CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Reagents

The details of chemicals and reagents are shown in Appendix.

2.2 Materials

2.2.1 Plasmid vectors

Plasmids pL0002 and pL0017 were kindly provided by Dr. Andrew Waters of Leiden University Medical Centre, Leiden, the Netherlands, (now at University of Glasgow, U.K.) (Franke-Fayard et al., 2004) (Figures 2.1 and 2.2).

2.2.2 Bacterial strains

E. coli DH5α and PMC103 cells were provided by Protein-Ligand Engineering and Molecular Biology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand. They were generally used as host cells for gene manipulation and amplification of recombinant plasmids.

2.2.3 Templates for gene amplification by PCR method

2.2.3.1 3'UTR of *Plasmodium berghei* dihydrofolate reductase-thymidylate synthase

The 1.0 kb of 3'UTR *P. berghei* dihydrofolate reductase (*Pbdhfr-ts*) was amplified by PCR from pL0002 plasmid (MR4; http//www.mr4.org/) (Figure 2.2) which contains the 3'UTR fragment.

2.2.3.2 Wild-type *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase

The wild-type *P. falciparum* dihydrofolate reductase-thymidylate synthase (*Pfdhfr-ts*) gene, 1.8 kb in size, cloned in pET-17b expression vector (pET-pfDHFR-TS) was kindly provided by Ms. Deanpen Japrung, Protein-Ligand Engineering and Molecular Biology Laboratory, BIOTEC, Thailand. The plasmid was used as a template for the creation of wild-type *Pfdhfr-ts* mutant libraries. The modified *Pfdhfr-ts* contains an *Afl*II restriction site in the junction region which serves as cloning site for libraries of *Pfdhfr* random mutations.

2.2.3.3 *Plasmodium falciparum* dihydrofolate reductase mutant genes

The known mutants of *P. falciparum* dihydrofolate reductase (*Pfdhfr*) with double mutation (C59R+S108N), triple mutation (C59R+S108N+I164L) and quadruple mutation (N51I+C59R+S108N+I164L) were constructed in pET-17b expression vector and named pETpfK1, pETpfCSL and pETpfV1S, respectively. All plasmids were provided by Protein-Ligand Engineering and Molecular Biology Laboratory, BIOTEC, Thailand.

2.2.4 Oligonucleotide primers

All primers used were synthesized by Biodesign, Thailand. The sequence of primers are shown in Table 2.1

Table 2.1 Sequence of primers used in this study

Primers	Length (bases)	Sequence (5'→3')	Utilities
Pb3'FNheI	26	GCGCTAGCTGATC CCGTTTTTCTTAC	Sense strand for amplification of 3'UTR of *Pbdhfr-ts*
Pb3'RKasI	26	TCGGCGCCCGGGG ATCAATTTATTTC	Antisense strand for amplification of 3'UTR of *Pbdhfr-ts*
PfFDHFRAgeI	25	GACCGGTATGATG GAACAAGTCTGC	Sense strand for amplification of *Pfdhfr-ts*
PfRDHFRNheI	24	CGGCTAGCATTAAG CAGCCAATCC	Antisense strand for amplification of *Pfdhfr-ts*
PfFDHFRAgeI BamHI	31	GACCGGTGGATCCAT GATGGAACAAGTCTGC	Sense strand for amplification of *Pfdhfr-ts* gene containing both *Age*I and *Bam*HI sites

Table 2.1 Sequence of primers used in this study (continued)

Primers	Length (bases)	Sequence (5'→3')	Utilities
FBAMHI	23	CGGTGGATCCAT GATGGAACAAG	Sense strand for amplification of *Pfdhfr-ts*
RAflII	23	CTTTGTCATCATT CTTAAGAGGC	Antisense strand for amplification of *Pfdhfr*
5'PbINTF	24	TTGAGCTACATA ACTTCCATACAT	Sense strand for amplification of endogenous 5'UTR of *Pbdhfr-ts*
3'PbINTR	18	CGATCTACACCT CTTCAT	Antiense strand for amplification of endogenous 3'UTR of *Pbdhfr-ts*
PbDTF	36	GGGGGGGGCATA TGGAAGACTTATC TGAAACATTCG	Sense Strand for amplification of *Pbdhfr-ts*
PbDTR	24	GGACTAGTTTAAG CTGCCATATCC	Antisense strand for amplification of *Pbdhfr-ts*
PbDRspeI	24	GGACTAGTGTACT TCCTCATTTGG	Antisense strand for amplification of *Pbdhfr*
Pb_a_tubulinF	20	GCATGCTGGGA GCTATTTTG	Sense strand for amplification of *P. berghei* alpha tubulin gene
Pb_a_tubulinR	22	GCTGGTTCAAATG CTGAGTTTG	Antisense strand for amplification of *P. berghei* alpha tubulin gene

2.2.5 DNA purification kits

Commercial DNA purification kits (Table 2.2.) were used in this study to purify plasmids, PCR products, DNA fragment and genomic DNA.

Table 2.2 DNA purification kits.

Kit	Company	Utlitility
QIAprep Spin Miniprep Kit	QIAGEN	purify small scale plasmid
QIAquick Gel Extraction Kit	QIAGEN	purify DNA fragments from agarose gel, PCR product, digested PCR product
QIAquick PCR Purification Kit	QIAGEN	purify DNA fragments from PCR product, digested PCR product
MinElute Gel Extraction Kit	QIAGEN	purify DNA fragment from agarose gel and digested PCR product to give high concentrations of DNA
Genomic DNA Mini Kit	Geneaid	extract genomic DNA from whole blood, red cell pellet containing *P. berghei*

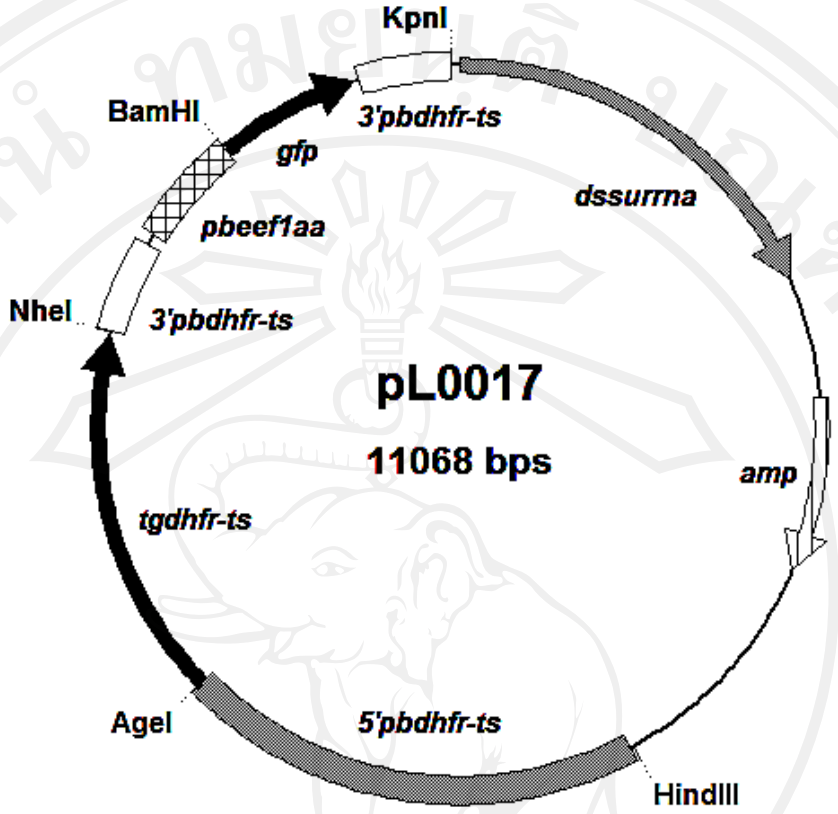

Figure 2.1 Circular map of pL0017 vector. This plasmid contains two expression cassettes of *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase (*Tgdhfr-ts*) under control of 2.3 kb of 5' and 0.5 kb of 3'UTR from *Pbdhfr-ts*, and GFP under control of 5'UTR of *Pbef-1αa* and 3'UTR of *Pbdhfr-ts*, and a sequence of double strand small subunit ribosomal RNA (*dssurrna*) for use as integration site at *dssurrna* locus, if needed.

2.3 Methods

2.3.1 Construction of *P. berghei* transfection plasmid

Plasmids for *P. berghei* transfection in this study were modified from the original plasmid pL0017 (Figure 2.1). This plasmid contains two expression cassettes of *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase (*Tgdhfr-ts*) under control of 2.3 kb of 5' and 0.5 kb of 3'UTR from *Pbdhfr-ts* and GFP under control of 5'UTR of *Pbef-1αa* and 3'UTR of *Pbdhfr-ts*, and a sequence of double strand small subunit ribosomal RNA (*dssurrna*) for use as integration site at *dssurna* locus, if needed.

