CHAPTER IV
MATERIALS AND METHODS

1 Study sites

1.1 *Plasmodium vivax* samples collection site for *in vitro* sensitivity study

The assessment of short term *in vitro* drug assay was conducted at Mae-Sot General Hospital, Tak Province during May-August, 2005. A total of 32 blood samples infected with *P. vivax* with parasitemia of 1,000-50,000 parasites *per* microliter were collected from patients attending the Vector Borne Disease Unit, Mae Sot District, Tak Province prior to treatment of a 3-day standard regimen of chloroquine and 14 days primaquine. All patients were briefed on the project and provided informed consent prior to collection of blood by venipuncture. Five milliliter of blood was collected in lithium heparin collecting tubes and spotted onto filter paper (Whatman No 3). Samples with mixed infection with other malaria species were excluded by microscopic examination **(Figure 11)**.

1.2 *Plasmodium vivax* samples collection sites for *dhfr-dhps* geographical distribution study

A total of 160 blood samples infected with *P. vivax* were collected during May-August 2005 from patients attending the Vector Borne Disease Unit in different geographical locations along the international border of Thailand, including Mae Hong Son, Chiang Mai, Tak, Kanchanaburi, Ratchaburi, Ranong, Trad, Chantaburi and Yala Provinces. Two to three hundred microliter of blood samples were collected from patient's finger-prick and dotted onto filter paper (Whatman No 3). Diagnosis and identification of malaria species were performed by microscopic examination of Giemsa-stained thick blood smear.

