CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 *P. vivax dhfr-ts* genes

Full-length *dhfr-ts* gene of *P. vivax* in both wild type and double mutant (S58R/S117N; SP21) forms were previously constructed in the vector pET-17b to obtain pETpvDHFR-TS and pETpvSP21, respectively (Bunyarataphan *et al*, 2006). These constructs were kindly provided by Dr. Ubolsree Leartsakulpanich, Protein-Ligand Engineering and Molecular Biology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Pathumthani, Thailand and used for sub-cloning into transfection plasmids.

2.1.2 Bacterial strain

Escherichia coli strain DH5α (*F⁻ Ø80d LacZDM15 Δ(LacZYA-argF) U169 deoR recA endA1 hsdR17(rk- mk+) supE44l- thi-1 gyrA96 relA1*) was obtained from Life Technology Incorporation, USA and was generally used as a standard host strain for plasmid transformation or general manipulation of recombinant plasmids.

2.1.3 Parasite strain

Plasmodium berghei 507m6cl1 parasite (Franke-Fayard *et al*, 2008) used in this study was a gift from Dr. Chris J. Janse at Leiden Malaria Research Group (LMRG), Department of Parasitology, Leiden University Medical Center (LUMC)??, Leiden, the Netherlands. This line is a genetically modified parasite of clone cl15cy1 of the ANKA strain to express GFP constitutively during the whole life cycle (Janse *et al*, 2006a). This line was selected by flow cytometric sorting, based on GFP fluorescence. As a stable transfectant with plasmid pL0023 (Malaria Research and Reference Reagent Resource Center: MR4), this line does not contain a drug selectable marker. The *gfp* gene is integrated into the genome by double cross-over integration under the control of *P. berghei* elongation factor 1α-a (*pbeef1aa*) promoter region. Gametocyte, ookinete, oocyst, sporozoite and liver stage development are comparable to wild type *P. berghei* ANKA (Janse *et al*, 2006a).

2.1.4 Laboratory animal

Pathogen-free, 4-week-old BALB/C mice were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand and kept for at least one week in a condition of 12 hr day/12 hr night cycle with tap water and pellet diet from CP diet 082, Perfect Companion Company, Bangkok, Thailand *ad libitum*. Experiments were started in 6- to 7-week-old animals. Procedures of the animal experiments were ratified by the Ethical Committee on Animal Experimentation, at Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand and at National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand, and

followed international guidelines for the humane use of animals in experimental studies.

2.1.5 Plasmids

pL0017 plasmid (11,068 bp, **Figure 2-1**) and pL0035 plasmid (5,961 bp, **Figure 2-2**) were gifts from Dr. Chris J. Janse at Leiden University Medical Center, the Netherlands, and were used as vectors for cloning of PCR product and in transfection experiments. pL0017 contains an incomplete copy of the *d-ssu-rrna* gene as a target region for integration, a pyrimethamine-resistant *Toxoplasma gondii dhfr-ts* (*tgdhfr-ts*) selectable cassette for use in selection of transgenic parasites under the control of 5' and 3'UTR of *P. berghei dhfr-ts* (*pbdhfr-ts*), and a GFP expression cassete which *gfp* gene is flanked by the *pbef1αa* promoter and the 3'UTR of *pbdhfr-ts* and used as a fluorescent marker (Janse *et al*, 2006a).

pL0035 plasmid contains an expression cassette of fusion gene of positive (human *dhfr*; *hdhfr*) and negative selectable marker (a fusion gene comprising of the open reading frames of the yeast *cytosine deaminase* and *uridyl-phosphoribosyltransferase*; *yfcu*) under the control of *pbef1αa* promoter (Braks *et al*, 2006).

All plasmids contain an ampicillin resistant gene that allows selection of ampicillin resistance colonies. The complete nucleotide sequences of the pL0017 and pL0035 plasmids was obtained from the Malaria Research and Reference Reagent Resource Center (MR4) and Leiden Malaria Research Group (LMRG).

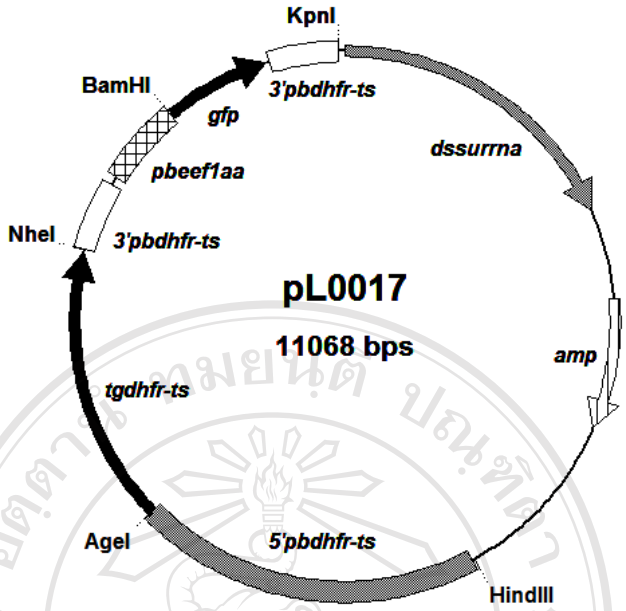


Figure 2-1 pL0017 plasmid (Janse *et al*, 2006a). This plasmid contains an incomplete copy of *d-ssu-rrna* unit as a target region for integration and the pyrimethamine-resistant *tgdhfr-ts* selectable marker cassette for selection of transgenic parasites. The *gfp* gene is flanked by the *pbef1αa* promoter and 3'UTR of *pbdhfr-ts*. Thin line indicates pUC19 plasmid sequence. *Age*I and *Nhe*I restriction sites were used as cloning sites for *pvdhfr-ts* gene to obtain pL17pvDHFR-TS and pL17pvSP21 transfection plasmids.