2.3.1.1 Plasmid isolation and purification

All of plasmid DNA in this study were amplified by transformation into *E. coli* strain DH5α and cultured at 37°C for 16 hr in LB media containing 100 μg/ml ampicillin. The plasmid was purified from bacterial culture using the QIAprep Spin Miniprep Kit according to manufacturer's protocol.

2.3.1.2 Plasmid analysis by agarose gel-electrophoresis

Agarose gel (0.8%) was prepared by dissolving 0.8 g of ultrapure agarose in 100 ml of 0.5X TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The gel suspension was heated until completely dissolved. The gel suspension was left to cool down to approximately 50°C and was poured into a casting chamber equipped with comb. The gel was allowed to solidify at room temperature at approximately 25°C before electrophoresis was carried out.

To perform gel-electrophoresis, DNA samples were mixed with 6X DNA dye (0.25% bromphenol blue, 0.25% xylene cyanol FF and 30% glycerol). Aliquots of

the dye-containing samples and DNA markers were loaded into the wells and electrophoresis was carried out at 100 volts using 0.5X TAE buffer as electrolyte. After electrophoresis, the gel was stained with ethidium bromide for 10 min and destained with tap water. The band of DNA in the gel was visualized under UV transluminator and the sizes of the DNA fragments were extrapolated based on the DNA standard markers.

2.3.1.3 Amplification of 3'UTR of *Pbdhfr-ts* 1.0 kb and ligation into pL0017 plasmid

The 1.0 kb of 3'UTR of *Pbdhfr-ts* was amplified by PCR using pL0002 (Figure 2) as a template, forward primer: Pb3'FNheI (5'-GCGCTAGCTGATCCCGTTTTTCTTAC-3') and reverse primer 1kb Pb3'RKasI (5'-TCGGCGCCCGGGGATCAATTTATTTC-3'). The underlined sequences are *Nhe*I and *Kas*I restriction sites, respectively which served as the cloning sites. The PCR mixture contained 1 µl of DNA template (pL0002, 10 ng/µl), 0.2 µl each of primer Pb3'FNheI (10 µM) and Pb3'RKasI (10 µM), 2 µl of 10X *Pfu* DNA polymerase buffer, 0.2 µl of *Pfu* DNA polymerase (3 U/µl), 0.2 µl of 10 mM dNTP, ddH_2O 15.4 µl to final volume of 20 µl. The PCR condition was performed as follows: the first cycle consisted of 95°C for 3 min then the subsequent 30 cycles were 95°C for 1 min, 55°C for 30 sec and 72°C for 2 min. The 1.0 kb of 3'UTR of *Pbdhfr-ts* PCR product was purified by QIAquick PCR Purification Kit according to manufacturer's protocol.

After purification, the PCR product was digested with *Nhe*I and *Kas*I. The digestion reaction composed of 20 µl of purified PCR product, 3 µl of 10X NEB buffer No. 2, 3 µl of BSA (1 mg/ml), 0.5 µl of *Nhe*I (10 U/µl), 1 µl of *Kas*I (4 U/µl)

and ddH_2O to the final volume of 20 µl. After incubation at 37°C for 2 hr, the digested PCR product was purified by QIAquick PCR Purification Kit.

For plasmid backbone preparation, the original plasmid, pL0017 (Figure 2.1) was digested with restriction enzymes *Nhe*I and *Kas*I to remove 0.5 kb of 3'UTR of *Pbdhfr-ts,* GFP cassette and *dssurrna* sequence. The digestion reaction composed of 10 µl of purified pL0017 plasmid, 2 µl of 10X NEB buffer No.2, 2 µl of 1 mg/ml BSA, 0.5 µl of *Nhe*I (10 U/µl), 1 µl of *Kas*I enzyme (4 U/µl) and ddH_2O to the final volume of 20 µl. After incubation at 37°C for 2 hr, the digested plasmid was separated on a 0.8% agarose gel. Thereafter, the gel was soaked in 0.05% methylene blue in 0.5X TAE buffer and allowed to stain for 5-10 min before the gel was destained with dH_2O until the DNA bands appeared. The expected band size of 6.6 kb of DNA on the gel was removed and further purified by QIAquick Gel Extraction Kit.

The ligation reaction consisted of 1 µl of purified *Nhe*I/*Kas*I digested pL0017 backbone (16 ng/µl), 7 µl of purified *Nhe*I/*Kas*I digested 3'UTR of *Pbdhfr-ts* PCR product (2.5 ng/µl), 1 µl of 10X T4 DNA ligase buffer, 1 µl of 50 mM ATP, 1 µl of T4 DNA ligase enzyme (400 U/µl) and ddH_2O to the total volume of 10 µl. T4 DNA ligase catalyzes the formation of covalent phosphodiester bond between neighboring 3' hydroxyl ends of one nucleotide with the 5' phosphate end of another. After incubation at 16°C for approximately 12-16 hr, the ligation product was transformed into *E. coli* PMC 103 cells by electroporation and plated onto LB agar containing 100 µg/ml ampicillin. The colonies grown after incubation at 37°C for 12-16 hr were randomly selected and checked for transformed plasmid DNA by colony PCR as

described in section 2.3.1.5. Only positive samples were extracted for plasmid DNA by QIAprep Spin Miniprep Kit. The corresponding size of plasmid was analysed by running digested plasmid through agarose gel-electrophoresis as described in section 2.3.1.2. The 1.0 kb 3'UTR *Pbdhfr-ts* insert of the plasmid was sent to by Biodesign, Thailand for DNA sequencing. The sequence showed complete homology with the 3'UTR of *Pbdhfr-ts* from pL0002. The modified plasmid was named pY001 (Figure 3.3).

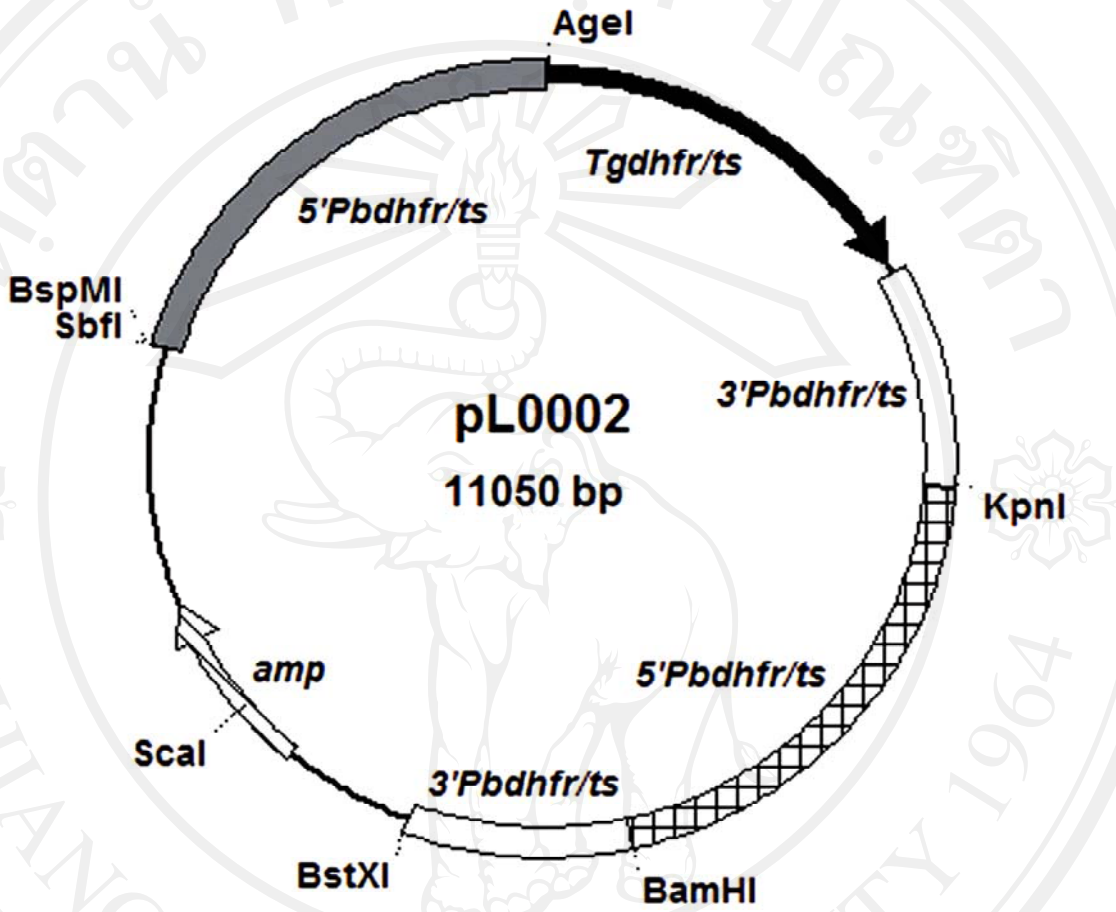

Figure 2.2 Circular map of pL0002 vector. This plasmid contains selection cassettes of *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase (*Tgdhfr-ts*) under control of 2.3 kb of 5' and 1.0 kb of 3'UTR from *Pbdhfr-ts*, and expression cassette under control of 2.3 kb of 5' and 1.0 kb of 3'UTR of *Pbdhfr-ts*.