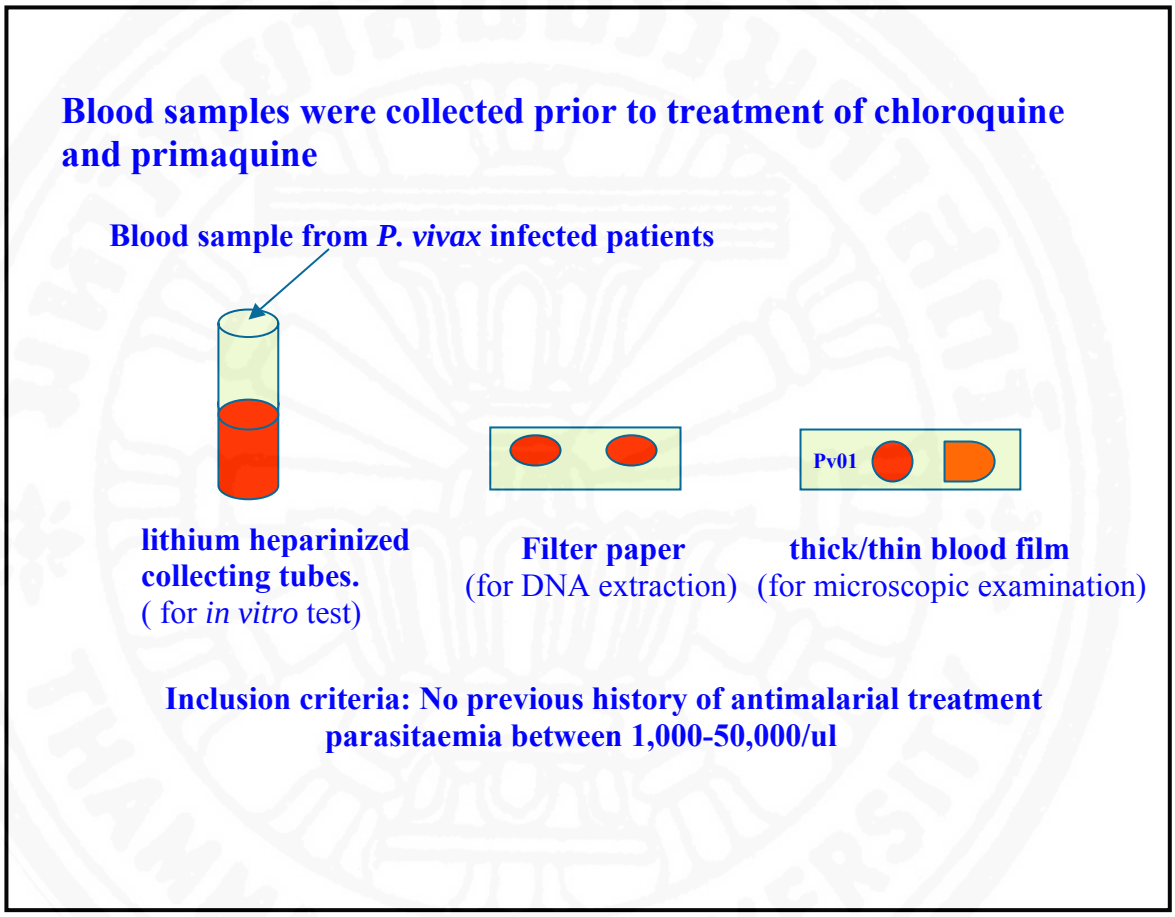

Figure 11 Collection of blood samples from *P. vivax* infected patients for *in vitro* drug sensitivity assay

2. Assessment of short term *in vitro* drug assay for *Plasmodium vivax*

2.1 Samples preparation

Figure 12 represents a schematic diagram summarizing the procedure for assessment of the short term *in vitro* assay for *P. vivax*. The short term *in vitro* culture assessment was performed with *P. vivax* field isolates using a modified method of Russell (Russell *et al.*, 2003). In brief, a 2 ml blood sample was mixed with phosphate buffered saline at the ratio of 1:1 and added to the CF11 column (10 ml of syringe tipped with glass wool and filled with CF11 cellulose powder (Whatman)). The supernatant was then removed and the pellet was resuspended in RPMI 1640 medium. The blood mixture was centrifuged and the supernatant was removed. The pellet was then resuspended in AB serum to obtain a hematocrit of 40%. Blood-serum suspension was mixed with McCoy's 5A medium at the ratio of 1:10. The concentrations of folic acid and para-aminobenzoic acid (*p*ABA) in the McCoy's medium were 10 mg/L and 1 mg/L, respectively. Fifty microliters of this mixture were added to each well of a 96-well microliter plates pre-dosed with tested drugs.

2.2 Preparation of pre-dosed plates

P. vivax field isolates were tested for their sensitivities against pyrimethamine, WR99210, chlorcycloguanil, sulfadoxine and dapsone. All of the antifolate drugs were obtained as a gift from Jacobus Pharmaceutical, Inc. (Princeton, NJ). Drug plates were prepared fresh to avoid possible degradation. A stock solution of each drug was prepared in 1% DMSO and was subsequently diluted in RPMI 1640 medium to obtain the desired drug concentrations for testing. The concentration ranges for each drug used were: 0-2,500 nM/well for pyrimethamine, 0-2,560 nM/well for WR99210 and 0-200,000 nM/well for chlorcycloguanil, sulfadoxine and dapsone. Fifty microliters of the final drug solution was added to each well of a 96 well microliter plate. This plate contained varying concentrations of drug in each column and well A was free of drug and served as control. Wells B-H contained ascending concentrations of drug, each concentration of which was tested in triplicate. The tested plate was incubated at 37.5^{o} C in a gas chamber containing

5% CO_2 for 24-36 hours depending on the stage of the parasite before culturing. After incubation, a thick blood film was prepared from each well and the number of normal schizonts (containing > 8 nuclei) *per* 200 asexual stage parasites was counted. The number of schizonts in the drug wells was compared with that in the control well and expressed as percentage. The dose-response curve was analyzed by Calcusyn™ software to obtain the IC_{50} value, the concentration that inhibits schizont maturation by 50% compared with the control.