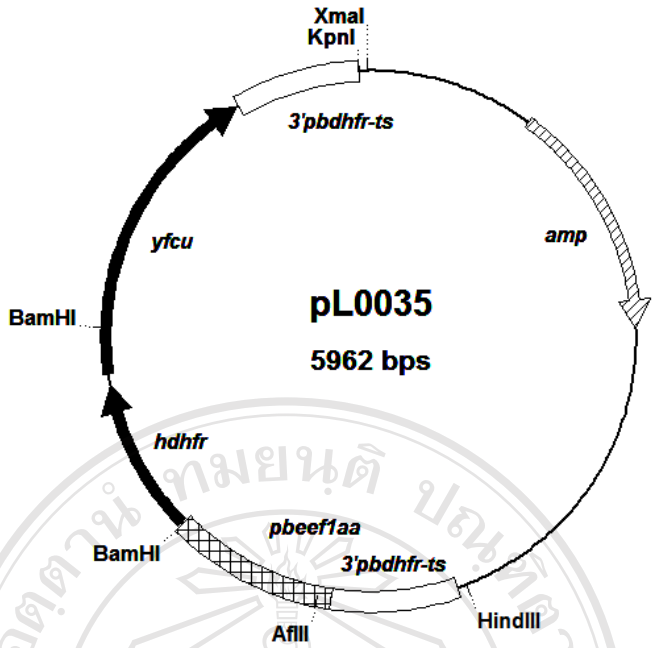


Figure 2-2 **pL0035 plasmid** (Braks *et al*, 2006). This plasmid contains an expression cassette of a fusion gene of the positive (*hdhfr*) and negative selectable markers (*yfcu*) under the control of *pbef1αa* promoter and 3'UTR of *pbdhfr-ts*. This plasmid was used to construct a transfection plasmid that contains *pvdhfr-ts* expression cassette. Wild type *pvdhfr-ts* flanked by 5' and 3'UTR of *pbdhfr-ts* was cloned into *Hind*III and *Afl*II restriction sites of the pL0035 plasmid to obtain pL35pvDHFR-TS.

2.1.6 Oligonucleotides

Oligonucleotide synthesis and DNA sequencing were performed at the BioService Unit, BIOTEC and Biodesign, Thailand. **Table 2-1** summarizes the list of oligonucleotides, their sequences, and usages. The restriction sites designed for cloning were underlined.

2.1.7 Chemicals and reagents

All chemicals and reagents used in this study were the analytical grade or the highest purity commercially available. Restriction endonucleases and other DNA-modifying enzymes were products from New England Biolabs (Beverly, MA, USA), and Promega (Madison, WI, USA) and were used according to the instruction given by the manufacturers. The plasmid miniprep kit and gel/PCR extraction kit were purchased from Qiagen (Qiagen GmbH, Hilden, Germany). Nitrocellulose membrane was purchased from Schleicher&Schuell (BioScience GmbH, Germany). Other reagents and chemicals were listed in the Appendix A.

Table 2-1 List of oligonucleotides, their sequences and usage

Oligo name	Length (bases)	Sequence (5'→3')	Usage
Sense-*Pv*DT-*Age*I	39	GGGGGGACCGGTATGGAGGACCTTTCAGATGTATTTGAC	For introducing *Age*I to 5' of *pvdhfr-ts* coding sequence (CDS)
Antisense-*Pv*DT-*Nhe*I	43	GGGGGGGCTAGCTTAGGCGGCCATCTCCATGGTTATTTTATCG	For introducing *Nhe*I to 3' of *pvdhfr-ts* CDS
*Pv*DTF	25	TTACGCCATCTGCGCATGCTGCAAG	Sense-strand for amplifying *pvdhfr-ts* in genomic DNA of *P. berghei*
*Pv*DTR	22	GTTGTTGTAGACGTGCGCGTTG	Antisense-strand for amplifying *pvdhfr-ts* in genomic DNA of *P. berghei*
*Pb*DTF	36	GGGGGGGGCATATGGAAGACTTATCTGAAACATTCG	Sense-strand for amplifying *pbdhfr-ts* in genomic DNA of *P. berghei*
PbDTR	24	GGACTAGTTTAAGCTGCCATATCC	Antisense-strand for amplifying pbdhfr-ts in genomic DNA of *P. berghei*
5'PvINTF	24	TTGAGCTACATAACTTCCATACAT	Sense-strand for amplifying 5' integrated fragment in genomic DNA of *P. berghei*

Table 2-1 List of oligonucleotides, their sequences and usage (continue)