2.3.1.4 Transformation of *E. coli* by high-voltage electroporation

The electroporation method was developed to introduce DNA into bacterial cell (Dower et al., 1988). The principle is based on the short electric impulses which above certain field strength can make biomembrane transiently more permeable without permanently damaging the membrane structure. The electric field pulse causes phospholipid membrane disturbance which leads to pore formation. The DNA is then delivered to bacterial cell through membrane pore. The electroporation of prokaryotes requires high voltage, low ionic strength and small volume of high resistance samples are used.

2.3.1.4.1 Preparation of competent *E. coli* cells for electroporation

Fresh overnight culture of *E. coli* PMC103 was inoculated in LB broth [1 ml into 100 ml of LB medium (1:100 V/V)]. The cell culture was grown at 37°C with shaking at 250 rpm to an OD_{600} of 0.6-0.7. The cells were harvested and centrifuged at 1,600 × *g*, 4°C for 5 min. The supernatant was discarded and the cell pellet was placed on ice for 1 hr. After that, the cell pellet was washed twice in 50 ml ice-cold ddH_2O by centrifugation at 1,600 × *g*, 4°C for 5 min. The cell pellet was then re-suspended in 40 ml ice-cold 10% glycerol and centrifuged at 1,600 × *g*, 4°C for 5 min. The cells were again re-suspended with 1 ml of ice-cold 10% glycerol, thereafter an aliquot of 100 µl of cell suspension was transferred into sterilized microcentrifuge tubes and stored frozen at -80°C. The competent cells were freshly thawed prior to each usage to reduce the amount of cell death due to freezing and thawing of the cells.

2.3.1.4.2 Transformation using electrocompetent cell

The ligation reaction was purified as follows: 3 M of NaOAc pH 4.8 (1:10 v/v) and 1 ml of cold absolute ethanol were added to the ligation reactions and incubated at -20°C for 4 hr to precipitate DNA. The reaction was centrifuged at 20,000 × *g* for 10 min and the supernatant was removed. The DNA pellet was washed again with 500 µl of cold 70% ethanol. After centrifugation and removal of all residual ethanol, DNA pellet was dried before it was re-suspended with 10 µl of ddH_2O.

The frozen competent cells from section 2.3.1.3.1 were allowed to thaw on ice for 10 min. The purified DNA pellet from ligation reaction was added to 100 µl of competent cells already dispersed in a microcentrifuge tube and was incubated on ice for 20 min. The cell suspension was then transferred to a cold 0.2-cm-gap electroporation cuvette and the electroporation was performed using the Gene Pulser® (Bio-Rad). The condition was set at 2.5 kV, the capacitor was set at 25 µF and 200 Ω resistance (time constant is 2.5 to 4.9 ms). After electroporation, 800 µl of LB broth was immediately added to the cell suspension which was placed on ice. To allow the restoration of growth and expression of antibiotic resistant marker encoded by the plasmid, the samples were incubated at 37°C with aeration and shaking at 250 rpm for 1 hr. The whole volume of each transformation culture was plated on LB agar plate containing 100 µg/ml of ampicillin for selection. The plates were left until the solution was completely absorbed to agar plates at room temperature. Thereafter, the plates with media side up were incubated in 37°C incubator for 12-16 hr.

2.3.1.5 Screening of bacterial colonies by PCR method

PCR mixture was prepared for amplification of the gene of interest. The mixture was divided in aliquot into PCR tubes according to the numbers of colonies to be screened. The bacterial colonies were randomly picked by sterile toothpick and inoculated into the PCR mixture. The PCR condition followed the appropriate condition for each interested gene. The PCR products were then analysed on 0.8% agarose gel. The gel electrophoresis procedure was as described in section 2.3.1.2.

2.3.1.6 Amplification of wild-type *Pfdhfr-ts* and ligation into pY001 plasmid

The 1.8 kb of wild-type *Pfdhfr-ts* was amplified by PCR using the template which contained *Pfdhfr-ts* in pET-17b expression vector (pET-pfDHFR) kindly provided by Ms. Deanpen Japrung, BIOTEC. The PCR reaction containing 1 µl of DNA template (10 ng), 0.5 µl each of forward primer PfFDHFRAgeI (5'-GACCGGTATGATGGAACAAGTCTGC-3') (10µM) and reverse primer PfRDHFRNheI (5'-CGGCTAGCATTAAGCAGCCAATCC-3') (10µM) (underlined sequences represent *Age*I and *Nhe*I restriction sites, respectively), 10 µl of 5X GoTaq® Flexi buffer, 5 µl of 25 mM $MgCl_2$ solution, 5 µl of GoTaq® DNA polymerase (5 U/µl), 0.5 µl of 10 mM dNTP, ddH_2O 32 µl to final volume of 50 µl. The PCR condition was performed as follows: the first cycle was set at 95°C for 3 min, then the subsequent 30 cycles were set at 95°C for 1 min, 58°C for 1 min and 72 °C for 2 min. The 1.8 kb of *Pfdhfr-ts* PCR product was purified by QIAquick PCR Purification Kit.

After purification, the PCR product was digested with *Age*I and *Nhe*I. The digestion reaction composed of 10 µl of purified PCR product, 3 µl of 10X NEB

buffer No. 1, 3 µl of 1 mg/ml BSA, 1 µl of *Age*I (5 U/µl), 0.5µl of *Nhe*I (10 U/µl), and ddH_2O to the final volume of 30 µl. After incubation at 37°C for 2 hr, the digested PCR product was purified by QIAquick PCR Purification Kit.

For plasmid backbone preparation, plasmid pY001 was digested with the restriction enzymes *Age*I and *Nhe*I to remove 1.8 kb of *Tgdhfr-ts*. The digestion reaction composed of 6 µl of purified pY001 plasmid (8 ng/µl), 5 µl of 10X NEB buffer No.1, 2 µl of 1 mg/ml BSA, 2 µl of *Age*I (5 U/µl), 1 µl of *Nhe*I (10 U/µl) and ddH_2O to the final volume of 50 µl. After incubation at 37°C for 2 hr, the digested plasmid was loaded on 0.8% agarose gel.

After gel electrophoresis, the gel was soaked in 0.05% methylene blue in 0.5X TAE buffer and allowed to stain for 5-10 min, then the gel was destained with dH_2O until the DNA bands appeared. The expected band size of 5.7 kb of DNA on the gel was removed and further purified by QIAquick Gel Extraction Kit.

The ligation reaction consisted of 3 µl of purified *Age*I/*Nhe*I digested pY001 backbone (2.6 ng/µl), 3.5 µl of purified *Age*I/*Nhe*I digested *Pfdhfr-ts* PCR product (16 ng/µl), 1 µl of 10X T4 DNA ligase buffer, 1 µl of 50 mM ATP, 1 µl of T4 DNA ligase enzyme (400 U/µl) and ddH_2O to the final volume of 10 µl. After incubation at 16ºC for approximately 12-16 hr, the ligation product was transformed into *E. coli* DH5α by heat shock method and plated onto LB agar containing 100 µg/ml ampicillin. The colonies grown after incubation at 37°C for 12-16 hr were randomly selected, and subjected to PCR reaction to screen for ligated plasmid as described in section 2.3.1.5. Only positive samples were extracted and cleaned up by QIAprep Spin Miniprep Kit. The corresponding size of plasmid was analysed by running

digested plasmid through agarose gel-electrophoresis as described in section 2.3.1.2. The 1.8 kb *Pfdhfr-ts* insert of the plasmid was sent to Biodesign, Thailand for DNA sequencing. The sequence showed complete homology with the wild-type *Pfdhfr-ts*. The modified plasmid was named pY003 (Figure 3.6).

2.3.1.7 Transformation using calcium chloride treatment/heat shock method

The aim of this method is to render cells permeable to DNA by treatment with $CaCl_2$ (Mandel and Higa, 1970). This method involves two steps. The plasmid DNA is mixed with the cells in the presence of $CaCl_2$ and presumably adheres to them. The mixture of DNA and cells is then heat shocked, which allows the DNA to efficiently enter the cells.

