CHAPTER 3

RESULTS

3.1 Construction of *P. berghei* transfection plasmid

3.1.1 Construction of pY001 plasmid

Plasmid for *P. berghei* transfection was constructed by modification from the original plasmid pL0017 (Figure 2.1) which is 11 kb in size. To reduce the plasmid size for further use in construction of *Pfdhfr* libraries, 0.5 kb of 3'UTR *Pbdhfr-ts*, GFP expression cassette and *dssurna* sequence were removed from pL0017 by digestion with restriction enzyme *Nhe*I and *Kas*I. The digested pL0017 which is now 6.6 kb in size served as backbone for the construction of pY001 plasmid (Figure 3.1, lane 1). The 1.0 kb 3'UTR of *Pbdhfr-ts* was amplified by PCR using pL0002 plasmid as template. The 1.0 kb 3'UTR *Pbdhfr-ts* PCR product was digested with restriction enzymes *Nhe*I and *Kas*I. The digested product served as insert for the ligation reaction pY001 (Figure 3.1, lane 3). The ligation product was transformed into *E. coli* PMC 103 competent cells by electroporation and the transformed cells were plated on selective LB agar containing 100 µg/ml ampicillin. After overnight incubation at 37 °C, approximately 15 colonies were obtained. To verify the positive clone containing inserted 1.0 kb 3'UTR *Pbdhfr-ts* sequence colonies were selected for colony PCR amplification. Three positive clones were selected for plasmid purification and further confirmed by digestion with various restriction enzymes. When digested with *Kas*I and *Nhe*I, the expected band size should be approximately 6.6 kb and 1.0 kb and when double digested with *Hind*III and *Kas*I the expected band size should be

approximately 5.1 kb and 2.5 kb. When the plasmid was single digested with *Xho*I, the expected band size should be about 7.6 kb. The correct patterns of digestion are shown in clone II and clone III (Figure 3.2 lanes 7-9 and lanes 10-12, respectively). Clone III was selected for further experiment. The inserted 1.0 kb 3'UTR *Pbdhfr-ts* sequence was verified to be the correct sequence by DNA sequencing. The corresponding size of the newly constructed plasmid was 7.6 kb and it was named pY001 as shown in Figure 3.3. The pY001 plasmid construction and the plasmid map are shown in Figure 3.3**.** The plasmid pY001 contains only expression cassette of *Toxoplasma gondii* dihydrofolate reductase thymidylate synthase (*Tgdhfr-ts*) under control of 2.3 kb of 5' and 1.0 kb of 3'UTR of *Pbdhfr-ts.*

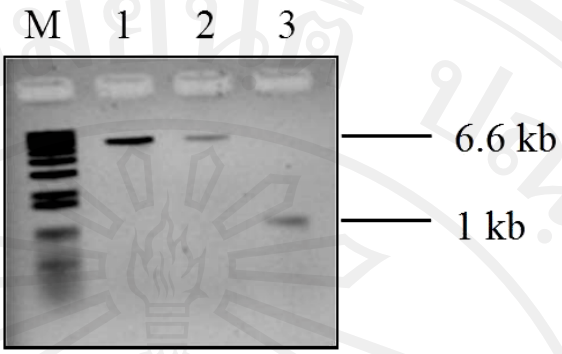


Figure 3.1 Agarose gel-electrophoresis of pL0017 plasmid backbone (lanes 1, 2) and 3'UTR 1.0 kb PCR product, both digested with *Nhe*I and *Kas*I (lane 3). The 1 kb plus molecular marker is shown in lane M.

[Original plasmid map pL0017 is shown in Figure 2.1 and pY001 plasmid construct map is shown in Figure 3.3]

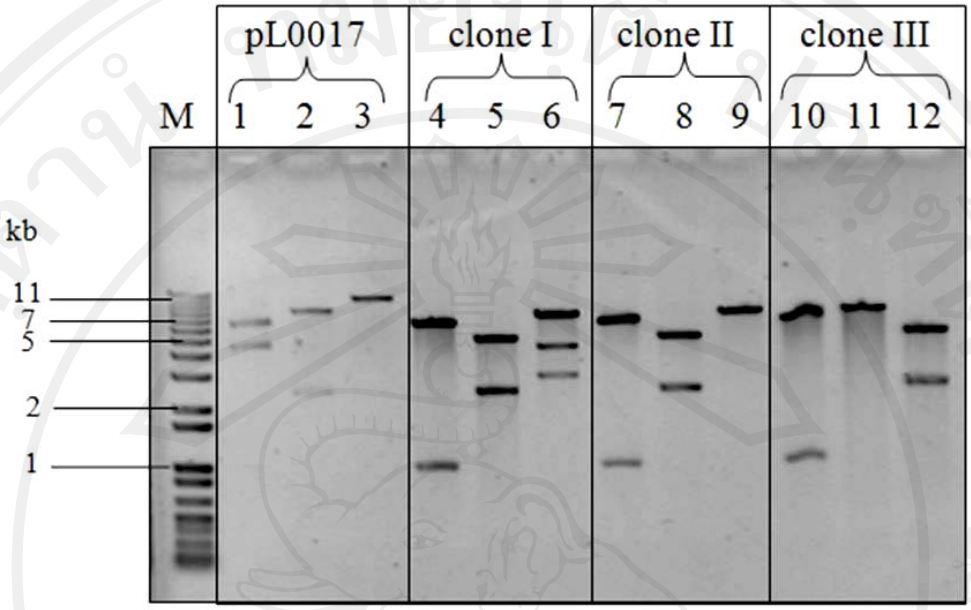
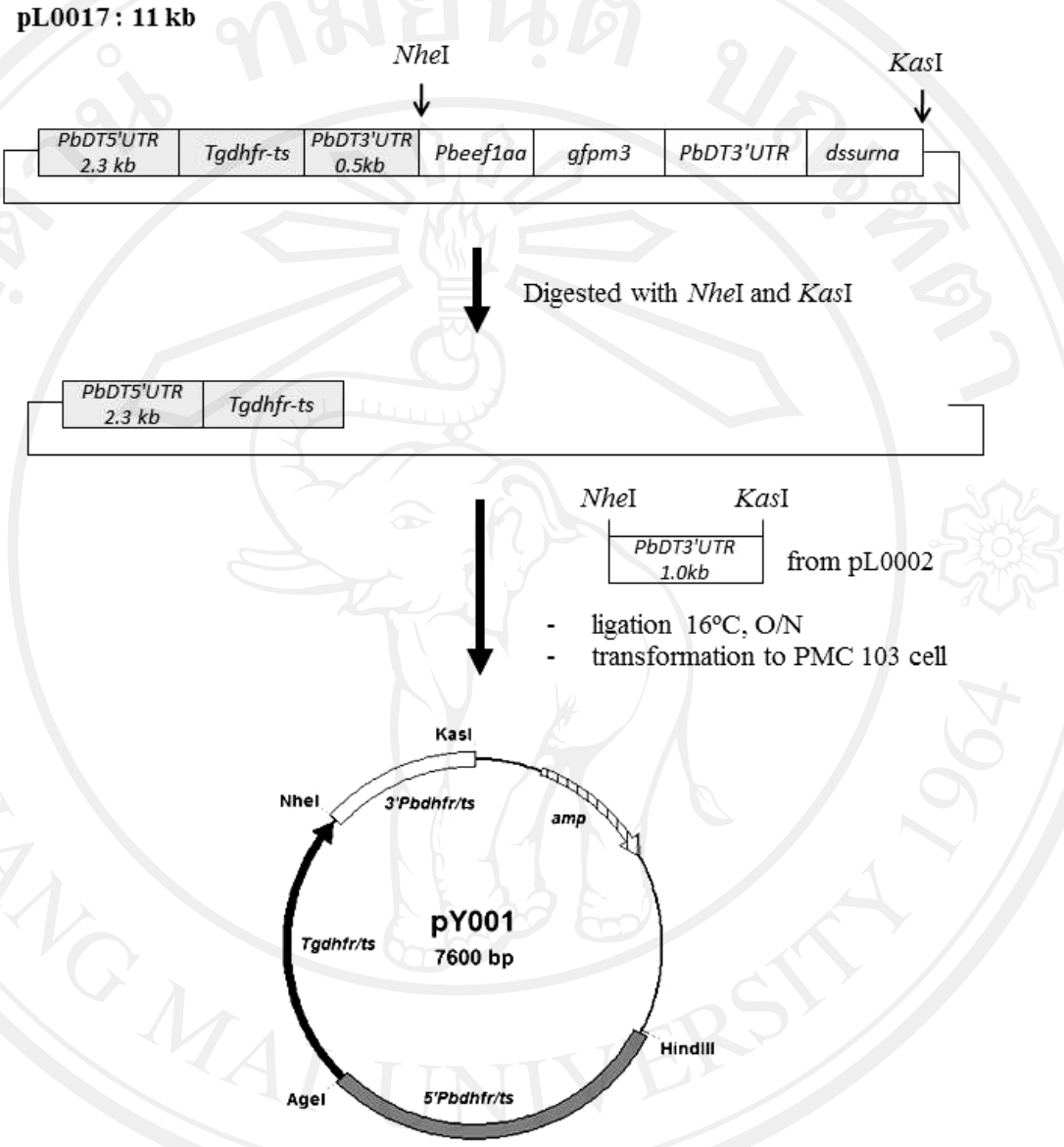