Oligo name	Length (bases)	Sequence (5'→3')	Usage
5'*Pv*INTR	20	AGCATGCGCAGATGGCGTAA	Antisense-strand for amplifying 5' integrated fragment in genomic DNA of *P. berghei*
3'*Pv*INTF	20	AACCATGTTGACAGCTTGAA	Sense-strand for amplifying 3' integrated fragment in genomic DNA of *P. berghei*
3'*Pv*INTR	18	CGATCTACACCTCTTCAT	Antisense-strand for amplifying 3' integrated fragment in genomic DNA of *P. berghei*
hDyFCUF	20	AACTGCATCGTCGCTGTGTC	Sense-strand for amplifying hdhfr-*yfcu* gene
hDyFCUR	22	GGCGGGATCGAGTTAAACACAG	Antisense-strand for amplifying *hdhfr-yfcu* gene
3'UTR*Pb*DT Probe	22	CGCATATCCTCTGCTCAATATC	Antisense-strand for using as a Southern blot probe at 3'UTR *pbdhfr-ts*

2.2 General molecular biological methods

2.2.1 Preparation of plasmid DNA

2.2.1.1 Isolation of plasmid DNA by alkaline lysis method

Small scale and rapid preparation of plasmid DNA was carried out by the alkaline lysis method. The principle of the technique involves lysis of *E. coli* cells by SDS at high pH followed by neutralization. Proteins and high molecular weight chromosomal DNA are denatured while covalently closed circular double-strand plasmid DNA remains in the solution. The prepared DNA was used for screening of the recombinant plasmids and further characterization by restriction analysis.

A single colony from the *E. coli* harboring plasmid DNA was selected and grown in 5 ml of LB (Luria Bertani) broth (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract and 1% w/v NaCl, pH 7.0) supplemented with 100 µg/ml of ampicillin. After overnight growth at 37^{o}C with shaking, the bacterial culture was harvested by centrifugation at a 20,000 x *g* for 1 minute in a microcentrifuge tube. The supernatant was discarded and the cell pellet was resuspended with 300 µl of ice-cold cell resuspension solution (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, and 100 µg/ml RNase A). The plasmid DNA was extracted by adding 300 µl of freshly prepared lysis solution (200 mM NaOH and 1% (v/v) SDS), followed by gently inversion until the solution became clear. The cellular debris was immediately neutralized by adding 300 µl of ice-cold neutralization solution (5 M KOac and 11.5% (v/v) acetic acid), followed by mixing thoroughly by repeating inversion. After centrifugation at 20,000 x *g* for 10 minutes, 850 µl of the clear supernatant containing plasmid DNA was transferred to a new microcentrifuge tube and 650 µl of

ice-cold isopropanol was added. The plasmid DNA was precipitated at -20°C for 15 min. After centrifugation at 20,000 x *g* for 15 min, the DNA was washed twice with 1 ml of ice-cold 70% ethanol. After air-drying to remove residual solvent, the pellet was dissolved in 50 µl of TE (50 mM Tris-HCl, pH 8.0, 10 mM EDTA) or sterile distilled water. The plasmid DNA was then kept at -20°C until use.

2.2.1.2 Isolation of plasmid DNA by QIAGEN kit

The method is used for extraction of the high purity plasmid DNA from small scale culture (5-10 ml overnight *E. coli* culture) using QIAprep Spin Miniprep Kit (Qiagen). The isolation of plasmid DNA was carried out according to the standard protocol by the manufacturer. The principle of this method is based on alkaline extraction and selective adsorption of plasmid DNA onto silica-membrane in the presence of high salt buffer while contaminants pass through the column. The plasmid DNA was eluted by applying 30-50 µl of elution buffer or sterile distilled water to the membrane and the purified plasmid DNA was collected. The concentration of the DNA was determined by measuring the absorbance at 260 nm. One OD unit of double stranded DNA is equal to 50 µg/ml of DNA. The ratio of $A_{260/280}$ at 1.8-2.0 was considered acceptable.

2.2.2 Agarose gel electrophoresis

Agarose gel (0.8%) was prepared by dissolving 0.8 g of ultrapure agarose (Seagem) in 100 ml of 0.5x TBE buffer (Tris Borate Buffer: 45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 8.0). The agarose suspension was melted in a microwave oven until completely dissolved, and allowed to be cooled to approximately 50°C prior to pouring into a casting chamber equipped with comb.

After allowing the agarose to be harden at room temperature, it was placed in the electrophoresis chamber and electrophoresis buffer (0.5x TBE) was added so that the gel surface was submerged. The DNA samples were mixed with 1/3 volume of the gel-loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol) and were loaded into the wells. The DNA marker was also loaded in the same gel as marker for DNA molecular size. Electrophoresis was carried out by applying constant voltage at 100 volts until bromophenol blue migrated to about three quarters of the gel length, at which time the electrophoresis was stopped. The gel was stained with ethidium bromide solution (0.5 µg/ml) for 10-15 min, and was subsequently destained with distilled water until the background was clear. The DNA patterns were visualized under UV transilluminator and the size of DNA fragment was estimated by comparing to the size of the DNA marker. The DNA bands were photographed using a Gel Documentation apparatus (Bio-Rad laboratories).