2.3.1.7.1 Preparation of competent *E. coli* cells using calcium chloride treatment

A single colony of *E. coli* DH5α cells was inoculated into 5 ml of LB broth and incubated overnight at 37°C with shaking at 250 rpm. The overnight culture was diluted 1:200 with new LB broth and incubated at 37°C with shaking at 250 rpm until OD_{600} is 0.4-0.6 (about 2-3 hr). The cell growth was stopped at linear log phase to maximize transformation efficiency. The culture was transferred to cold 50 ml sterile polypropylene tube and left on ice for 15 min. The cells were subjected to centrifugation at 1,600 × *g* at 4°C for 7 min. The supernatant was discarded and the cell pellets were gently re-suspended in 1/5 volume of ice-cold sterile 0.1 M $MgCl_2$. The cell suspension was kept on ice for 15-20 min and then centrifuged at 1,600 × *g* 4°C for 7 min. The supernatant was discarded and the cell pellets were re-suspended in ice-cold sterile 0.1 M $CaCl_2$ at 1/50 the original culture. Competency was

established by gently incubating cells on ice for 1 hr. Finally, 2 ml of ice-cold sterile 75% glycerol were added into the cell suspension and stored frozen at -80°C.

2.3.1.7.2 Transformation of $CaCl_2$ competent cells

The frozen competent cells from 2.3.1.7.1 were allowed to thaw on ice for 10 min. Each plasmid DNA sample from stock of intact plasmid or ligation reaction was added to 100 µl of competent cells already dispersed in microcentrifuge tube and was incubated on ice for 20 min. Each tube of transformation mixture was subjected to heat shock for exactly 45 sec at 42°C and then immediately chilled on ice for 2 min before 800 µl LB broth was added to the reaction mixture. To allow the restoration of growth and expression of the antibiotic resistant marker encoded by the plasmid, the samples were incubated at 37°C with aeration and shaking at 250 rpm for 1 hr. The appropriate volume of each transformation culture was plated on LB agar plate containing 100 µg /ml of ampicillin for selection. The plates were left until the solution was completely absorbed at room temperature. After that, the plates with media side up were incubated in 37°C incubator for 12-16 hr.

2.3.1.8 Amplification of mutant *Pfdhfr-ts* and ligation into pY003 plasmid

All known mutant *Pfdhfr* (double, triple, quadruple mutation) were amplified by PCR using the template pETpfK1, pETpfCSL, pETpfV1S which contains each of mutant *Pfdhfr*, respectively. The PCR reaction contained 1 µl of DNA template (10 ng/µl), 0.5 µl of the forward primer PfFDHFRAgeI (5'-GACCGGTATGATGGAACAAGTCTGC-3') (10 µM) and 0.5 µl reverse primer PfRDHFRNhe (5'-CGGCTAGCATTAAGCAGCCAATCC-3') (10 µM) (underlined sequences represents *Age*I and *Nhe*I restriction sites respectively), 5 µl of 10X *Pfu*

buffer, 0.3 µl of *Pfu* DNA polymerase (3 U/µl), 0.2 µl of GoTaq® DNA polymerase (5 U/µl), 0.5 µl of 10 mM dNTP, ddH_2O 42 µl to final volume of 50 µl. The PCR condition was set as follows: the first cycle was set at 95°C for 3 min then the subsequent 30 cycles were 95°C for 1 min, 58°C for 1 min and 72°C for 2 min. The 1.8 kb of all mutant *Pfdhfr* PCR product was purified by QIAquick PCR Purification Kit.

Thereafter, each of the PCR products was digested with *Age*I and *Nhe*I. The digestion reaction composed of 10 µl of purified PCR products (50 ng/µl), 3 µl of 10X NEB buffer No.1, 3 µl of 1mg/ml BSA, 1 µl of *Age*I (5 U/µl), 0.5 µl of *Nhe*I (10 U/µl) and ddH_2O to the final volume of 30 µl. After incubation at 37°C for 2 hr, the digested PCR product was purified by QIAquick PCR Purification Kit.

For plasmid backbone preparation, plasmid pY003 was digested with the restriction enzymes *Age*I and *Nhe*I to remove 1.8 kb of *Pfdhfr-ts*. The digestion reaction composed of 4 µl of purified pY003 plasmid (250 ng/µl), 5 µl of 10X NEB buffer No.1, 5 µl of 10X BSA, 2 µl of *Age*I (5 U/µl), 1 µl of *Nhe*I (10 U/µl) and ddH_2O to the final volume of 50 µl. After incubation at 37°C for 5 hr, the digested plasmid was loaded and run through 0.8% agarose gel. The expected band size of 5.7 kb of DNA on the gel was removed and further purified with the QIAquick Gel Extraction Kit.

Each tube of ligation reaction consisted of 4 µl of purified *Age*I/*Nhe*I digested pY003 backbone (7.5 ng/µl), 2.5-4 µl of each purified *Age*I/*Nhe*I digested mutant *Pfdhfr* PCR product (7-11 ng/µl), 1 µl of 10X T4 DNA ligase buffer, 1 µl of 50 mM ATP, 1 µl of T4 DNA ligase enzyme (400 U/µl) and ddH_2O to the final volume of 10

µl. After incubation at 16°C for approximately 12-16 hr, the ligation product was transformed into *E. coli* DH5α by $CaCl_2$ heat shock method and plated onto LB agar containing 100 µg/ml ampicillin. The colonies grown after incubation at 37°C for 12-16 hr were randomly selected and plasmid DNA were randomly selected, and subjected to PCR reaction to screen for ligated plasmid, as described in section 2.3.1.5. Only positive samples were extracted and cleaned up by QIAprep Spin Miniprep Kit. Thereafter, the agarose gel was analysed by running through agarose gel-electrophoresis as described in section 2.3.1.2. The clone was verified as the appropriate DNA sequence by DNA sequencing. These plasmids were named pY003K1 (double mutation), pY003CSL (triple mutation), pY003V1S (quadruple mutation) as shown in Figure 3.8.

2.3.1.9 Amplification of wild-type *Pfdhfr-ts* containing *Bam*HI cloning site and ligation into pY001 plasmid

pY003 plasmid contains cloning sites *Age*I *and Afl*II for construction of mutant *Pfdhfr* libraries; however, after digestion with *Age*I, a non-specific band which may affect the construction of *Pfdhfr* libraries was found. Thus, the cloning site was changed from *Age*I to *Bam*HI by using forward primer PfFDHFRAgeIBamHI (5'-GACCGGTGGATCCATGATGGAACAAGTCTGC-3') containing *Age*I and *Bam*HI restriction sites as shown with underlined sequence, respectively, and PfRDHFRNheI (5'-CGGCTAGCATTAAGCAGCCAATCC-3') to amplify *Pfdhfr-ts*. The PCR mixture contained 1 µl of DNA template (pET-pfDHFR, 10 ng/µl), 0.5 µl each of primer PfFDHFRAgeIBamHI and PfRDHFRNheI (10µM each), 5 µl of 10X *Pfu* DNA polymerase buffer, 0.3 µl of *Pfu* DNA polymerase (3 U/µl), 0.2 µl of

GoTaq® DNA polymerase (5 U/µl), 0.5 µl of 10 mM dNTP and ddH_2O 42 µl to final volume of 50 µl. The PCR condition was performed as follows: the first cycle was set at 95°C for 3 min, then the subsequent 30 cycles were 95°C for 1 min, 58°C for 1 min and 72°C for 2 min. The 1.8 kb of *Pfdhfr-ts* PCR product was purified by QIAquick PCR Purification Kit.

After purification, the PCR product was digested with *Age*I and *Nhe*I. The digestion reaction composed of 6 µl of purified PCR product (30 ng/µl), 5 µl of 10X NEB buffer No.1, 5 µl of 1 mg/ml BSA, 1 µl of *Age*I (5 U/µl), 0.5 µl of *Nhe*I (10 U/µl), and ddH_2O to the final volume of 50 µl. After incubation at 37°C for 1 hr, the digested PCR product was purified by QIAquick PCR Purification Kit.

For plasmid backbone preparation, plasmid pY001 was digested with the restriction enzymes *Age*I and *Nhe*I to remove 1.8 kb of *Tgdhfr-ts*. The digestion reaction composed of 2 µl of purified pY001 plasmid (370 ng/µl), 5 µl of 10X NEB buffer No. 1, 5 µl of 1 mg/ml BSA, 2 µl of *Age*I (5 U/µl), 1µl of *Nhe*I (10 U/µl) and ddH_2O to the final volume of 50 µl. After incubation at 37°C for 3 hr, the digested plasmid was loaded on 0.8% agarose gel. After that, the gel was soaked in 0.05% methylene blue in 0.5X TAE buffer and allowed to stain for 5-10 min then the gel was destained with dH_2O until the DNA bands appeared. The expected band size of 5.7 kb of DNA on the gel was removed and further purified with the QIAquick Gel Extraction Kit.