Figure 3.2 Restriction analysis of the selected 3 recombinant clones and pL0017 plasmid control. Lanes 1, 4, 7, 10, digested with *Kas*I and *Nhe*I (expected band size = 6.6 kb and 1.0 kb). Lanes 2, 5, 8, 12, digested with *Hind*III and *Kas*I (expected band size = 5.1 kb and 2.5 kb). Lanes 3, 6, 9, 11, digested with *Xho*I, (expected band size = 7.6 kb). The 1 kb plus molecular marker is shown in lane M.



Circular map of pY001

Figure 3.3 Construction of pY001 plasmid.

3.1.2 Construction of pY003 plasmid

In order to construct plasmid containing *Pfdhfr-ts* under control of 5' and 3'UTR of *Pbdhfr-ts*, plasmid pY001 was further modified by digestion with *Age*I and *Nhe*I to remove *Tgdhfr-ts* sequence. The remaining 5.8 kb fragment (Figure 3.4, lane 1), served as backbone for ligation reaction of new plasmid construction. The sequence of *Pfdhfr-ts* was PCR amplified using pET-PfDHFR plasmid as template. The *Pfdhfr-ts* PCR product was digested with *Age*I and *Nhe*I enzymes and the digested product served as insert in the ligation reaction (Figure 3.4, lane 3). The ligation product was transformed into *E. coli* DH5α competent cell by heat shock method and the transformed cells were plated on selective LB agar containing 100 µg/ml ampicillin. After overnight incubation at 37°C, approximately 210 colonies were obtained. To verify the positive clone, containing *Pfdhfr-ts* gene colonies were selected for colony PCR amplification. Two positive clones were selected for plasmid purification and further confirmed by digestion with various restriction enzymes. The plasmids were double digested with *Hind*III and *Afl*II enzymes, of which the expected band size should be approximately 4.6 kb and 3.0 kb, and double digested with *Afl*II and *Kas*I enzymes of which the expected band size should be 5.5 kb and 2.1 kb. The correct patterns of digestion were shown in the clones as shown in Figure 3.5. The inserted *Pfdhfr-ts* sequence was verified to be of correct sequence by DNA sequencing. The corresponding size of the plasmid was 7.6 kb and named pY003 as shown in Figure 3.6. The pY003 plasmid construction and plasmid map are shown in Figure 3.6. The plasmid pY003 contains one expression cassette of *Pfdhfr-ts* under control of 2.3 kb of 5' and 1.0 kb of 3'UTR of *Pbdhfr-ts*. The *Pfdhfr-ts* contained *Afl*II restriction site

in junction region which served as cloning site for libraries of *Pfdhfr* random mutations.

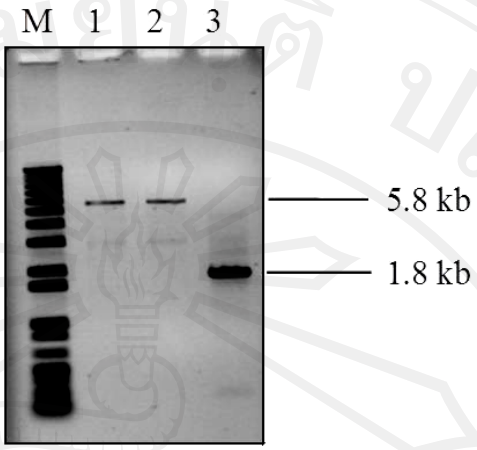


Figure 3.4 Agarose gel-electrophoresis of pY001 plasmid backbone (lanes 1, 2) and 1.8 kb *Pfdhfr-ts* PCR product (lane 3) both digested with *Age*I and *Nhe*I. The 1 kb plus molecular marker is shown in lane M.