2.2.3 Digestion of plasmid DNA with restriction endonuclease

The plasmid DNA prepared as described above (**Section 2.2.1**) was digested with desirable restriction endonucleases for characterization of the desired transformants sub-cloning into the plasmid vectors. Approximately 1 µg DNA was digested with 5 U of restriction enzymes in an appropriate 1x restriction buffer. The buffers for specific restriction enzymes were followed to the manufacturer's recommendation. The digestion reaction was incubated at suitable temperature of each enzyme for 2 hr. The second digestion was performed after the first digestion was completed for another 2 hr. The digested DNA was then analyzed on agarose gel electrophoresis (**Section 2.2.2**).

2.2.4 Purification of DNA fragment from agarose gel

To purify a DNA fragment of the PCR products after restriction enzyme digestion, the DNA was first analyzed by agarose gel electrophoresis (**Section 2.2.2**). After ethidium bromide staining, the DNA fragments were visualized under a long-wavelength UV transilluminator. The desired band was excised from the gel using a clean razor blade and the excised DNA was chopped into small pieces. The DNA fragment was then recovered using QIAquick Gel Extraction Kit according to manufacturer's instructions (QIAGEN).

2.2.5 Ligation of DNA fragments

After digestion with appropriate restriction enzymes, the digested vector and the insert DNA fragment were ligated using T4 DNA ligase. The standard ligation reaction comprised of the digested vector and the insert DNA fragment at 1:20 molar ratio, 1.5 U of T4 DNA ligase (Promega, 3 U/µl), 1x ligation buffer (30 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP) and sterile distilled water in a final volume of 10-20 µl. Control reaction contained all components except for the insert DNA fragment. The ligation reactions were incubated at 14^{o}C for overnight and were used to transform the appropriate strain of competent *E. coli* cells.

2.2.6 Introduction of plasmid DNA into *E. coli* cells

2.2.6.1 Preparation of *E. coli* competent cells

Competent *E. coli* cells were prepared by $MgCl_2/CaCl_2$ treatment protocol. A single colony of *E. coli* strain DH5α was inoculated into 5 ml of LB broth and incubated at 37^{o}C with shaking overnight. The overnight culture was used as

inoculum (1% inoculum size) to inoculate 250 ml of LB broth. The culture was shaked at 37°C until A_{600} reached about 0.4, at which time the cell culture was harvested by centrifugation at 150 x *g* for 5 min at 4°C. After removing the supernatant, the cell pellet was gently resuspended in 1/5 original culture volume of ice-cold 100 mM $MgCl_2$. The cell suspension was kept on ice for 20 min before centrifugation at 150 x *g* for 5 min at 4°C to pellet the cells. After removing the supernatant, the cell pellet was gently resuspended with 1/50 original culture volume of ice-cold 100 mM $CaCl_2$ and was allowed to be on ice for 1 hr. Sterile glycerol was added to the cell suspension at 15% final concentration with gentle swirling. The resulting competent cells were stored in aliquots in pre-chilled sterile microcentrifuge tubes, followed by freezing at -80°C until use. The known concentration of standard plasmid DNA was used to test the transformation efficiency of the competent cells.

2.2.6.2 Transformation of recombinant plasmid DNA into *E. coli* cells

The frozen $MgCl_2/CaCl_2$-treated competent cells prepared as described in the previous section (**Section 2.2.6.1**) were thawed on ice for 15 min and aliquots of 100 µl were dispensed into pre-chilled 1.5-ml microcentrifuge tube. The ligation reaction (**Section 2.2.5**) or purified plasmid DNA (100 ng, see **Section 2.2.1**) was gently mixed with 100 µl of competent cells and incubated on ice for 30 min. The tube was transferred to a water bath set at 42°C for 2 min without agitation and immediately chilled on ice for additional 5 min. Nine hundred microliters of LB broth was added into the tube, and the transformed cells were then shaken at 37°C for 1 hr to allow the cells to recover and resume the growth. Appropriate fractions of the

transformation reaction were plated onto LB agar (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% w/v NaCl, pH 7.0, and 1.5% bacto-agar) supplemented with 100 µg/ml of ampicillin. The plates were then incubated at 37°C for overnight.

2.2.6.3 Characterization of recombinant clones

The isolated bacterial colonies appeared on the LB plates supplemented with ampicillin were randomly picked and grown in 5 ml of LB broth containing 100 µg/ml of ampicillin. The plasmid DNA was extracted by alkaline lysis method as previously mentioned in the **Section 2.2.1**. To analyze the recombinant clones containing the desired insert gene, the putative recombinant DNA was characterized by digestion with suitable restriction enzymes (**Section 2.2.3**), and analyzed by agarose gel electrophoresis (**Section 2.2.2**). Further validation of the recombinant plasmids was carried out by DNA sequencing.

2.2.7 Preparation of linearized transfection plasmids

In order to integrate the construct into the targeted locus, complete digestion to obtain linearized plasmid is required. The targeting plasmids were digested with appropriate restriction enzymes. After incubation at 37°C for overnight, ice-cold isopropanol (1 ml) was added to precipitate the linearized plasmid DNA by incubation at -20°C for overnight. The DNA was harvested by centrifugation at 20,000 x *g* for 10 min followed by washing with ice-cold 70% ethanol. The DNA pellet was then dried by incubation at 50°C for 5 min or until all of ethanol evaporated and no fluid was visible. The pellet was then dissolved in 10 µl of TE buffer pH 8.0 and kept at -20°C for transfection experiment.