The ligation reaction composed of 8 µl of purified digested pY001 backbone (4 ng/µl), 3 µl of purified digest wild-type *Pfdhfr* PCR product (9 ng/µl), 1µl of 10X T4 DNA ligase buffer, 1 µl of 50 mM ATP, 1 µl of T4 DNA ligase enzyme and ddH_2O to

the final volume of 15 µl. After incubation at 16°C for approximately 12-16 hr, the ligation product was transformed into *E. coli* DH5α by heat shock method and plated onto LB agar containing 100 µg/ml ampicillin. The colonies grown after incubation at 37°C for 12-16 hr were randomly selected, thus plasmid DNA were randomly selected and subjected to PCR reaction to screen for ligated plasmid as described in section 2.3.1.5. Only positive samples were extracted and cleaned up by QIAprep Spin Miniprep Kit. The corresponding size of plasmid digested with *Hind*III (20 U/µl) and *Afl*II (20 U/µl), *Afl*II (20 U/µl) and *Kas*I (4 U/µl), *Bam*HI (20 U/µl) and *Nhe*I (10 U/µl). Then, the gel was analysed by agarose gel-electrophoresis followed section 2.3.1.2. The 1.8 kb of wild-type *Pfdhfr-ts* insert of plasmid was sent to Biodesign, Thailand for DNA sequencing. The modified plasmid was named pY005 (Figure 3.13). The plasmid contained wild-type *Pfdhfr-ts* under control of 5' and 3'UTR *Pbdhfr-ts*. In junction region of the *Pfdhfr-ts*, there is *Afl*II restriction site which serves as cloning site for library of *Pfdhfr* random mutations.

2.3.1.10 Construction *Pfdhfr* mutant library by using wild-type or mutant *Pfdhfr* as template

Pfdhfr mutant library was generated by error prone PCR method (Chusacultanachai et al., 2002). The PCR reaction composed of 1 ng of wild-type *Pfdhfr* in pY005 plasmid or mutant *Pfdhfr* plasmid as a template, 10 µM of the forward primer FBAMHI (5'-CGGTGGATCCATGATGGAACAAG-3') and reverse primer RAflII (5'-CTTTGTCATCATTCTTAAGAGGC-3') (underlined sequences represent *Bam*HI and *Afl*II restriction sites respectively), 0.1 mM dGTP, 0.1 mM dATP, 0.5 mM dCTP, 0.5 mM dTTP, 5 µl of 1X Arnold's buffer and 1 µl of GoTaq

DNA polymerase (5 U/µl) and ddH_2O to the final volume of 50 µl. The condition of this reaction is as follows: denaturation 95°C for 1 min, annealing 50°C for 1 min, extension 72°C for 1 min for 30 cycles. The 0.7 kb of *Pfdhfr* PCR product was purified by QIAquick PCR Purification Kit.

Afterwards, the PCR product was digested with *Bam*HI and *Afl*II. The digestion reaction composed of 6 µl of purified PCR product, 5 µl of 10X NEB buffer No.1, 5 µl of 1 mg/ml BSA, 1 µl of *Bam*HI (20 U/µl), 0.5 µl of *Afl*II (10 U/µl), and ddH_2O to the final volume volume of 50 µl. After incubation at 37°C for 1 hr, the digested PCR product was purified using QIAquick PCR Purification Kit.

For plasmid backbone preparation, plasmid pY005 was digested with the restriction enzyme *Bam*HI and *Afl*II to remove 0.7 kb of wild-type *Pfdhfr*. The digestion reaction composed of appropriate volume of purified pY005 plasmid, 10X NEB buffer No.1, 10X BSA, *Bam*HI (20 U/µl), *Afl*II (20 U/µl) and ddH_2O to the final volume of 50 µl. After incubation at 37°C for 2-3 hr, the digested plasmid was run on 0.8% agarose gel. Then digested plasmid on agarose gel was soaked in 0.05% methylene blue in 0.5X TAE buffer and allow it to stain for 5-10 min then the gel was destained with dH_2O until the DNA band appeared. The expected band size of 6.8 kb of DNA on the gel was removed and further purified using QIAquick Gel Extraction Kit.

The ligation reaction consisted of *Bam*HI/*Afl*II purified digested pY005 backbone, purified digested mutant library of *Pfdhfr* PCR product, 1 µl of 10X T4 DNA ligase buffer, 1 µl of 50 mM ATP, 1 µl of T4 DNA ligase enzyme and ddH_2O to final volume of 10-15 µl. After incubation at 16°C for approximately 12-16 hr, the

ligation product was transformed into *E. coli* DH5α by $CaCl_2$ heat shock method and plated onto LB agar containing 100 μg/ml ampicillin. The colonies that grew after incubation at 37°C for 12-16 hr were randomly selected and subjected to PCR reaction to screen for ligated plasmid as described in section 2.3.1.5. Only positive samples were extracted and cleaned up by QIAprep Spin Miniprep Kit. The corresponding size of plasmid was analysed by agarose gel-electrophoresis followed section 2.3.1.2. The variant clones were verified DNA sequence by DNA sequencing.

2.3.2 Experimental animals

For all experiments, female BALB/c mice (National Laboratory Animal Center, Mahidol University) age between 4-6 weeks, weight 20-25 g were used for *P. berghei* parasite infection. These mice were maintained in a clean animal room with the controlled conditions of 12 hr day/12 hr night cycle at 25°C and 40-70% humidity. All animal experiments were approved by a local ethics committee following national guidelines on the use of experimental animals.

2.3.3 Parasite manipulation

2.3.3.1 Parasite strain

The transgenic *P. berghei* parasite line MRA-867 expressing green fluorescent protein without drug-resistant selectable marker (PbGFP) was used in this study (Janse et al., 2006b) and was provided by Drs. Andrew Waters and Chris Janse of Leiden University Medical Centre, Leiden, the Netherlands. PbGFP parasite had been modified from the reference clone (clone 15cy1; wild-type parasite) of the ANKA strain of *P. berghei* to contain GFP. The *gfp* gene was introduced into *c*-or *d-rrna* gene on chromosome 5 via single-crossover recombination, and was used as

fluorescence marker under fluorescence microscope. In addition, GFP was used to detect the parasite by flow cytometry.

2.3.3.2 Infection of experimental animal with blood stage parasites

The *P. berghei* parasite was maintained and propagated *in vivo* by inoculating with infected erythrocytes (blood stage) via intraperitoneal (i.p.) or intravenous (i.v.) injection. Blood stage parasites were either obtained from cryo-preserved stock or directly from heart- or tail-blood of infected animals.

2.3.3.3 Giemsa stained blood film

The course of parasitemia (= percentage of infected cells) is determined in Giemsa stained blood film made from tail blood. The thin blood film was made on a microscope slides, air-dried, fixed with methanol, and stained with a fresh Giemsa working solution for 10 min. After washing with tap water and air dried, the slide was observed under a light microscope to determine the parasitemia.

2.3.3.4 Cryopreservation of blood stage parasites

The blood containing *P. berghei* parasite from infected mice was collected by cardiac puncture into cryotube with 30% glycerol/PBS solution and stored in liquid nitrogen tank.

2.3.4 *Plasmodium berghei* transfection (Janse et al., 2006c)

2.3.4.1 DNA preparation for transfection

2.3.4.1.1 Preparation of DNA for episomal plasmid transfection

A) pY003K1 plasmid

The plasmid containing *Pfdhfr* double mutant (C59R+S108N), (pY003K1 plasmid) was inoculated into 5 ml of LB broth containing 100 µg/ml ampicillin. The

Plasmid was purified from bacterial culture using the QIAprep Spin Miniprep Kit according to manufacturer's protocol. The plasmid was precipitated by 1 ml of cold isopropanol then incubated in -80°C for 2 hr. Thereafter, the reactions mix was centrifuged at 15,000 × *g* at 4°C for 30 min, then the supernatant was removed and washed with 500 µl of 70% ethanol. After centrifugation, DNA pellet was re-suspended with 10 µl of TE buffer.

B) Mixture of pY005, pY003K1, pY003CSL, pY003V1S plasmid

Each of the plasmids pY005, pY003K1, pY003CSL, pY003V1S was inoculated into 5 ml of LB broth containing 100 µg/ml ampicillin. The Plasmid was purified from bacterial culture using the QIAprep Spin Miniprep Kit according to manufacturer's protocol. Equal amount (500 ng/µl) of each plasmid was mixed together to a final volume of 20 µl for transfection.

C) Plasmid containing mutant libraries of *Pfdhfr*

The transformants of *Pfdhfr* mutation libraries were used to inoculate 100 ml of LB broth containing 100 µg/ml ampicillin. The culture was grown at 37°C with shaking for 12-16 hr. All plasmids were extracted by QIAprep Spin Miniprep Kit, precipitated by isopropanol then incubated in -80°C for 2 hr. Thereafter, the reactions mix was centrifuged at 15,000 × *g* at 4°C for 30 min, then the supernatant was removed and washed with 500 µl of 70% ethanol. After centrifugation, DNA pellet was re-suspended with 10 µl of TE buffer.