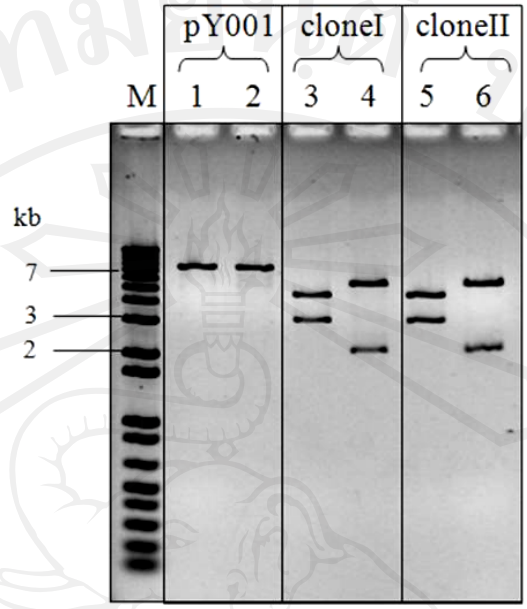
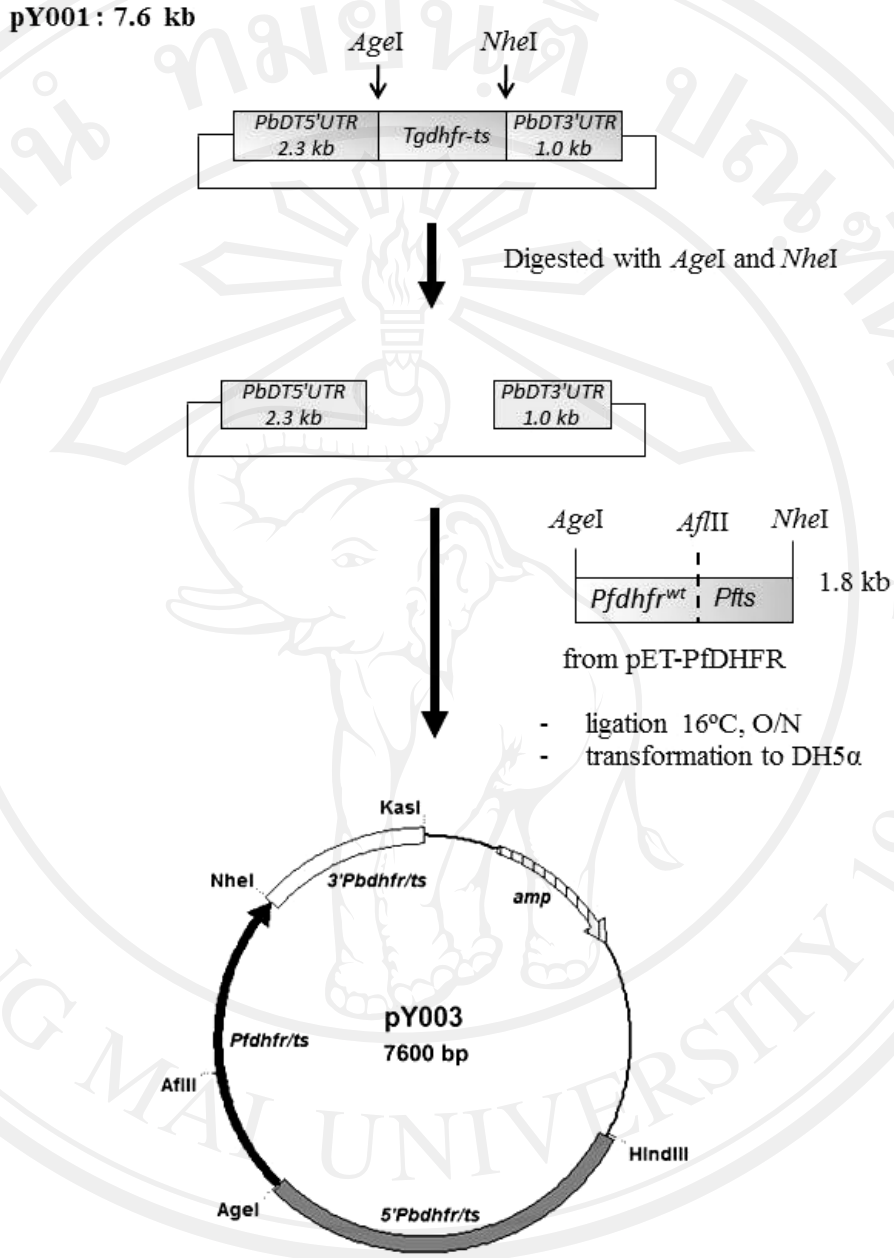


Figure 3.5 Restriction analysis of the selected 2 recombinant clones and pY001 plasmid control. Lanes 1, 3, 5, digested with *Hind*III and *Afl*II (expected band size = 4.6 kb and 3.0 kb). Lanes 2, 4, 6 digested with *Afl*II and *Kas*I (expected band size = 5.5 kb and 2.1 kb). The 1 kb plus molecular marker is shown in lane M.



Circular map of pY003

Figure 3.6 Construction pY003 plasmid.

3.1.3 Construction of pY003K1, pY003CSL and pY003V1S plasmids

To construct *P. berghei* transfection plasmids containing known drug resistant *Pfdhfr* mutant (double, triple, quadruple mutation), the *Pfdhfr* mutant genes bifunctional with *ts* gene were PCR amplified by using plasmids pETpfK1, pETpfCSL, pETpfV1S, respectively, as templates. The PCR products were digested with *Age*I and *Nhe*I. The digested *Pfdhfr-ts* PCR product of 1.8 kb band size is shown in Figure 3.7 (lanes 3-5) and served as insert for ligation reactions. Plasmid pY003 which contains *Pfdhfr-ts* wild-type gene was digested with *Age*I and *Nhe*I to remove *Pfdhfr-ts*. The expected band size of digested pY003 is 5.8 kb as shown in Figure 3.7 (lane 1) and served as backbone for ligation reactions with *Pfdhfr-ts* mutant inserts. Each ligation product was transformed into *E. coli* DH5α competent cells by heat shock method and cells were plated on LB selective agar containing 100 μg/ml ampicillin. After overnight incubation at 37°C, colonies grew on the selective agar medium with little inter-spatial distribution. Three recombinant clones of each mutant were selected for plasmid purification. These plasmids were named pY003K1 (double mutant), pY003CSL (triple mutant) and pY003V1S (quadruple mutant). The corresponding size of the plasmids is 7.6 kb. The plasmid construction and plasmid map of all the plasmids are shown in Figure 3.8. All plasmids contain one expression cassette of known *Pfdhfr-ts* mutant under control of 2.3 kb of 5' and 1.0 kb of 3'UTR of *Pbdhfr-ts*. The plasmids were confirmed by digestion with various restriction enzymes. The plasmids were double digested with *Hind*III and *Afl*II enzymes which would give the expected band size of approximately 7.6 kb, and when double digested with *Afl*II and *Kas*I, the expected band size should be 7.6 kb. The correct patterns of digestion were shown in all selected clones as shown in Figures 3.9 and 3.10. The

inserted *Pfdhfr-ts* mutant sequence was verified to be of appropriate sequence by DNA sequencing.

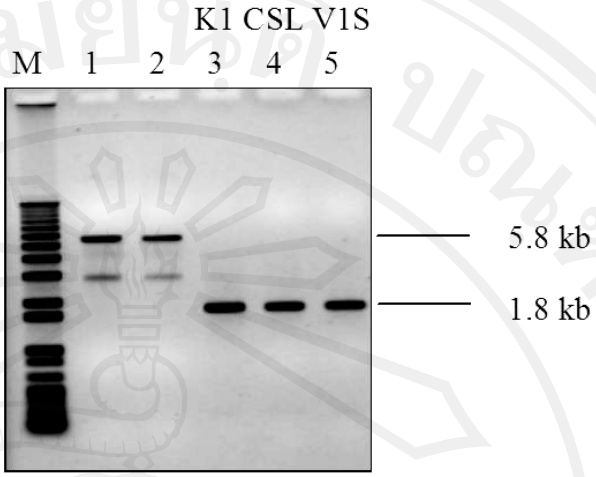
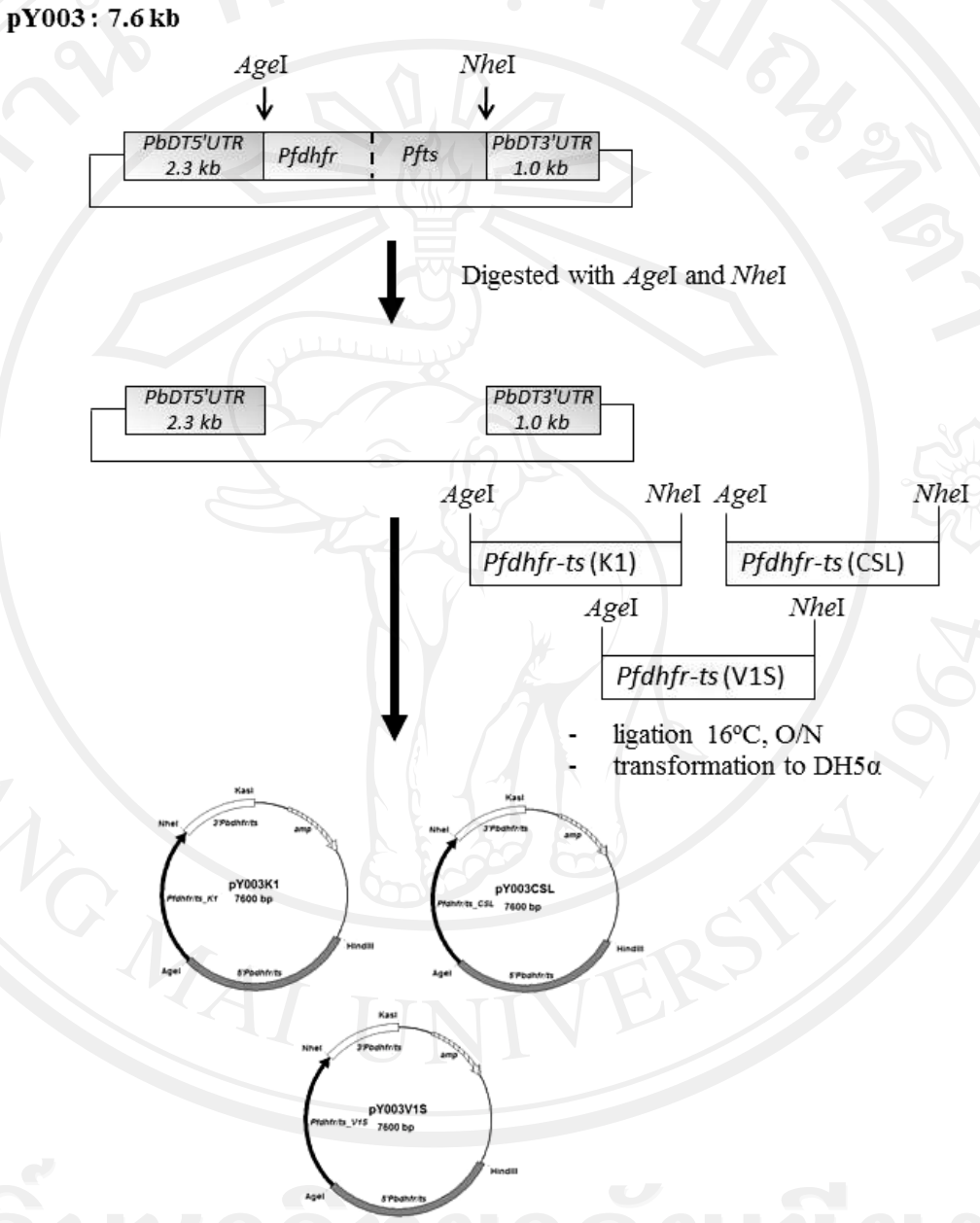