2.3 Parasitological methods

2.3.1 Cryopreservation of blood stage parasites

P. berghei infected mouse blood with a parasitemia of 1-20% was collected by cardiac puncture using 21-G needle with 1-ml tuberculin syringe containing 500 U/ml of heparin. Cryopreservation was carried out by mixing 0.5 ml of the infected mouse blood with 0.5 ml of freezing solution (30% v/v glycerol/PBS) containing 500 U/ml of heparin. Then, 0.5 ml of the mixture was transferred to 1.2-ml cryogenic vial (Nunc, USA) and frozen directly in liquid nitrogen (Waters *et al*, 1997).

2.3.2 Infection of laboratory animals

2.3.2.1 Infection with cryopreserved blood stage parasites

Cryopreserved blood stage parasites in cryogenic vials (**Section 2.3.1**) were thawed at room temperature. Thereafter, one or two naïve BALB/C mice were injected intraperitoneally (i.p.) with 0.5 ml of the thawed cryopreserved blood stage parasites using 27-G needle with 1-ml tuberculin syringe (Waters *et al*, 1997).

2.3.2.2 Infection with blood stage parasites

Mice could directly be infected with blood stage parasites from infected mouse blood as a mechanical passage. One microliter of mouse blood contains approximately $5x10^6$ erythrocytes. For maintenance and propagation of the parasites, a mouse was infected with $1x10^6$ parasitized erythrocytes. Infected mouse blood with a parasitemia of 1-10% was collected from either heart or tail blood, then resuspended and diluted to $1x10^6$ parasitized erythrocytes in 200 µl of PBS. Naïve

BALB/C mice were subsequently injected intraperitoneally with 200 µl of this suspension using 27-G needle with 1-ml tuberculin syringe (Waters *et al*, 1997).

2.3.3 Determination of parasitemia by microscopic enumeration of Giemsa stained thin blood smear

The level of parasitemia was estimated by counting the number of parasitized erythrocytes on Giemsa stained thin blood film. Thin blood smear was made from tail blood of infected mouse on a standard microscopic slide. After air-dried, the slide was fixed in methanol for 2 sec. Giemsa solution was freshly prepared in Giemsa staining buffer (0.067 M sodium phosphate buffer, pH 7.2) for a concentration of 10% (v/v). The fixed slide was stained with the Giemsa solution for 10 min, and rinsed carefully with tap water. The stained slide was left in an upright position to air-dry. The parasitemia (percentage of parasitized erythrocytes) in the thin blood film was examined under a standard light microscope with 1000x magnification.

2.3.4 Analysis of blood stage parasites by flow cytometry

The relative GFP-fluorescence intensity of blood stage parasites was analyzed by flow cytometry using a flow cytometer machine (CYTOMICS FC 500 MPL, Beckman Coulter, USA). Excitation of cells was performed at a wavelength of 488 nm and emission of the green fluorescence was detected using a band pass filter of 530/30 nm. Tail blood (5-10 µl) from mice infected with *P. berghei* expressing GFP was collected in 1 ml of PBS supplemented with 500 U/ml of heparin. Two hundred microliters of the blood suspension was transferred to a 96-well microplate and analyzed by the flow cytometer. In flow cytometry, logarithmic green fluorescence signal in the FL-1 channel was detected. Background fluorescence signal of the non-

fluorescent population was adjusted to plot between 10^0 and 10^1 in the flow cytometric scale (Janse *et al*, 2006b).

2.3.5 Leucocyte depletion from *Plasmodium berghei* infected mouse blood

Leucocytes were removed from infected mouse blood by using CF11 cellulose (Whatman, UK). After collection of blood from *P. berghei* infected mouse, blood cells were harvested by centrifugation at 20,000 x *g* for 30 sec, and washed twice with 1 ml of PBS. One milliliter of PBS was used to resuspend the cell pellet and leukocytes were then depleted by passing the blood cell suspension through CF11 cellulose column. The CF11 cellulose column was freshly prepared by adding CF11 cellulose powder in a 15-ml centrifuge tube (2 ml of CF11 cellulose powder for 1 ml of blood cell suspension) and resuspended with 10 ml of PBS by vortexing. The column was set up in a 10-ml tuberculin syringe and washed twice with 5 ml of PBS. Following passing of blood cell suspension through the column, 5 ml of PBS was then applied to wash the column. Thereafter, erythrocyte pellet was harvested by centrifugation at 750 x *g* for 5 min and washed once with 1 ml of PBS. The erythrocyte pellet was used for parasite genomic DNA or total RNA extractions (Thathy & Menard, 2002).

2.3.6 Genomic DNA isolation from malaria parasites

Blood from mice harboring *P. berghei* was collected from the tail bleed or by cardiac puncture using a 21-G needle with 500 U/ml of heparin in 1-ml tuberculin syringe, and leucocytes were then excluded by CF11 cellulose column as described in the previous section (**Section 2.3.5**). The erythrocyte pellet was resuspended in

approximately 5 volumes of 0.2% saponin (w/v) in PBS and incubated at 37^{o}C for 10 min in order to lyse the erythrocytes. Parasite pellet was then harvested by centrifugation at 20,000 x *g* for 10 min, and washed once with 1 ml of PBS. Total parasite genomic DNA was isolated using Genomic DNA Mini Kit (Blood/Culture cell) (Geneaid Biotech, Taiwan) according to the standard protocol by the manufacturer. Parasite genomic DNA was resuspended in sterile distilled water or TE buffer pH 8.0, and kept at -20^{o}C for further usage.