2.3.4.1.2 Preparation of linearized DNA for transfection

A single colony of plasmid containing *Pfdhfr* mutant from the library was inoculated into 5 ml of LB broth containing 100 µg/ml ampicillin. The culture was

grown at 37°C with shaking for 12-16 hr. The plasmids were extracted by Plasmid extraction Mini kit. The extracted plasmids were linearized with restriction enzyme. The reaction contained the appropriate concentration and volume of mutant plasmid, 1 mg/ml BSA, *Hind*III (20 U/µl), *Kas*I (4 U/µl) enzyme and ddH_2O to the final volume of 120-150 µl. The reaction was incubated at 37°C overnight. Thereafter, the linearized plasmid was run on a 0.6% agarose gel, 100 V for 50 min. The digested plasmid on agarose gel was then soaked in 0.05% methylene blue in 0.5X TAE buffer and allowed to stain for 5-10 min, and the gel was destained with dH_2O until the DNA band appeared. The expected size of 5.2 kb of DNA on the gel was excised and further purified using MinElute Gel Extraction Kit. The linearized mutated *Pfdhfr* plasmid was introduced into *P. berghei* parasites. The 5' and 3'UTR positions served as the homologous recombination sites for replacement of the endogenous *P. berghei dhfr-ts* on chromosome 7 with *P. falciparum dhfr-ts* (Figure 3.28).

2.3.4.2 Blood stage *P. berghei* parasite culture

Normally, *P. berghei* parasites are maintained *in vitro* for only one developmental cycle. Ring forms or young trophozoites are allowed to develop into mature schizonts during a period of 16-23 hr. The schizonts of *P. berghei* parasites containing merozoites can survive for several hours and can be manipulated without bursting or loss of viability. Infected mouse blood with a parasitemia of 10-15% were collected by cardiac puncture under CO_2-anaesthesia in 10 ml of complete RPMI1640 medium (containing 20% FBS), neomycin (10 mg/ml). Erythrocytes were harvested by centrifugation at 1,600 × *g* for 5 min at room temperature and complete RPMI1640 medium containing was used to re-suspend the cell pellet. Then this suspension was

transferred into a culture flask. The flask was flushed with gas mixture (5%CO_2, 5%O_2 and 90%N_2). After gassing, the flask was closed tightly and put in the orbital incubator shaker at 36.5°C for 16 hr. The shaking was adjusted to a speed enough to keep the cells in suspension.

2.3.4.3 Mature schizont purification

One millilitre of overnight *P. berghei* parasite culture was collected in microcentrifuge tube and spun for 5 sec before the morphology of parasites was observed by Giemsa stained smear. The appropriate viable mature schizont containing 12-16 free merozoites ranged from 70-80% of parasites. The cells from the culture were harvested and centrifuge at 1,600 × *g* for 5 min at 25°C. Sixty percent Nycodenz solution in PBS was freshly prepared and the parasite suspension was overlay with the Nycodenz solution. The cell suspensions was centrifuged at 450 × *g* without brake for 30 min at 25ºC. Then the brown layer containing schizonts at the interface between the two suspensions was collected into a new 50 ml tube. The collected cells were made up with complete RPMI1640 medium to 40 ml. The schizonts were harvested by centrifugation at 450 × *g* for 8 min and then gently re-suspended with 1 ml of complete RPMI1640 medium for one transfection.

2.3.4.4. Electroporation of healthy schizonts by Amaxa device

Schizont pellet suspension was harvested by centrifugation at maximum speed for 5 sec. For each transfection, 100 µl of the human T-cell Nucleofector solution and 1-5 µg of circular or linear DNA were used to re-suspend the schizont pellet. The electroporation was performed using Amaxa® device with program U-33. Two hundred microlitres of complete RPMI1640 medium was added immediately to the

electroporated cells, then complete transfection solution was injected into the tail vein of a mouse that has been placed under the heat lamp for 30 min to warm and make tail vein swollen and visible for easy injection.

2.3.5 *In vivo* antimalarial drug testing

The efficacy dose of pyrimethamine to inhibit wild-type PbGFP parasite and transfected resistant parasites was determined by *P. berghei* rodent malaria 4-day suppressive test (Peters, 1975). This protocol determines the efficacy of four daily doses of candidate antimalarial compounds, using the rodent parasite *P. berghei*. The percentage parasitemia was determined by counting infected erythrocytes on Giemsa stained smear or by flow cytometric analysis. The difference between the mean value of the control group (taken as 100%) and those of the experimental groups was calculated and expressed as percent inhibition using the following equation.

$$\% \text{ inhibition} = 100-\{[\text{mean parasitemia treated}/\text{mean parasitemia}] \times 100\}$$

2.3.5.1 Determination of pyrimethamine sensitivity of PbGFP parasites

The efficacy of pyrimethamine to inhibit PbGFP parasite was determined by 4-day suppressive test. Groups of five BALB/c mice were infected by i.p. injection with 1×10^7 parasitized erythrocytes. Four hours after infection, the experimental groups were treated with pyrimethamine at different concentration (0.0015, 0.015, 0.05, 0.15, 0.25, 0.5 mg/kg) by i.p. injection. The control group was treated with 5%DMSO in PBS pH 4.0. The treatment was repeated with the same dose and same route at 24, 48, 72 hours post-infection. Twenty-four hours after the last treatment,

the percentages of parasitemia were determined by counting of infected erythrocytes on Giemsa stained smear. Experiments were carried out three times on this group of study and results were recorded as mean values.

2.3.5.2 Determination of pyrimethamine sensitivity of transgenic (*PbPf*K1) parasite (episomal form)

Groups of five BALB/c mice were infected by i.p. with 1×10^7 of *PbPf*K1 parasites. After 4 hr, the treatment groups were treated with pyrimethamine at different concentration (1.5, 5, 15, 30 mg/kg). Unfortunately, the 30 mg/kg pyrimethamine treated group died soon after drug injection as a result of the toxicity of pyrimethamine at high level to the mice. The concentration of pyrimethamine was reduced to a regimen of (20, 12, 6 and 3 mg/kg) for all the experiments in these groups and drug was administered at 24, 48, 72 hr post-infection. The control group was treated with 5%DMSO in PBS pH 4.0. Twenty-four hours after the last treatment, the percentages of parasitemia were determined by counting of infected erythrocytes on Giemsa stained smear.

2.3.5.3 Determination of pyrimethamine sensitivity of transgenic *P. berghei* expressing *Pfdhfr* mutant.

Groups of four BALB/c mice were infected by i.p. with 1×10^7 parasitized erythrocytes. After infection for four hours, the experimental groups were treated with pyrimethamine at different concentration (0.3, 1, 3, 9, 18 mg/kg) by i.p. injection. The control group was treated with 5%DMSO in PBS pH 4.0. The treatment was repeated with the same dose and same route of administration at 24, 48, 72 hours post-infection. Twenty-four hours after the last treatment, the percentages of

parasitemia were determined by counting of infected erythrocytes on Giemsa stained smear. Experiments were carried out three times on this group of study and results were recorded as mean values.

2.3.6 Selection and identification of resistant *Pfdhfr* in transfected *P. berghei* parasites

The efficacy dose of pyrimethamine was previously established for the selection of resistant parasite by the 4-day suppressive test. Twenty-four hours after transfection, the ED_{95} of PbGFP parasite, 0.25 mg/kg pyrimethamine was used in treating the mice infected with transgenic parasites expressing *Pfdhfr* mutant by i.p. injection daily. Pyrimethamine-resistant parasites were obtained after 7-10 days, the genomic DNA was extracted from whole blood using Genomic DNA Mini Kit and then transformed to *E. coli* DH5α. The selected *Pfdhfr* mutant DNA sequences were verified by DNA sequencing.

2.3.7 Cloning of transgenic *P. berghei* parasites

The transgenic parasite clone was obtained by limiting dilution method. Percentage of parasitemia was examined by Giemsa stained blood film. The number of erythrocytes was calculated with hemocytometer. The dilution calculation was done such that 1-2 parasites were delivered per animal in 200 µl by i.v. injection into a tail vein of 10 mice. The successful cloning experiment showed less than 50% of the mice were positively infected.

2.3.8 Genetic analysis of transgenic *P. berghei* parasite

The correct integration of *Pfdhfr-ts* mutants replacing endogenous *Pbdhfr-ts* were analyzed by PCR and Southern blot analysis.

2.3.8.1 PCR analysis of transgenic *P. berghei* parasite

Transgenic *P. berghei* with a parasitemia of 10-15% was collected from an infected mouse by cardiac puncture under CO_2-anaesthesia. After that, the parasite genomic DNA was extracted from parasite pellet by using Genomic DNA Mini Kit.