Figure 3.7 Agarose gel-electrophoresis of pY003 plasmid backbone (lanes 1, 2) and 1.8 kb PCR product of known *Pfdhfr-ts* mutants, both digested with *Age*I and *Nhe*I (lanes 3, 4, 5). The 1 kb plus molecular marker is shown in lane 1.



Circular maps of pY003K1, pY003CSL, pY003V1S

Figure 3.8 Construction pY003K1, pY003CSL and pY003V1S plasmids.

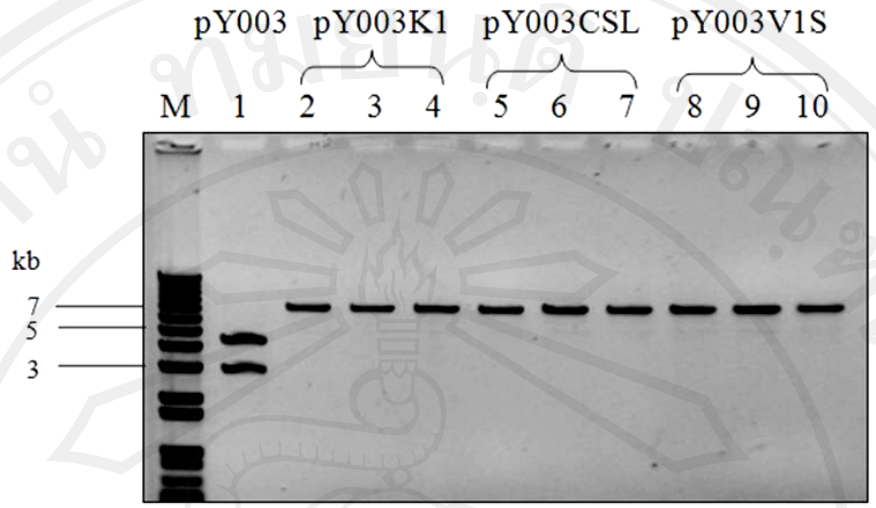


Figure 3.9 Restriction analysis of the selected 3 recombinant clones of each known *Pfdhfr-ts* mutant digested with *Hind*III and *Afl*II enzymes. pY003 plasmid control, (expected band size = 4.6 kb and 3.0 kb), pY003K1, pY003CSL, pY003CSL (expected band size = 7.6 kb). The 1 kb plus molecular marker is shown in lane M.

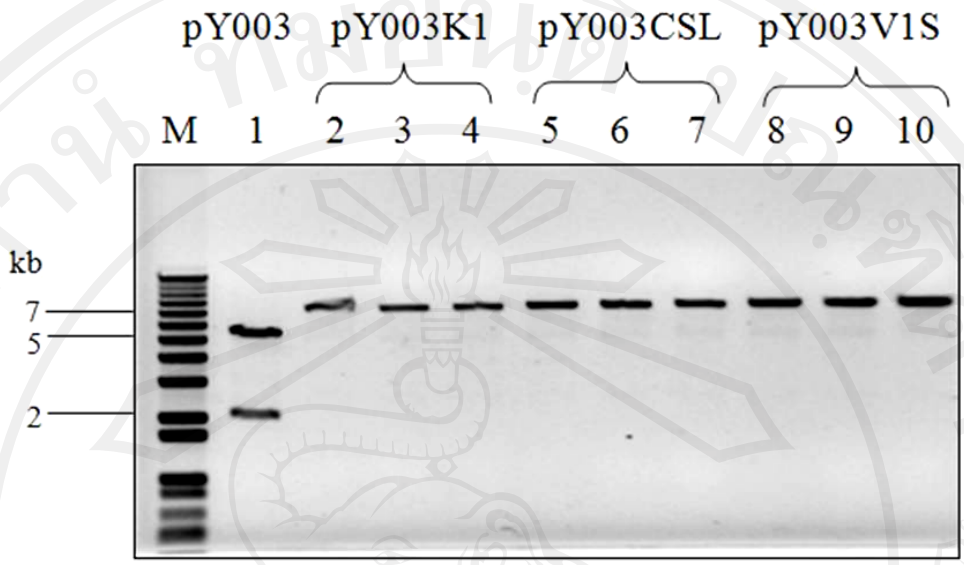


Figure 3.10 Restriction analysis of the selected 3 recombinant clones of each known mutant *Pfdhfr-ts* digested with *Afl*II and *Kas*I enzymes. pY003 plasmid control, (expected band size = 5.6 kb and 2.0 kb), pY003K1, pY003CSL, pY003CSL (expected band size = 7.6 kb). The 1 kb plus molecular marker is shown in lane M.