2.4 Transfection of *P. berghei* parasites

All transfection experiments in this study were performed using a standard protocol employing the non-viral Nucleofector technology (Janse *et al*, 2006a; Janse *et al*, 2006c) for genetic transformation of the rodent malaria parasite *P. berghei*. The improvement using this method over standard methodologies (Menard & Janse, 1997; Waters *et al*, 1997) results in transfection efficiencies in the range of 10^{-3}-10^{-4} (targeted integration of DNA plasmids into the genome). The Nucleofector technology is based on electroporation of purified mature schizonts with constructs that contain selectable marker cassettes (Janse *et al*, 2006a; Janse *et al*, 2006c). Transformed parasites are usually selected *in vivo* by treating mice with drugs.

2.4.1 Standard *in vitro* culture of asexual blood stage *P. berghei* and schizont purification

In vitro cultures were initiated from mouse blood infected with *P. berghei*. BALB/C mice were infected with PbGFP507m6cl1 from either cryopreserved or fresh parasites isolated from an infected mouse. In this study, the infection was always initiated with 6x10^{6} parasitized erythrocytes by i.p. injection. Parasitemia was

subsequently monitored daily. On day 4-5, infected mouse blood with parasitemia between 5 and 15% was collected and transferred to *in vitro* culture for the cultivation of schizonts.

RPMI1640 medium containing 25 mM HEPES, 0.2% $NaHCO_3$ and 50 IU neomycin was freshly prepared with adding FBS to a concentration of 30% (v/v). Infected mouse blood with a parasitemia of 5-10% was collected by cardiac puncture using a 21-G needle with 100 µl of heparin (500 U/ml) in 1-ml tuberculin syringe. The blood was transferred into a 50-ml centrifuge tube containing 10 ml of complete culture medium. Blood cells were harvested by centrifugation at 450 x *g* for 5 min and supernatant was discarded. Then, blood cell pellet was resuspended with 10 ml of complete culture medium and subsequently transferred into 250-ml cell culture flask containing 50 ml of complete culture medium. After adding gas mixtures (5% CO_2, 5% O_2 and 90% N_2) at 1 bar for 2 min, the culture flask was incubated at 37^{o}C with slightly shaking for 20 hr (Janse *et al*, 2006c).

By the end of culture period, quality of the culture was determined by Giemsa stained thin blood smear of the erythrocyte pellet obtained from a 0.5 ml aliquot of culture suspension, by which mature schizonts should be visible at a frequency of 3-6 schizonts per field. Thereafter, the mature schizonts were separated from uninfected erythrocytes on density gradients in the following manner. Briefly, 60% Nycodenz/PBS solution was freshly prepared and 10 ml aliquot of the solution was transferred into a sterile 50-ml centrifuge tube. Next, 35 ml of the parasite culture was gently overlayered the Nycodenz solution. The tube was centrifuged at 450 x *g* for 20 min without brake. Then, the brown-grayish layer which contains schizonts at

the interface between the two suspensions was carefully collected using a Pasteur pipette into a 50-ml centrifuge tube. The complete culture medium was subsequently added to the collected cell suspension up to a volume of 45 ml and centrifugation was performed at 450 x *g* for 5 min to obtain schizont pellet. The pellet was carefully resuspended with an appropriate volume of the complete culture medium and 1 ml of the schizont suspension was distributed to a 1.5-ml microcentrifuge tube. The schizonts from one tube were used for one transfection experiment (Janse *et al*, 2006c).

2.4.2 Small-scale *in vitro* culture of asexual blood stage *P. berghei* and schizont purification (Somsak *et al*, 2011)

For one transfection experiment, 20 µl of tail blood at parasitemia of 10% was collected in a 1.5-ml microcentrifuge tube containing 1 ml of RPMI1640 medium and 500 U/ml of heparin, and resuspended gently by inverting the tube. The infected blood pellet was collected by centrifugation at 450 x *g* for 2 min before it was resuspended again in complete RPMI1640 medium supplemented with fetal bovine serum (30%), neomycin (50 IU/mL) and Hepes (25 mM). The infected blood suspension was briefly gassed with gas mixture (5%CO_2, 5%O_2 in N_2) for 15 sec before incubation at 37^{o}C with continuous agitation, with tube lying down on its side, to keep the culture as suspension for 20 hr in an incubator equipped with an orbital shaker.

Cell pellet from the overnight small-scale culture was collected by centrifugation at 450 x *g* for 2 min, resuspended in complete RPMI1640 medium, and subsequently layered on top of 60% Nycodenz/PBS solution in a new 1.5-ml

microcentrifuge tube. After centrifugation at 450 x *g* for 20 min, without brake during deceleration, using a centrifuge machine with a swinging rotor, an interface layer containing mature schizonts could be observed. The fully developed mature schizonts were carefully collected, washed once with complete medium and then resuspended in complete medium until using in transfection experiment.