2.3.8.1.1 PCR analysis for checking 5' and 3'UTR integration

The correct integrations at 5' and 3'UTR were verified by PCR from genomic DNA of transgenic *P. berghei* parasite. The DNA fragment of 4.0 kb between endogenous 5'UTR *Pbdhfr-ts* gene and *Pfdhfr* in the inserted vector was detected using the forward primer 5'PbINTF (5'-TTGAGCTACATAACTTCCATACAT-3') and reverse primer RAflII (5'-CTTTGTCATCATTCTTAAGAGGC-3'). The DNA fragment of 3.0 kb between *Pfdhfr-ts* in the inserted vector and endogenous 3'UTR *Pbdhfr-ts* was detected using the forward primer FBAMHI (5'-CGGTGGATCCATGATGGAACAAG-3') and 3'PbNTR (5'-CGATCTACACCTCTTCAT-3'). The reactions contained 0.5 µl of dNTP, 5 µl of 1 mg/ml BSA, 5 µl of 10X *Pfu* buffer, 0.1 µl *Pfu* polymerase (3 U/µl), 0.4 µl GoTaq® DNA polymerase (5 U/µl), and ddH_2O to the final volume of 50 µl. PCR was performed with 1 cycle of 95°C for 2 min, followed by 30 cycles of 95°C or 45 sec, 45ºC for 45 sec and 62ºC for 4 min. The PCR products were run on 0.8% agarose gel. The band of DNA in the gel was visualized under UV transluminator and the sizes of the DNA fragments were extrapolated based on the DNA standard markers.

2.3.8.1.2 PCR analysis for checking *Pbdhfr-ts* gene

Since *Pbdhfr-ts* was replaced by *Pfdhfr-ts* via double homologous event, the *Pbdhfr-ts* should be absence in transgenic parasite. However, a mixture between

episomal form and integrated form of *Pfdhfr-ts* expression cassette in transgenic parasite was found. Then PCR amplification of *Pbdhfr-ts* was used to verify the correct integration of gene. The PCR reaction contained 5 µl of template genomic DNA from transgenic parasite, 0.5 µl of forward primer PbDTF (5'-GGGGGGGGCATATGGAAGACTTATCTGAAACATTCG-3') (10 mM), 0.5 µl of reverse primer PbDTR (5'-GGACTAGTTTAAGCTGCCATATCC-3') (10 mM) 0.5 µl of dNTP, 5 µl of 1 mg/ml BSA, 5 µl of 10X *Pfu* buffer, 0.1 µl *Pfu* polymerase, 0.4 µl Go Taq® DNA polymerase, and adjusted volume to 50 µl with ddH_2O and PCR was performed with 1 cycle of 95°C for 3 min, followed by 30 cycles of 95°C for 45 sec, 45°C for 45 sec and 62°C for 2 min. The PCR products were run on 0.8% agarose gel. Thereafter, the gel was stained with ethidium bromide and visualized under UV transluminator. The absence of *Pbdhfr-ts* in PCR product showed non-episomal form in the transgenic parasite.

2.3.8.1.3 PCR analysis for checking *Pfdhfr* gene

The presence of *Pfdhfr* in transgenic mutant *P. berghei* parasites were verified by PCR from 5 µl genomic DNA template, using 0.5 µl of forward primer FBamHI (5'-CGGTGGATCCATGATGGAACAAG-3') (10mM), 0.5 µl of reverse primer RAflII (5'-CTTTGTCATCATTCTTAAGAGGC-3') (10 mM), 0.5 µl of dNTP, 5 µl of 1mg/ml BSA, 5 µl of 10X *Pfu* buffer, 0.1 µl *Pfu* polymerase (3 U/µl), 0.4 µl GoTaq® DNA polymerase (5 U/µl), and ddH_2O to the final volume of 50 µl. The PCR condition is the same as described in section 2.3.1.10. The PCR products were run on 0.8% agarose gel 100 V for 40 min. The corresponding size was 0.7 kb on the gel after staining with ethidium bromide and visualized under UV transluminator.

2.3.8.2 Southern analysis of transgenic *P. berghei* parasite

The correct integration of the mutant *Pfdhfr* in transgenic *P. berghei* parasites was further confirmed by Southern analysis.

2.3.8.2.1 Leukocyte removal from parasitized red blood cells (Janse et al., 1994)

The transgenic *P. berghei* with a parasitemia of 10-15% was collected from infected mice by cardiac puncture under CO_2-anaesthesia. The red cell pellet was collected by centrifugation at 10,000 × *g* for 5 min and re-suspended with 1 ml of PBS. Leukocytes were removed by passing the blood suspension through CF11 column and the flow through containing infected RBC was collected into the new 15-ml tube and centrifuged at 1500 × *g* for 10 min. The pellet was resuspended with 300 µl of PBS before 500 µl of 0.2% saponin was added to the suspension, and incubated on ice for 5 min. Thereafter, the suspension was centrifuged at 10,000 × *g* for 5 min at room temperature. The pellet was re-suspended with RBC lysis solution. The parasite genomic DNA was extracted from parasite pellet by using Genomic DNA Mini Kit.

2.3.8.2.2 Genomic DNA restriction digestion and Southern probe hybridization

Genomic DNA of transgenic mutant parasites was digested with *Eco*RI restriction enzyme. The episomal plasmid contained *Eco*RI site in *Pfdhfr* gene whereas the integrated parasite contained *Eco*RI sites in both endogenous gene of 5'UTR *Pbdhfr-ts* and *Pfdhfr* gene. *Eco*RI digestion showed different sizes of DNA fragments between integrated and episomal transgenic parasite (Figure 3.32). The

digestion reaction composed of 50 µg of genomic DNA, 10 µl of 10X NEB buffer No.1, 10 µl of 1 mg/ml BSA, 3 µl of *Eco*RI (20 U/µl) and ddH_2O to the final volume of 100 µl. After incubation at 37°C 12-16 hr, digested genomic DNA was concentrated by added 10 µl of 3M NaOAc pH8.0 and 300 µl of cold isopropanol then incubated in -80°C for 2 hr. Thereafter, the reaction mix was centrifuged at 15,000 × *g* at 4°C for 30 min then the supernatant was removed and washed with 500 µl of 70% ethanol. After centrifugation, DNA pellet was re-suspended with 20 µl of TE buffer. The DNA was loaded on 0.6% agarose gel, run at 50 V for 60 min and then 100 V for 30 min. The DNA in the gel was soaked in ethidium bromide for 10 min then the UV picture of gel with a ruler next to it was taken. The DNA was depurinated in 0.125 M HCl for 20 min, denatured in 0.5 M NaOH/1.5 M NaCl for 30 min and neutralized in 0.5 M Tris/1.5 M NaCl pH 8.0 for 30 min. The DNA was transferred overnight from the gel to a nitrocellulose membrane by capillary action.

Thereafter, the membrane was rinsed in ddH_20 and placed on Whatman paper soaked with 2X SSC. The DNA on wet membrane was covalently fixed to the membrane by UV-crosslinking (700x100 µJ/cm^2 on UV cross-linker, Biorad) before the membrane was put into a hybridization bottle with prehybridization solution and incubated in 37°C for 30 min. After heating the *Pfdhfr* probe at 95°C for 10 min, and placed on ice for 2 min, the probe was put into the bottle and hybridize the DNA on the membrane by incubation at 37°C overnight.

2.3.8.2.3 Preparation of Digoxigenin-labelled hybridization probe

Digoxigenin-11-dUTP labeled *Pfdhfr* probe was prepared following manufacturer's protocol (DIG High Prime DNA Labeling and Detection Starter Kit II,

Roche Applied Science). DIG-labeled DNA probes are generated with DIG-High Prime according to the random primed labeling technique and all reagents necessary for random primed labeling, including Klenow enzyme, premixed in an optimized 5X reaction buffer concentrate in 50% glycerol. The 0.7 kb of wild-type *Pfdhfr* was amplified by PCR using 1 µl of pETpfTM4 (containing wild-type *Pfdhfr*, 10 ng/µl) as a template. The PCR reaction contained 10 µM each of the forward primer PfDTF_ageI (5'-GACCGGTATGATGGAACAAGTCTGC-3') and reverse primer RAflII (5'-CTTTGTCATCATTCTTAAGAGGC-3'), 5 µl of 10X *Pfu* buffer, 0.3 µl of *Pfu* DNA polymerase (3 U/µl), 0.2 µl of GoTaq® DNA polymerase (5 U/µl), 0.5 µl of 10 mM dNTP, ddH_2O 42 µl to final volume of 50 µl. The PCR condition was set as described in section 2.3.1.10. The template of PCR reaction was removed by adding 1 µl of *Dpn*I (20 U/µl) and incubating at 37°C for 1 hr. After purification, *Pfdhfr* PCR product served as a template for DIG-labeled *Pfdhfr* probe preparation. Distilled water was added to 1 µg of DNA template to a final volume of 16 µl in a reaction vial. The reaction was heated in a boiling water bath for 10 min and quickly placed on ice for 5 min. Four microliters of Mix DIG-High Prime (vial 1) was added to the denatured DNA, mixed and incubated overnight at 37°C. Thereafter, the reaction was stopped by heating at 65°C for 10 min. DIG labeled were used for hybridization to membrane blotted DNA. The hybridized probes were immunodetected with anti-Digoxigenin-AP, and are then visualized with the chemiluminescence substrate CSPD. Enzymatic dephosphorylation of CSPD by alkaline phosphatase leads to a light emission at a maximum wavelength of 477 nm on X-ray films.