3.1.4 Construction of pY005 plasmid

Plasmid pY003 contains cloning sites *Age*I and *Afl*II which were design as sites for construction of mutant *Pfdhfr* libraries. However, after digestion with *Age*I, a non-specific band was found which may affect the construction of *Pfdhfr* libraries. Thus, the cloning site was changed from *Age*I to *Bam*HI. After digestion with *Age*I and *Nhe*I to remove *Tgdhfr-ts*, plasmid pY001 served as backbone for ligation reaction. The digested pY001 is 5.8 kb band size as shown in Figure 3.11 (lane 1). *Pfdhfr-ts* was amplified by PCR reaction using forward primer containing both *Age*I and *Bam*HI restriction sites. *Pfdhfr-ts* PCR product was digested with *Age*I and *Nhe*I, and the digested PCR product served as the insert for ligation reaction as shown with the band size of 1.8 kb in Figure 3.11 (lane 2). The ligation product was transformed into *E. coli* DH5α competent cells by heat shock method and cells were plated on selective agar containing 100 μg/ml ampicillin. After overnight incubation at 37°C, colonies grew on the selective agar medium with little inter-spatial distribution. To verify the positive clone, colonies were selected for colony PCR screening. Three positive clones were selected for plasmid purification and further confirmation by restriction mapping with various restriction enzymes. The plasmids were double digested with *Hind*III and *Afl*II, with the expected band size of approximately 4.6 kb and 3.0 kb (Figure 3.12). Another double digestion with *Afl*II and *Kas*I, shows the expected band size of approximately 5.5 kb and 2.1 kb (Figure 3.12). Finally, the plasmids were double digested with *Bam*HI and *Nhe*I and the expected size of about 5.8 kb and 1.8 kb were detected. The correct patterns of digestion were achieved from clone III as shown in figure 3.12. The inserted *Pfdhfr-ts* was verified to be of

appropriate sequence by DNA sequencing. The construction of pY005 plasmid construction is shown in Figure 3.13. The corresponding size of the plasmid was 7.6 kb and named pY005 as shown in plasmid map (Figure 3.13). The plasmid pY005 contains one expression cassette of *Pfdhfr-ts* under control of 2.3 kb of 5' and 1.0 kb of 3'UTR of *Pbdhfr-ts*. This version of *Pfdhfr-ts* sequence contains *Afl*II restriction site in the junction region which serves as cloning site for libraries of *Pfdhfr* random mutations.

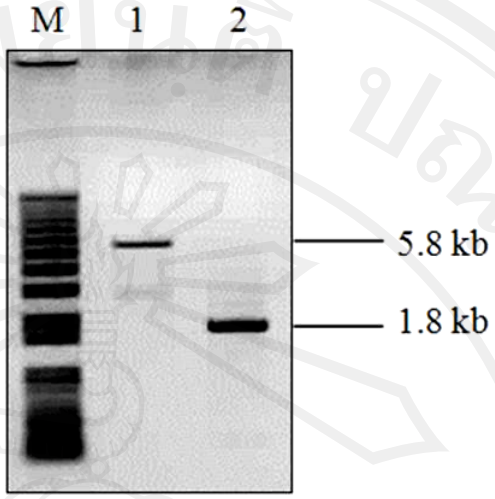


Figure 3.11 Agarose gel-electrophoresis of pY001 plasmid backbone (lane 1) and *Pfdhfr-ts* 1.8 kb PCR product (lane 2), both digested with *Age*I and *Nhe*I. The 1 kb plus molecular marker is shown in lane M.

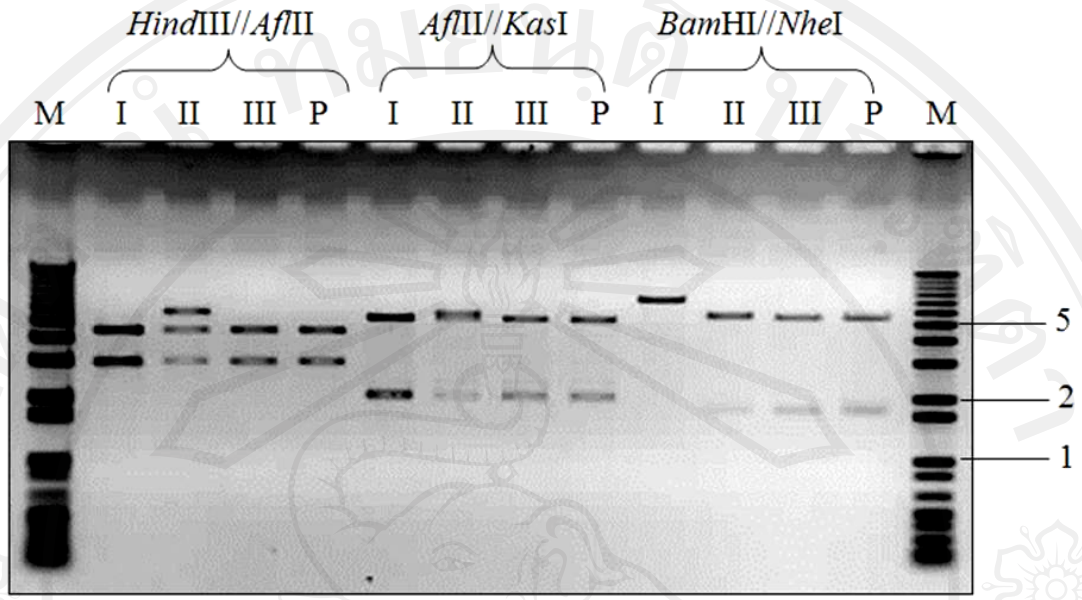
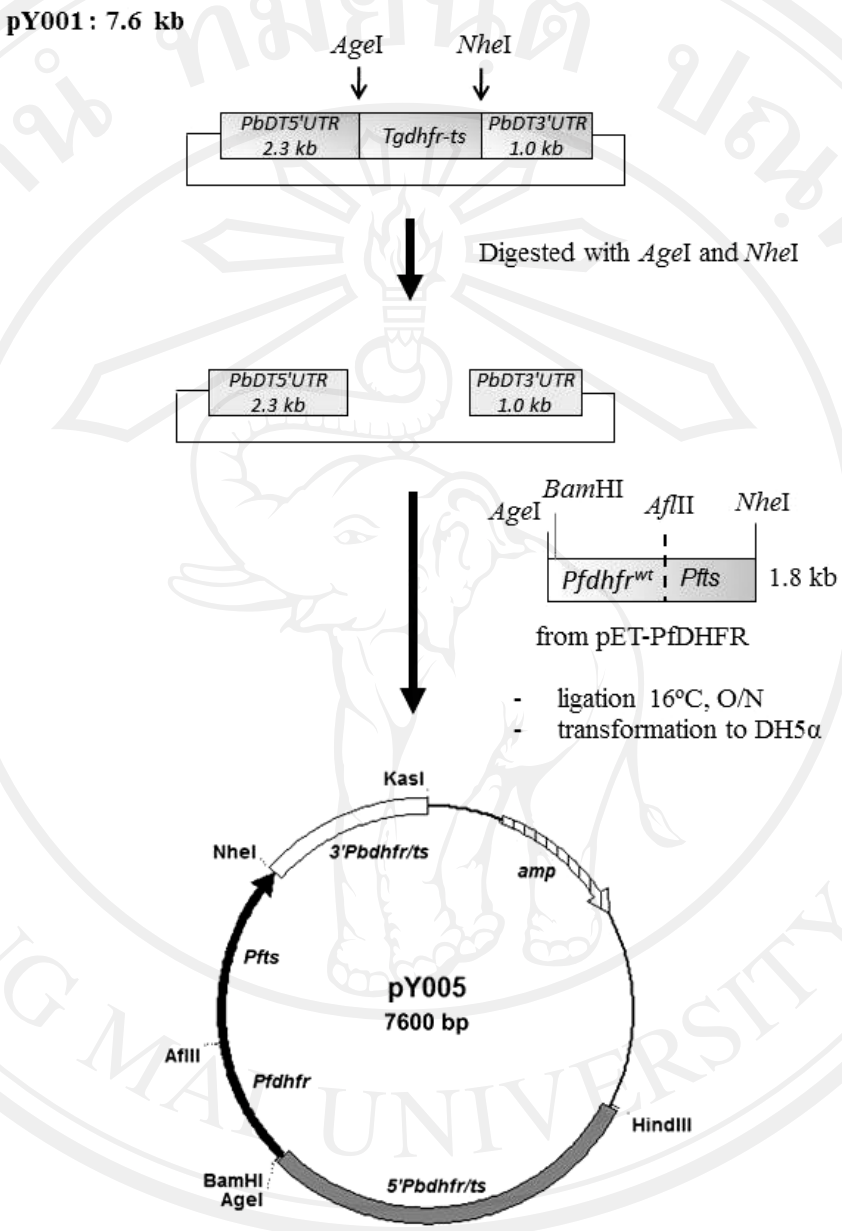