2.4.3 Electroporation of purified schizonts

The schizont pellet was then resuspended with 100 µl of the Nucleofector solution 88A6 (non-viral Human T-cell solution) containing 5 µg of the linearized DNA, prepared as described in **Section 2.4.1 and 2.4.2**. The mixture was transferred into an electroporation cuvette (Amaxa), which was then placed in the Amaxa Nucleofector device. The transfection was carried out by electroporation using the pre-set program U33. Immediately after electroporation, 200 µl of the complete culture medium was added to the electroporated cells in the cuvette and all solution was transferred into a 1.5-ml microcentrifuge tube using a plastic pipette.

2.4.4 Selection of transfected parasites

2.4.4.1 Positive selection procedure

The electroporated schizont solution was then inravenously (i.v.) injected into a tail vein of a naïve BALB/C mouse prewarmed under a heat lamp for 5-10 min before the injection. The parasites were allowed to complete one cycle of asexual division for 24 hr in the absence of drug pressure. The injected mice were then feed with drinking water containing freshly prepared pyrimethamine (7 mg/ml in DMSO and diluted a hundred times with drinking water to a final concentration of 70

µg/ml) for a period of 4-9 days up to the collection of infected blood (Janse *et al*, 2006a; Janse *et al*, 2006c).

2.4.4.2 Negative selection procedure

Selection of transgenic parasites was performed using negative selection after the positive selection. Mice transfected with plasmid pL35pvDHFR-TS were injected intraperitoneally with 5-fluorocytosine (5-FC) in 0.9% NaCl solution at a dose of 1 g/kg body weight twice a day for a period of 4 consecutive days. For the period of negative selection, the parasitemia declined to undetectable levels. Three to five days after the treatment period, the surviving parasites were present at a parasitemia between 3-5% (Braks *et al*, 2006).

2.4.5 Parasite cloning

The transfected parasites after positive and negative selection were cloned by limiting dilution technique when parasitemia reached between 0.1 and 1%. Briefly, 5 µl of infected blood was collected from the donor mice and subsequently diluted in 1 ml of complete culture medium in a 1.5-ml microcentrifuge tube. The number of erythrocytes and parasites per µl of blood were then calculated in a hemocytometer. Then, the suspension was serially diluted with complete culture medium to a final concentration of 1 parasite/0.2 ml. The cloning was carried out by i.v. injection of serially suspensions into groups of naïve BALB/C mice. Parasites became detectable in a blood smear 8 days after injection. Cloning was considered successfully if less than 50% of injected mice showing positive blood smear (Menard & Janse, 1997; Waters *et al*, 1997).

2.4.6 Genotypic analysis of transgenic parasites

2.4.6.1 Polymerase chain reaction analysis

Leucocytes in infected blood were removed by CF11 cellulose column (**Section 2.3.5**) and parasite genomic DNA was isolated (**Section 2.3.6**). The PCR amplification reaction was set up in a total volume of 25 µl containing 10-100 ng of genomic template DNA, 0.8 µM of each primer, 400 µM of dNTPs, 1x *Pfu* buffer, 1 µg/µl of BSA, 1 U of *Taq* DNA polymerase (Promega), and 1 U of *Pfu* DNA polymerase (Promega). Temperature cycling parameters used for PCR reaction were as follows: (1 cycle) 95^{o}C for 2 min, (30 cycles) 95^{o}C for 45 sec, 40^{o}C for 45 sec, 60^{o}C for 2-7 min, (last cycle) 95^{o}C for 45 sec, 55^{o}C for 45 sec, and 60^{o}C for 7-12 min. Oligonucleotide primers, *Pv*DTF and *Pv*DTR, were used to amplify *pvdhfr-ts* gene. In order to confirm the deletion of endogenous *dhfr-ts* of *P. berghei*, oligonucleotide primers, *Pb*DTF and *Pb*DTR were used. In addition, oligonucleotide primers, 5'*Pv*INTF/5'*Pv*INTR and 3'*Pv*INTF/3'*Pv*INTR, were used to confirm integration events at 5' and 3' flanking regions of *pbdhfr-ts* locus, respectively. Moreover, in order to confirm the presence of positive-negative selectable gene, oligonucleotide primers, hDyFCUF and hDyFCUR, were used. All oligonucleotide primers used in this section are listed in **Table 2-1**.

The amplified PCR products were then run on agarose gel electrophoresis and analyzed under UV transilluminator after ethidium bromide staining (**Section 2.2.2**).

2.4.6.2 Southern analysis

Synthetic oligonucleotides used as probes in Southern blot analysis were 5'UTRPbDT probe and 3'UTRPbDT probe (**Table 2.1**). The DNA was randomly labeled with Digoxigenin-11-dUTP in order to prepare probe for Southern blot analysis using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science) according to the manufacturer's instructions. The DIG-labeled probes were estimated for a concentration by comparing with DIG-labeled control DNA as provided by the Kit.

Parasite genomic DNA (10-15 µg) was prepared as previously described in **Section 2.3.5-2.3.6**, and digested with *Hind*III and *Kpn*I restriction enzymes overnight at 37^{o}C. After electrophoresis and ethidium bromide staining, the digested genomic DNA was visualized under UV light and then the gel picture was taken with a ruler lying alongside of the gel. Consequently, the stained gel containing digested genomic DNA was depurinated with 0.5 M HCl for 10 min followed by DNA denaturation with Denaturing buffer (0.5 M NaOH and 1.5 M NaCl) at room temperature for 30 min with gentle shaking on an orbital shaker. DNA blotting was performed using 20x SSC solution as transferring buffer by capillary action. Buffer-soaked Hybond N+ membrane (Amersham, UK) was placed on top of a stack of paper towels. After placing the gel on top of the membrane, a weight was then placed on top of the gel to ensure good and even contact between gel and membrane. The blot was incubated at room temperature overnight.

