83.

Gramer MJ, Poeschl DM, Conroy MJ, Hammer BE. Effect of harvesting protocol on performance of a hollow fiber bioreactor. *Biotechnol Bioeng* 1999; 65: 334-40.

Hatabu T, Kawazu S, Kojima S, *et al*. *In vitro* susceptibility and genetic variations for chloroquine and mefloquine in *Plasmodium falciparum* isolates from Thai-Myanmar border. *Southeast Asian J Trop Med Public Health* 2005 ; 36 (suppl 4): 73-9.

Inoue Y, Arita N, Teruya K, Katakura Y, Shirahata S. Efficient production of recombinant human erythropoietin by replenishment of microcarriers in the hollow fiber culture cassette. *Biosci Biotechnol Biochem* 1999; 63: 1624-6.

Kim HS, Okuda Y, Begum K, *et al*. Analysis of *Pfmdr 1* gene in mefloquine-resistant *Plasmodium falciparum*. *Nucleic Acids Res Suppl* 2001; 1: 231-2.

Kreutz FT, Jafarin A, Biggs DF, Suresh MR. Production of highly pure monoclonal antibodies without purification using a hollow fiber bioreactor. *Hybridoma* 1997; 16: 485-6.

Lamers CH, Gratama JW, Luider-Vrieling B, Bolhuis RL, Bast EJ. Large-scale production of natural cytokines during activation and expansion of human T lymphocytes in hollow fiber bioreactor cultures. *J Immunother* 1999; 22: 299-307.

Li T, Glushakova S, Zimmerberg J. A new method for culturing *Plasmodium falciparum* shows replication at the highest erythrocyte densities. *J Infect Dis* 2003; 187: 159-62.

Malone CC, Schiltz PM, Mackintosh AD, Beutel LD, Heinemann FS, Dillman RO. Characterization of human tumor-infiltrating lymphocytes expanded in hollow-fiber bioreactors for immunotherapy of cancer. *Cancer Biother Radiopharm* 2001; 16: 381-90.

Oduola AM, Alexander BM, Weatherly NF, Bowdre JH, Desjardins RE. Use of non-human plasma for *in vitro* cultivation and antimalarial drug susceptibility testing of *Plasmodium falciparum*. *Am J Trop Med Hyg* 1985; 34: 209-15.

Pan D, Whitley CB. Closed hollow-fiber bioreactor: a new approach to retroviral vector production. *J Gene Med* 1999; 1: 433-40.

Peel SA, Merritt SC, Handy J, Baric RS. Derivation of highly mefloquine-resistant lines from *Plasmodium falciparum in vitro*. *Am J Trop Med Hyg* 1993; 48: 385-97.

Piret JM, Cooney CL. Immobilized mammalian cell cultivation in hollow fiber bioreactors. *Biotechnol Adv* 1990; 8: 763-83.

Potter K, Butler JJ, Adams C, *et al*. Cartilage formation in a hollow fiber bioreactor studied by proton magnetic resonance microscopy. *Matrix Biol* 1998; 17: 513-23.

Schuster FL. Cultivation of *Plasmodium* spp. *Clin Microbiol Rev* 2002; 15: 355-64.

Serirom S, Raharjo WH, Chotivanich K, Loareesuwan S, Kubes P, Ho M. Anti-adhesive effect of nitric oxide on *Plasmodium falciparum* cytoadherence under flow. *Am J Pathol* 2003; 162: 1651-60.

Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976; 193: 673-5.

Udomsangpetch R, Somsri S, Panichakul T, *et al*. Short-term *in vitro* culture of field isolates of *Plasmodium vivax* using umbilical cord blood. *Parasitol Int* 2007; 56: 65-9.

Vande Waa JA, Jensen JB, Akood MA, Bayoumi R. Longitudinal study on the *in vitro* immune response to *Plasmodium falciparum* in Sudan. *Infect Immun*1984; 45: 505-10.