Figure 3.12 Restriction analysis of the selected 3 recombinant clones. Plasmids were digested with various restriction enzymes. Lanes 1-4 digested with *Hind*III and *Afl*II. Lanes 5-8 digested with *Afl*II and *Kas*I. Lanes 9-12 digested with *Bam*HI and *Nhe*I. The 1 kb plus molecular markers are shown in lane M. I = clone I, II = clone II, III = clone III, P = pY003 control plasmid.



Circular map of pY005

Figure 3.13 Construction of pY005 plasmid.

3.2 Determination of pyrimethamine sensitivity of wild-type PbGFP parasites

The efficacy of pyrimethamine to inhibit wild-type PbGFP parasite was determined by 4-day suppressive test as described in section 2.3.5 Materials and Methods. The wild-type PbGFP-infected mice were treated with different concentration of pyrimethamine at 0.0015, 0.015, 0.05, 0.15, 0.5 mg/kg. The percentages of parasitemia were determined by counting of infected erythrocytes on Giemsa stained slide. The experiments were performed in three independent studies and the data represents mean values ± SD of percentage of growth inhibition as shown in Table 3.1. As shown in Figure 3.14, *in vivo* ED_{50} and ED_{95} of pyrimethamine against wild-type PbGFP are 0.02 mg/kg and 0.25 mg/kg, respectively. The ED_{95} concentration of pyrimethamine was then used for selection of transgenic resistant parasites in subsequent experiments.

Table 3.1 Inhibition of wild-type PbGFP parasite by pyrimethamine in mice.

Group	Pyrimethamine (mg/kg)	%Growth inhibition			Mean of % inhibition ± SD.
		Exp. I	Exp. II	Exp. III	
1	0.50	100	100	100	100 ± 0.00
2	0.15	94.89	95.40	95.55	95.28 ± 0.33
3	0.05	80.76	70.18	67.31	72.57 ± 7.08
4	0.015	55.50	42.75	39.51	45.92 ± 8.45
5	0.0015	30.34	12.62	15.87	19.61 ± 9.43

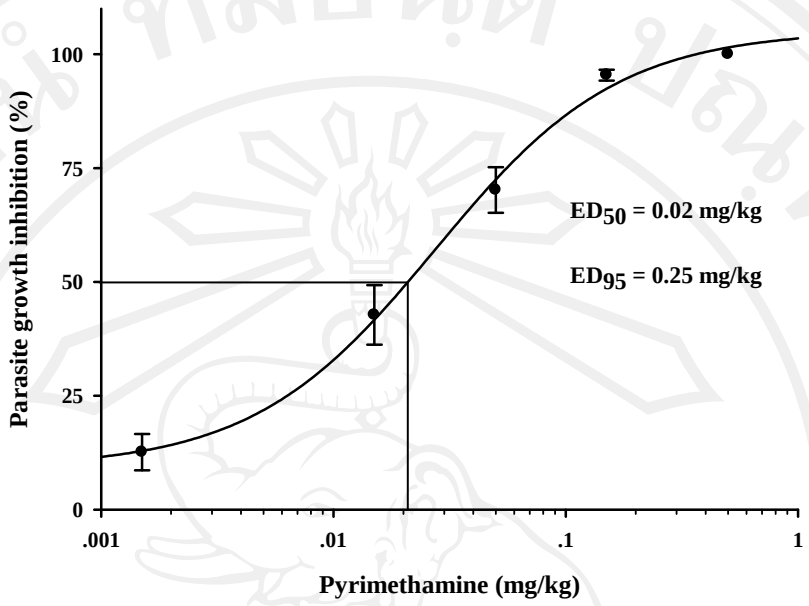


Figure 3.14 Dose-inhibition curve of pyrimethamine against wild-type PbGFP parasite. The experiments were performed in three independent studies and the data represents mean values of percentage of growth inhibition ± standard deviation.

3.3 Introduction of known *Pfdhfr* mutants to *P. berghei* parasite by transfection

To test whether *P. berghei* system can be used for selection and identification of mutant library of *Pfdhfr*, the equal amount of plasmids containing *Plasmodium* expression cassette of wild-type *Pfdhfr* and known *Pfdhfr* mutants (double mutant (C59R+S108N), triple mutant (C59R+S108N+I164L), quadruple mutant (N51I+C59R+S108N+I164L)) were episomally transfected to *P. berghei* parasite. The resistant parasites were selected by pyrimethamine at 0.25 mg/kg (ED_{95}) injected intraperitoneally everyday. The parasitemia of transfected *P. berghei* parasite was monitored as shown in Figure 3.15. On day 14 post transfection, heart blood was collected from the infected mouse before the genomic DNA was extracted and purified. The percentage of transfected *Pfdhfr* within the parasite genome was confirmed by PCR amplification using FBamHI and RAflII primer. In addition, the extracted genomic DNA was transformed into *E. coli* DH5α strain to recover the transfected plasmid. After overnight incubation at 37°C, approximately 17 colonies were derived. The colonies were randomly picked and prepared for plasmid. The *Pfdhfr* genes in the extracted plasmids were sequenced with RAflII primer. The sequencing results showed that all types of *Pfdhfr* mutants were obtained without any deletion of wild-type *Pfdhfr* sequence. The sequences were aligned with the wild-type *Pfdhfr* sequence as shown in Figure 3.16.

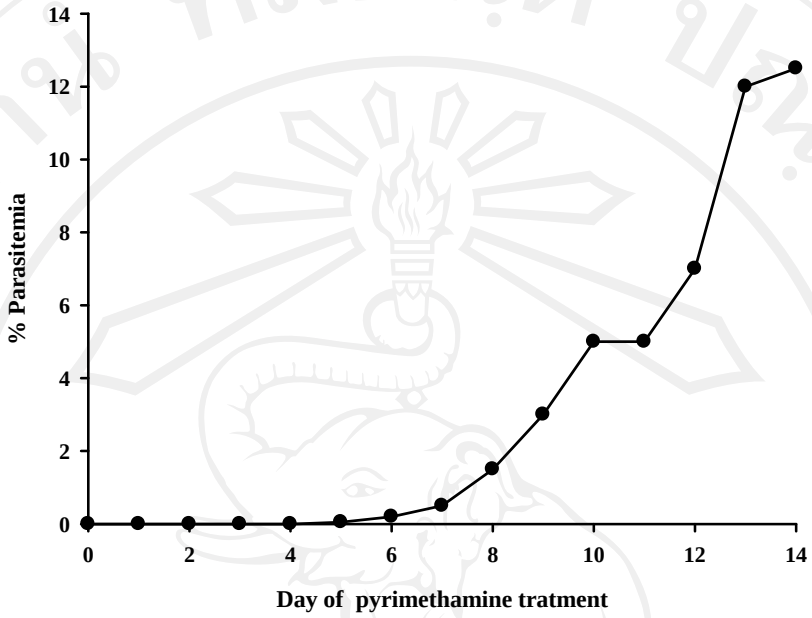


Figure 3.15 Development of transgenic parasites transfected with a mixture of plasmids containing wild-type and known *Pfdhfr-ts* mutant under 0.25 mg/kg pyrimethamine selection. The graph shows a representative result of growth development.