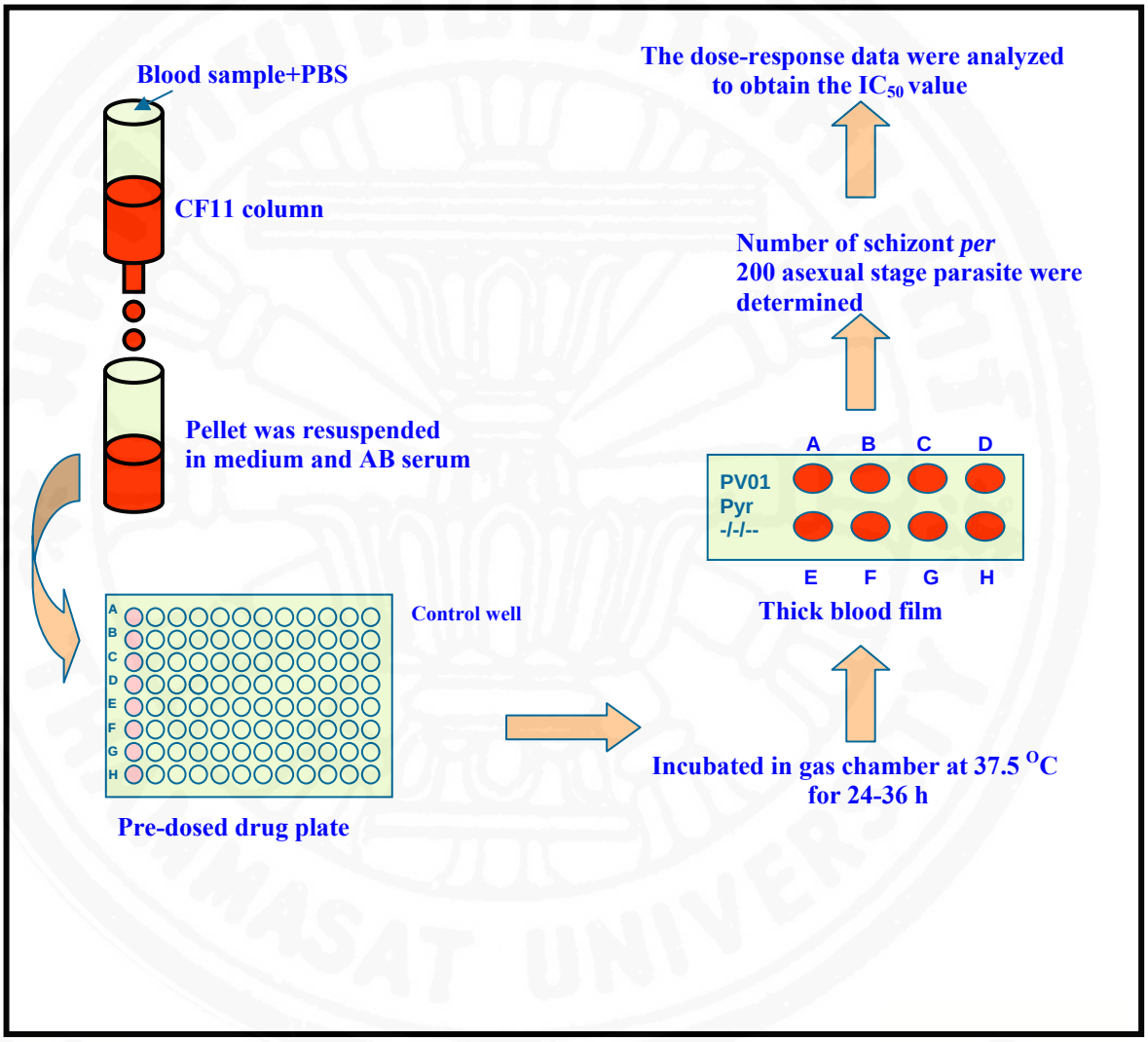

Figure 12 Procedure for assessment of short term *in vitro* drug sensitivity for *P. vivax*