2.3.8.2.4 Digoxigenin chemiluminescent detection using DIG Kit

After washing the membrane with 2X SSC at room temperature for 5 min with shaking twice, the membrane was equilibrated in 15 ml washing buffer for 1 min. Then the membrane was incubated in 100 ml of 1% blocking solution provided by the kit and 18 ml maleic acid buffer at room temperature for 30 min. The anti-Digoxigenin-alkaline phosphatase conjugated antibody was centrifuged at 10,000 × *g* for 5 min, diluted 1:10,000 in 1% blocking solution (2 µl in 20 ml) before the membrane was incubated in it, at room temperature for 15 min. The membrane was washed in 100 ml washing buffer at room temperature twice before it was equilibrated in 20 ml of detection buffer for 2 min. The excess liquid on the membrane were removed by dripping one corner onto a tissue paper. Then the membrane was transferred into a hybridization bag before two millilitre of CSPD solution were added onto membrane. The excess liquid was squeezed out and the air-bubbles were removed. The bag was sealed and put in exposure cassette and placed at 37°C for 10 min. The membrane was exposed to x-ray film for up to 30 min. The expected bands were visualized after washing with developer solution, water and fixer solution, respectively.

2.3.9 *Pf*DHFR-TS expression profile by transgenic mutant parasite

The transgenic parasites express *Pf*DHFR-TS under control of 5' and 3'UTR *Pbdhfr-ts.* To clarify whether this promoter efficiently promoted *Pfdhfr-ts* mRNA expression, the synthesis of cDNA from mRNA of *Pfdhfr* by reverse transcriptase PCR (RT-PCR) was carried out. Traditionally RT-PCR involves two steps: the RT reaction and PCR amplification.

2.3.9.1 Total RNA isolation

The transgenic *P. berghei* with a parasitemia of 10-15% was collected from infected mice by cardiac puncture under CO_2-anaesthesia. Leucocytes were removed as described in section 2.3.8.2.1. Total RNA of each transgenic parasite was isolated from parasite pellet by re-suspending with 1 ml of Trizol® reagent (Invitrogen) and incubating at room temperature for 10 min. For purification step, the samples were centrifuged at 15,000 × *g* for 3 min at room temperature, and supernatant was transferred into a new RNase-free micro-centrifuge tube. Two hundred microliters of chloroform was added, mixed well and incubated at room temperature for 10 min. To separate the phase, the samples were centrifuged at 4°C, 15,000 × *g* for 10 min. The upper phase was transferred into a new RNase-free tube. The RNA was precipitated by adding 500 µl of isopropanol and incubating at -20°C overnight. After that, the samples were centrifuged at 4°C, 15,000 × *g* for 15 min. The RNA pellet was washed with 70% ethanol and centrifuged for 10 min, 15,000 × *g* at 4°C and re-suspended with 30-50 µl of RNase-free water.

2.3.9.2 cDNA preparation

To further purify total RNA, DNA contamination was removed using DNaseI. The reaction contained as follows: 20 µl of total RNA (1 µg/µl), 10 µl of GoTaq® buffer, 2 µl of DNase I and adjusted the volume to 50 µl by RNase-free water. After incubation at 37°C for 1 hr, the RNA samples were purified by phenol:chloroform (1:1 v/v), then re-precipitated by cold isopropanol (1:1 v/v) at -80°C for 30 min and washed with 70% ethanol. After centrifugation at 15,000 × *g* for 30 min, the RNA pellet was re-suspended with 20 µl of RNase-free water.

The cDNA of each transgenic mutant parasite was synthesized by RT-PCR using specific primers as following information. PfRDHFRNheI primer (5'-CGGCTAGCATTAAGCAGCCAATCC-3') was used to create *Pfdhfr-ts* cDNA. The cDNA of *Pbdhfr-ts* was used as negative control and created by PbDTR primer (5'-GGACTAGTTTAAGCTGCCATATCC-3'). For the positive control, *P. berghei* alpha tubulin housekeeping gene that expresses all parasite life cycle was used. The cDNA were synthesized by Pb_a_tubulin primer (5'-GCTGGTTCAAATGCTGAGTTTG-3'). The reaction of master mix 1 for cDNA construction composed of 1 µg of purified total RNA, 2 µl of each specific primer (10 mM) and adjusted volume to 25 µl. The master mix 1 reaction was heated at 70°C for 10 min to denature the RNA, afterwhich the mix was placed on ice for 5 min. The reaction mix was divided into two equal parts of 12 µl. The 12 µl mix served as template with primers ready for RT-PCR reaction. The RT-PCR reactions composed of the template and primer from master mix 1 with and without the reverse transcriptase enzyme as shown below in Table 2.3 and Table 2.4, respectively.

Table 2.3 RT-PCR reactions composed of the template and primer from master mix 1 with reverse transcriptase enzyme (+RT)

Component	**Per Reaction**
Template (master mix 1)	12 µl
25 mM $MgCl_2$	3.6 µl
5X RT buffer	6 µl
RNase inhibitor	0.5 µl
10 mM dNTP	1.5 µl
In Prom II (reverse transcriptase)	1 µl
dH_2O	5.4 µl
Total volume	30 µl

Table 2.4 The RT-PCR reactions composed of the template and primer from master mix 1 without reverse transcriptase enzyme (-RT)

Component	**Per Reaction**
Template (master mix 1)	12 µl
25 mM $MgCl_2$	3.6 µl
5X RT buffer	6 µl
RNase inhibitor	0.5 µl
10 mM dNTP	1.5 µl
dH_2O	6.4 µl
Total volume	30 µl

The reactions were incubated at 37°C overnight. Thereafter, the samples were heated at 70°C for 15 min to inactivate reverse transcriptase enzyme. The newly synthesized cDNA was placed on ice and ready for RT- PCR amplification. Alternatively, cDNA was stored at -20°C until used.

2.3.9.3 PCR amplification of cDNA

Synthesized cDNA (diluted 1:10) was used as template for amplification with primer specific for *Pfdhfr* [*Pfdhfr-ts*cDNA using FBamHI (5'-CGGTGGATCCATGATGGAACAAG-3') and RAflII (5'-CTTTGTCATCATTCTTAAGAGGC-3')], *Pbdhfr* [*Pbdhfr-ts*cDNA using PbDTF (5'-GGGGGGGGCATATGGAAGACTTATCTGAAACATTCG-3') and PbDR speI (5'-GGACTAGTGTACTTCCTCATTTGG-3')] and *P. berghei* alpha tubulin gene [PbalphatubulinecDNA using Pb_a_tubulinF (5'-GCATGCTGGGAGCTATTTTG-3') and Pb_a_tubulinR (5'-GCTGGTTCAAATGCTGAGTTTG-3'). The PCR master mix reaction contained 5 µl of cDNA template, 0.5 µl of forward primer (10 mM), 0.5 µl of reverse primer (10mM), 0.5 µl of 10 mM dNTP, 5 µl of *Pfu* buffer, 5 µl of 1 mg/ml BSA, 0.1 µl of *Pfu* polymerase (3 U/µl), 0.4 µl of GoTaq® DNA polymerase (5 U/µl) and ddH_2O to the final volume of 50 µl. The conditions of each PCR reaction are as shown below.

A) For *Pfdhfr*

Initial denaturation 95°C for 3 min

Cycling

Denaturation	95°C for 3 min	30 cycles
Annealing	50°C for 1 min	
Extension	72°C for 1 min	

Final extension 72°C for 5 min

B) **For *Pbdhfr***

Initial denaturation 95°C for 3 min

Cycling

Denaturation 95°C for 3 min
Annealing 50°C for 45 sec
Extension 62°C for 1 min
} 30 cycles

Final extension 62°C for 5 min

C) For *P. berghei* alpha tubulin

Initial denaturation 95°C for 3 min

Cycling

Denaturation 95°C for 30 sec
Annealing 52°C for 30 sec
Extension 60°C for 1 min 30 sec
} 30 cycles

Final extension 60°C for 5 min

The PCR products were run on 0.8% on agarose gel, 100 V for 40 min. The corresponding sizes were shown on the gel after staining with ethidium bromide and visualized under UV transluminator.

2.4 Data analysis

The non-linear regression for sigmoidal dose-inhibition (variable slope) was used to calculate the 50%, 90% and 95% effective dose (ED_{50}, ED_{90} and ED_{95}) values. Unpaired *t*-test was used to compare the mean ED_{50} value. All the data were subjected to statistical analysis using the SigmaPlot software.