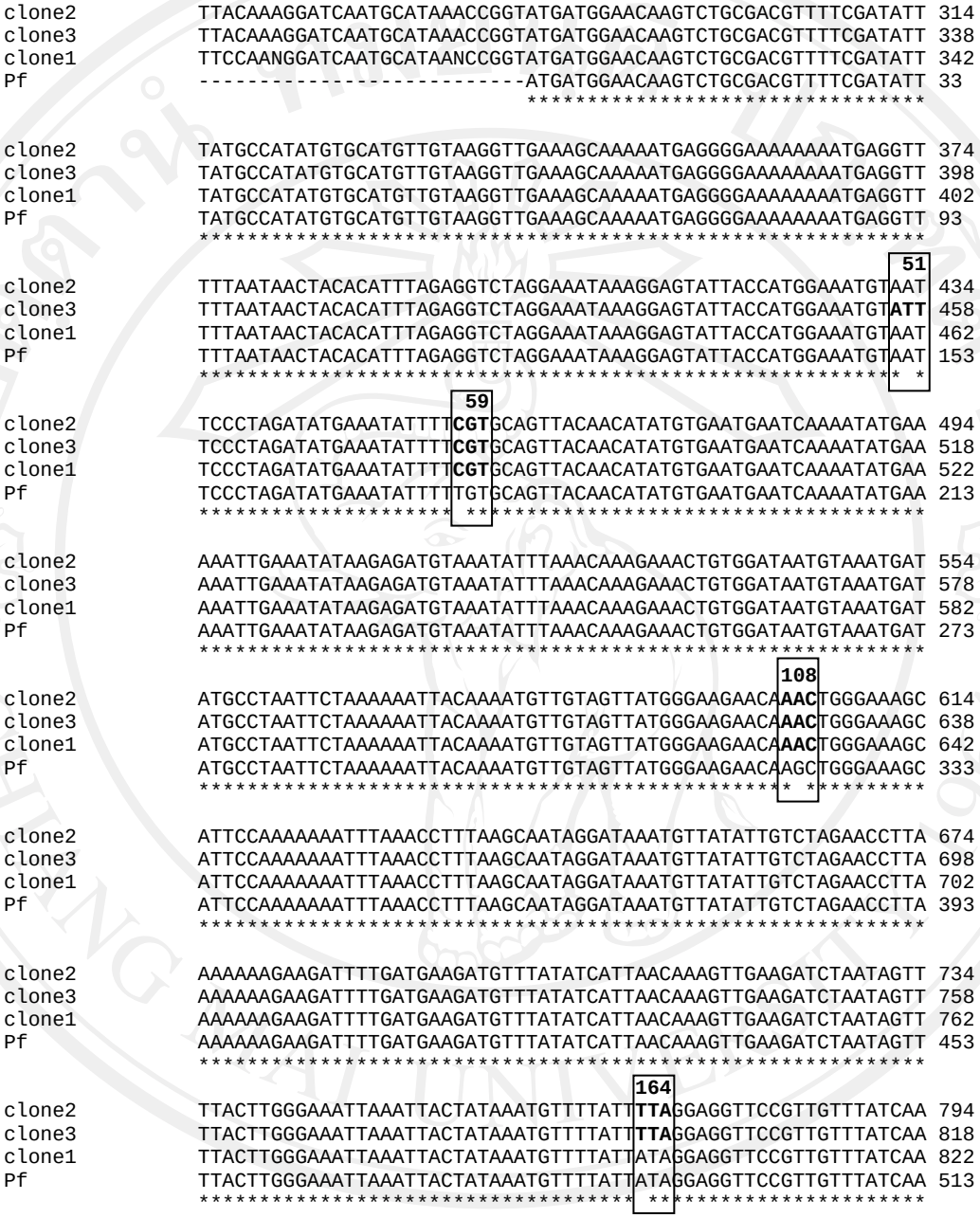


Figure 3.16 Sequence alignment of known mutant *Pfdhfr* (clone1 = double mutant (C59R+S108N), clone2 = triple mutant (C59R+S108N+I164L), clone3 = quadruple mutant (N51I+C59R+S108N+I164L)) and wild-type *Pfdhfr* (Pf). Codons with base substitution are boxed. The amino acid positions are indicated above the aligned DNA sequences. The alignments were performed using ClustalW sequence alignment program.

3.4 Construction of *Pfdhfr* random mutant library using wild-type *Pfdhfr* as template

A random mutant library of *Pfdhfr* was constructed by error-prone PCR, by using plasmid containing wild-type *Pfdhfr* as a template. The *Pfdhfr* random mutation was carried out using GoTaq®Flexi DNA polymerase which lacks proof-reading property, therefore the accumulation of mis-incorporation of nucleotides was allowed during DNA amplification cycles. Randomly mutated *Pfdhfr* PCR product digested with *Bam*HI and *Afl*II and served as insert for the ligation as shown in Figure 3.17 (lane 2). Plasmid pY005 was also digested with *Bam*HI and *Afl*II to remove *Pfdhfr*. The 6.9 kb fragment of digested pY005 (Figure 3.17, lane 1) served as backbone for the ligation reaction. The ligation product was transformed into *E. coli* DH5α strain by heat shock method and the transformed cells were plated on LB agar containing 100 μg/ml ampicillin. After overnight incubation at 37°C, colonies containing plasmid with random mutant *Pfdhfr* were obtained. The clones were verified by randomly picking colonies from the agar plates for PCR amplification. The positive variant clones were selected for plasmid purification. *Pfdhfr* genes in plasmids were subjected to sequence determination by DNA sequencing. The representative variations of *Pfdhfr* mutation are shown in Table 3.2. Approximately 14,000 colonies were collected from each transformation of ligation product.

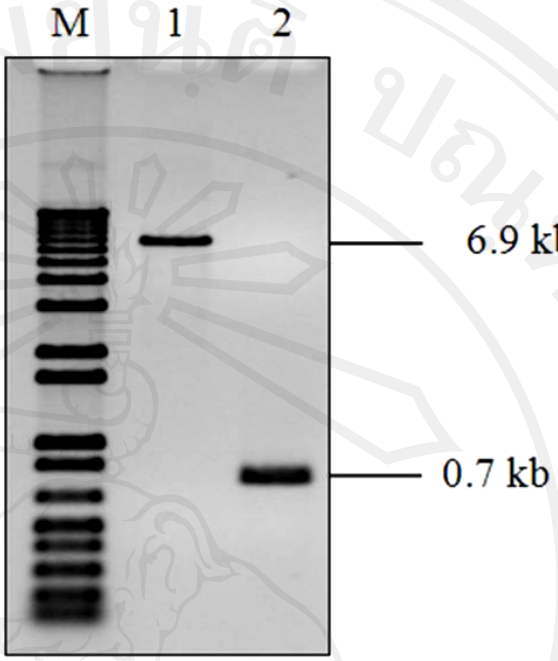


Figure 3.17 Agarose gel-electrophoresis of pY005 plasmid backbone (lane 1) and random mutant *Pfdhfr* 0.7 kb PCR product (wild-type *Pfdhfr* as template, lane 2) both digested with *Bam*HI and *Afl*II. The 1 kb plus molecular marker is shown in lane M.