3. Detection of mutations in the *Pvdhfr* and *Pvdhps* genes

Parasite DNA was extracted from dried blood spots on filter paper using a QIAamp DNA extraction mini-kit (QIAGEN) and used as template for PCR amplification. Primers were designed according to the published sequence of *dhfr-ts* (GenBank accession no. X98123) and *dhps* gene (GenBank accession no. AY186730) of *P. vivax*. *Pvdhfr* was amplified with a pair of primers (forward: 5'-atg gag gac ctt tca gat gta ttt gac att -3' and reverse: 5'-cca cct tgc tgt aaa cca aaa agt cca gag -3'). PCR was carried out in a total volume of 50 µl with the following reaction mixture: 0.1 µM of each primer, 2.5 mM $MgCl_2$, 100 mM KCl, 20 mM Tris-HCl pH 8.0, 100 µM deoxynucleotides (dNTPs), 15-20 µl of genomic DNA and 0.5 unit of *Taq* DNA polymerase (Promega). PCR cycling parameters were as follows: initial denaturation at 94°C for 3 min, followed by 5 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min then followed by 5 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and finally with 20 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. *Pvdhps* was amplified by nested PCR with a first round pair of PCR primers (forward: 5'-aaa gcg tag cga cag aag aac- 3' and reverse: 5'-ttg aaa cac gca tta tgg tat cg- 3') and in the second round pair of PCR primers (forward: 5'-ctc gcc atg ctc gta att tt-3' and reverse: 5'-gag att acc cta agg ttg atg tat c-3'). PCR was carried out in a total volume of 50 µl with the following reaction mixture: 0.1 µM of each primer, 2.5 mM $MgCl_2$, 100 mM KCl, 20 mM Tris-HCL pH 8.0, 100 µM deoxynucleotides (dNTPs), 15-20 µl of genomic DNA and 0.5 unit of *Taq* DNA polymerase (Promega). PCR was performed using 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. PCR products were fractioned by 1.5% agarose gel electrophoresis, purified with QIAquick PCR purification kit (QIAGEN) and sequenced using the fluorescent dye chemical system (MegaBACE; Amersham Pharmacia Biotech).

4. Multiclonal detection by PVMSP-3α

Detection of multiclonal infection of the PVMSP-3α gene was performed using the PCR-RFLP method described by Bruce and colleadge (Bruce *et al.*, 1999). The

PVMSP-3α was amplified by nested PCR and the size of the PCR products were estimated on a 1.8% agarose gel. Five µl of the PCR product was digested with *Hha I* (BioLabs) and analyzed on 1.8% agarose gel electrophoresis. Multiple infections were distinguished when the summed size of the DNA fragments resulting from *Hha I* digestion exceeded the size of the uncut PCR product (Bruce *et al.*, 1999; Cui *et al.*, 2003).

5. Cloning and expression of *Pvdhfr* allele in yeast expression vector

The TH5 (*Mata ura3-52 leu2-3,112trp1 tup1 dfr1::URA3) Saccharomyces cerivisiae* strain, which lacks endogenous DHFR activity and requires supplemental dTMP for growth (Huang *et al.*, 1992) was used for expression of *Pvdhfr* as previously described (Hastings and Sibley, 2002; Sibley *et al.*, 1997; Wooden *et al.*, 1997). The shuttle plasmid **(Figure 13)** that can be propagated in both *E. coli* and *S. cerivisiae* was used as vector for expression of the *Pvdhfr* coding region (Sikorski and Hieter, 1989). PCR primers for amplification of the *Pvdhfr*, plus 19 downstream nucleotides were designed for homologous recombination in yeast (Hastings and Sibley, 2002). PCR cycling parameters used were the same as for *Pvdhfr* amplification. Yeast were transformed using a high efficiency lithium acetate protocol and plated onto medium lacking tryptophan and dTMP to select for the plasmid and functional DHFR activity. **Figure 14** represents a schematic diagram summarizing the procedure for PCR amplification and exogenous *Pvdhfr* expression process. This procedure produced a series of genetically matched yeast strains dependent upon different alleles of *Pvdhfr*.