For DNA hybridization, the membrane was rinsed with distilled water, and UV cross-linking of the membrane was then performed. The membrane was

incubated with pre-warmed DIG Easy Hyb solution (5 ml/100 cm^2 filter) at 37^oC in hybridization oven with gently agitation in hybridization bottle for 30 min. DIG-labeled DNA probe (25 ng/ml DIG Easy Hyb) was denatured by boiling in a water bath for 5 min, and immediately chilled on ice. The denatured probe was subsequently added into the hybridization bottle containing pre-warmed DIG Easy Hyb solution and the membrane. The hybridization was carried out by incubation at 37^oC in hybridization oven overnight with gently agitation.

For chemiluminescence detection, the membrane was washed twice with 2x SSC solutions for 5 min and equilibrated in washing buffer for 1 min. Thereafter, blocking solution was added to the membrane and incubated at room temperature for 30 min with gentle shaking on an orbital shaker followed by incubation with Anti-DIG-AP (alkaline phosphatase) antibody (1:10,000) for further 30 min. Consequently, the membrane was washed twice with washing buffer for 15 min and equilibrated in detection buffer at room temperature for 5 min. The membrane was then placed on a plastic bag, and 2 ml of CSPD ready-to-use solution was then applied. The membrane was covered immediately and incubated at room temperature for 5 min. After the excess liquid was squeezed out, the plastic bag containing the membrane was sealed and incubated at 37^oC for 10 min. The chemiluminescent signal was carried out by exposing the membrane with X-ray film for 5-30 min at room temperature.

2.4.7 Phenotypic analysis of asexual blood stage development *in vivo*

A group of 5 naïve BALB/C mice were i.p. infected with either 1x10^6 *P. berghei* wild type or transgenic parasites. Total blood parasitemia was determined by

microscopic examination of Giemsa stained thin blood smear or flow cytometric analysis of tail blood every day during a period of 10 days.

2.4.8 *In vivo* drug susceptibility assays

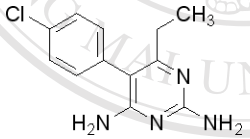
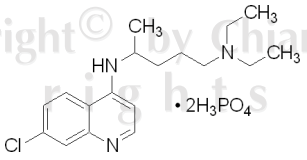
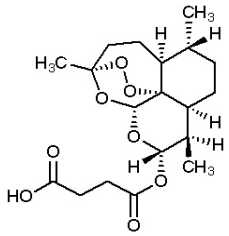
Three known antimalarial drugs (**Table 2.2**) were used to test the drug susceptibility of transgenic *P. berghei* parasites: pyrimethamine (PYR; Sigma-Aldrich, USA), chloroquine diphosphate salt (CQ; Sigma-Aldrich, USA), and artesunate (ARTESUNAT® tablet, 50 mg, Mekophar Chemical Pharmaceutical Joint-stock Company, Vietnam). The drugs were freshly prepared in 100% DMSO (PYR and CQ) or in distilled water (DW) (artesunate). Drug doses (mg/kg) were adjusted at the time of administration according to the weight of the mice by DMSO or DW. Untreated controls were given DMSO or DW only.

For a standard 4-day suppressive test (Peters *et al*, 1975), infected mouse blood with parasitemia between 10-30% was collected and diluted in RPMI1640 medium to obtain $1x10^8$ parasitized erythrocytes per ml. An aliquot of 0.2 ml (containing $2x10^7$ parasitized erythrocytes) was injected intraperitoneally into experimental groups of 5 mice. Four hours after injection, the experimental groups were administered orally by gavage with a single dose of drug. Twenty four, 48, and 72 hr after injection, the experimental groups was treated again with the same dose and the same route as done on the first day. Twenty-four hours after the last treatment, parasitemia was calculated by either Giemsa stained thin blood smear or flow cytometric analysis. The difference of the mean value between control group and experimental groups was calculated and presented as percentage of inhibition using the following equation:

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{Mean parasitemia of experiment group}}{\text{Mean parasitemia of control group}}\right) \times 100$$

The mean parasitemia of the control group was set up at 100%, and the mean parasitemia of the treated groups with highest drug concentration were set up at 0% for calculation of the percentage of inhibition. Growth inhibitory (non-linear regression function for sigmoidal dose-responses) curve and ED_{50} values were calculated using SigmaPlot software (Systat Software, USA).

Table 2-2 Structure of known antimalarial drugs used in this study

Antimalarial	**Structure**	**Company**	**MW**
Pyrimethamine		Sigma-Aldrich Cat.no. P7771 or 46706	248.71
Chloroquine diphosphate salt		Sigma-Aldrich Cat.no. C6628	515.86
Artesunate		ARTESUNAT® tablet, 50 mg, Mekophar Chemical Pharmaceutical Joint-stock Company, Ho Chi Minh City, Vietnam	384.42