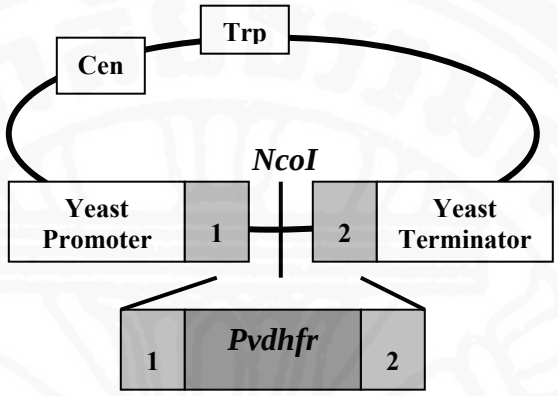

Figure 13 The shuttle plasmid GR7. It has a yeast centromere (Cen) for maintaining approximately one copy *per* cell, and *trp1* gene (Trp) for selection of transformant. The yeast promoter derives from the 600 bp 5' region of the *Saccharomyces cerevisiae dfr1* coding sequence, the yeast terminator is the 400 bp 3' region of *S. cerevisiae dfr1* coding sequence, region 1 and 2 contains 20 bp of DNA sequence upstream of *Pfdhfr* coding sequence, the frist 34 bp of the *Pfdhfr* coding sequence, and 75 bp from the 3' end of the *Pfdhfr* sequence. *Nco I* is a restriction site to allow generation of the linearized vector.

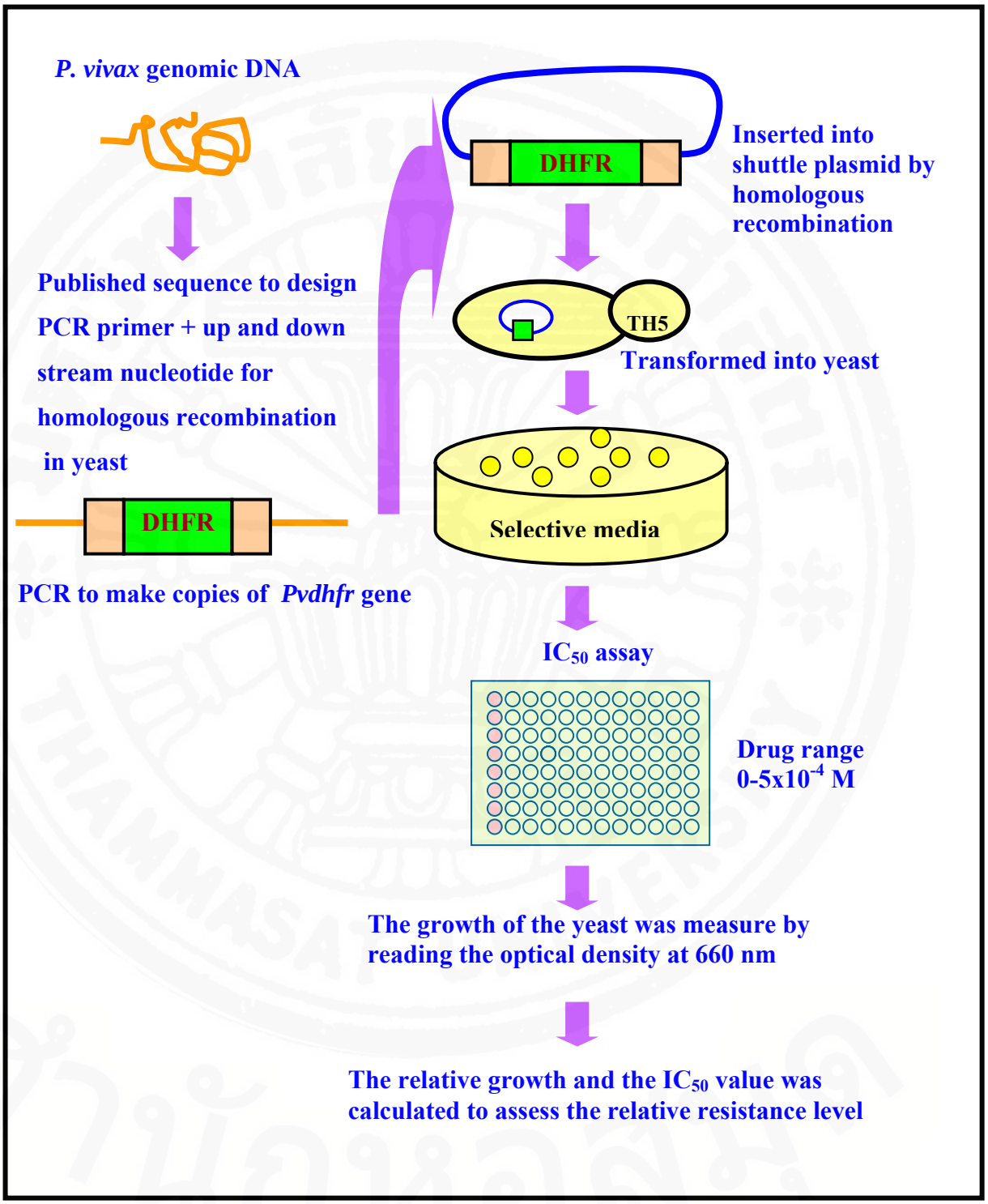

Figure 14 Overview of the PCR amplification and exogenous *Pvdhfr* expression process.

6. Determination of drug sensitivity in yeast expression system

Sensitivity assays in the yeast expression system were performed in 96 well microtiter plates (Hastings *et al.*, 2005; Hastings and Sibley, 2002). Each strain of transformed yeast was grown for 18-24 hours in complete medium lacking dTMP and 0- 5 x 10^{-4} M pyrimethamine, chlorcycloguanil or WR99210 **(Figure 15)**. The growth of the yeast in each well was measured by reading the optical density at 660 nm. The growth of each yeast strain at each drug concentration was used to plot the percent growth relative to the yeast in the control well that contained only the DMSO solvent. The IC_{50} value was calculated from the slope and intercept of the line defined by the two data points that bracket 50% relative growth. Comparisons of the IC_{50} values of yeast dependent upon the mutant alleles to that dependent upon the wild-type allele were used to assess the relative resistance level of each *Pvdhfr* allele to the drug.

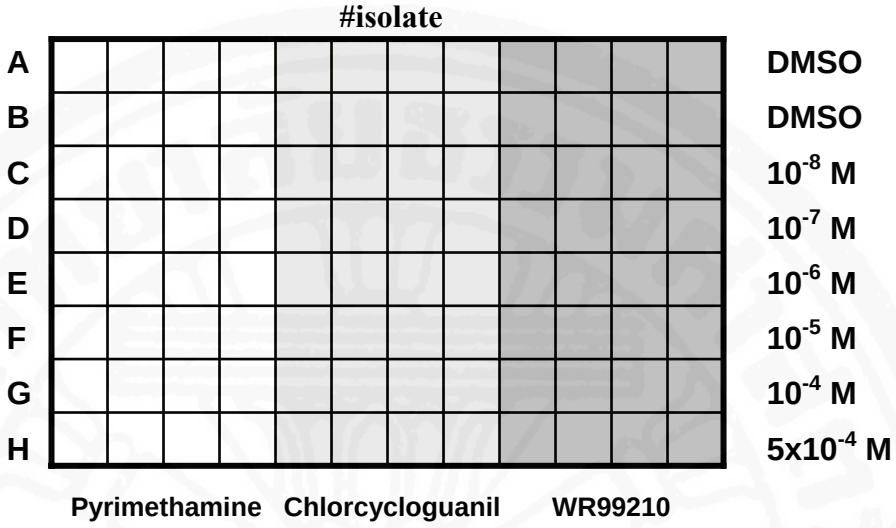

Figure 15 Diagram of a typical 96 well culture dish arrangement for the IC_{50} determination

7. Statistical analysis

Statistical analysis to investigate the association between number of point mutations in *Pvdhfr* and *Pvdhps* and *in vitro* sensitivity of *P. vivax* isolates (IC_{50}) was performed using a two sided Student's t-test. Statistical significance level was set at α= 0.05 for all tests.