Table 3.2 Variation of *Pfdhfr* mutation constructed in bacteria compared with wild-type *Pfdhfr* template. The amino acids are shown in alphabetical symbol, whereas the codon that codes for the amino acid is shown in the brackets.

Clone	Amino acid Postion	Base substitution conferring change in amino acid	
		Template (wild-type)	Generated mutant
1	23	L (AAA)	I (ATA)
	62	T (ACA)	T (ACG)
	218	N (AAT)	D (GAT)
2	11	I (ATT)	I (ATA)
	14	I (ATA)	V (GTA)
	75	Y (TAT)	Stop (TAA)
	112	I (ATT)	V (GTT)
3	59	C (TGT)	R (CGT)
	110	E (GAA)	D (GAC)
	198	P (CCA)	S (TCA)
	219	T (ACA)	K (AAA)
4	17	C (TGT)	W (TGG)
	151	V (GTT)	A (GCT)
5	-	wild-type	-
6	145	K (AAA)	K (AAG)

Table 3.2 Variation of *Pfdhfr* mutation construction on bacteria compared with wild-type *Pfdhfr* template. The amino acids are shown in alphabetical symbol, whereas the codon that codes for the amino acid is shown in the bracket (continued).

Clone	Amino acid Position	Base substitution conferring change in amino acid	
		Template (wild- type)	Generated mutant
7	-	wild-type	-
8	34	N (AAC)	D (GAC)
	36	T (ACA)	A (GCA)
9	53	L (CTA)	P (CCA)
10	-	wild-type	-
11	33	N (AAT)	D (GAT)
	101	V (GTT)	V (GTC)
	184	F (TTT)	F (TTC)
12	87	D (GAT)	G (GGT)
	181	L (AAA)	N (AAT)

3.5 Transfection and selection of transfected resistant mutant parasite from wild-type *Pfdhfr* random libraries

An approximately 14,000 colonies were pooled and plasmid DNA containing random mutant *Pfdhfr* library was prepared. After plasmid extraction and precipitation to small volume, 23 µg/µl of circular containing different *Pfdhfr* mutants were transfected to *P. berghei* parasites using protocol as described on section 2.3.4.2 of Chapter II. Transfected parasites were then injected into the tail vein of a mouse. The parasites expressing drug resistant *Pf*DHFR-TS were selected by pyrimethamine at 0.25 mg/kg (ED_{95}) injected i.p. everyday. The parasitemia of transfected drug resistant *P. berghei* parasite increased on day 13 post transfection, thereafter the parasitemia was monitored daily as shown in Figure 3.18. The tail blood of infected mouse was collected on alternate days. The genomic DNA of collected parasites was extracted and verified for *Pfdhfr* by PCR using FBamHI and RAflII primer as shown in Figure 3.19. In addition, the extracted genomic DNA was transformed to *E. coli* DH5α strain to recover the plasmids for overnight culture. Colonies were picked before they were extracted for plasmid. On day 20 post transfection, the heart blood was collected from the mouse and extracted genomic DNA that was also transformed to *E. coli* DH5α strain to recover the plasmids for overnight culture before they were extracted. Colonies were picked for overnight culture before they were extracted for plasmid. The extracted plasmids were subjected to sequence determination by DNA sequencing. The sequence was aligned with wild-type *Pfdhfr* sequence. From this experiment, two variations of *Pfdhfr* were found. One sequence contained a single mutation at amino acid position 108 whereas the other one had amino acid changes at

position 108 and 196. At position 108, amino acid serine (AGC) was changed to asparagine (AAC) while at position 196, phenyalanine showed a silent mutation (TTT to TTC). The DNA sequence alignments are shown in Figure 3.20.

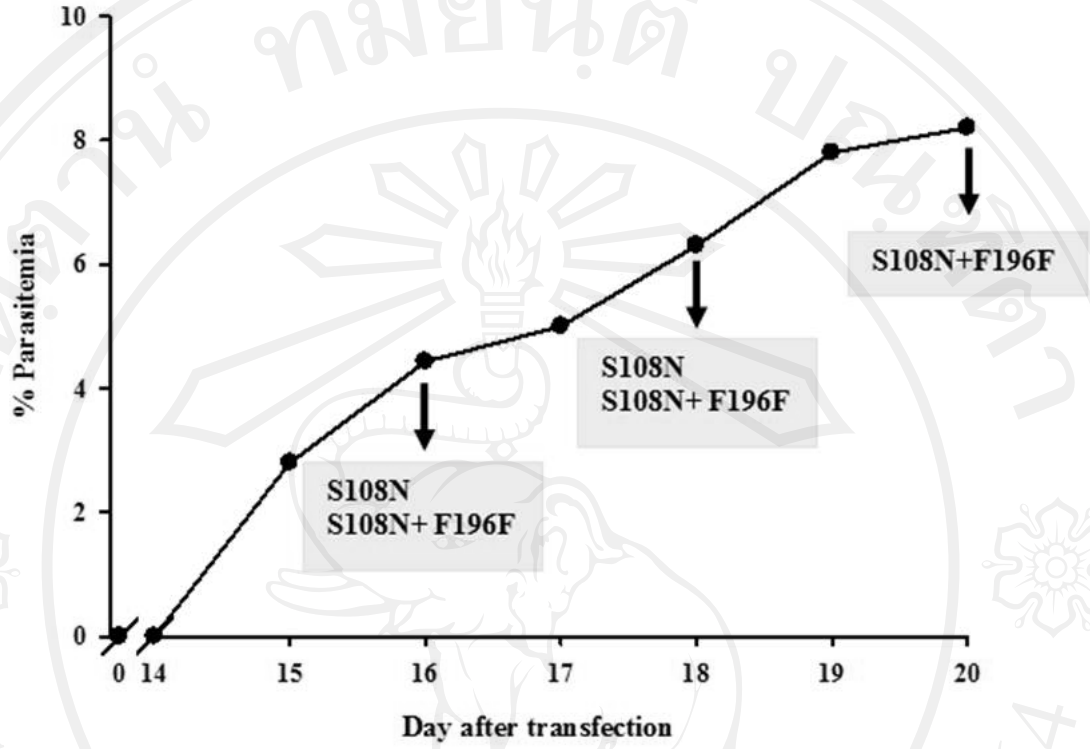


Figure 3.18 Development of transfected *P. berghei* parasite harbouring random mutant *Pfdhfr* library (wild- type *Pfdhfr* template). Genomic DNA from transfected parasite was extracted on days 16, 17 and 20 after transfection. Amino acid substitutions are show in alphabetical symbol.

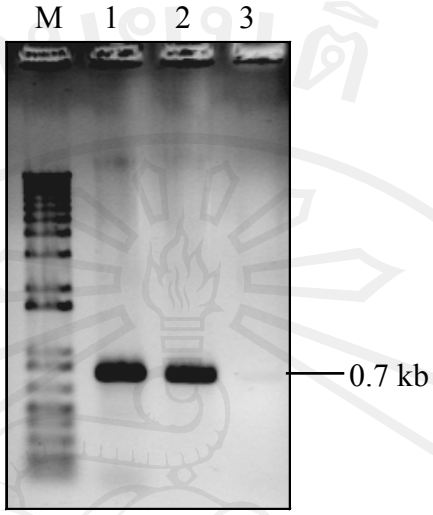


Figure 3.19 Agarose gel-electrophoresis of PCR product of 0.7 kb from genomic DNA of parasites transfected with mutant *Pfdhfr* library (lane 1). pY003 plasmid (lane 2) and wild-type *P. berghei* genomic DNA (lane 3) served as positive and negative control, respectively. The 1 kb plus molecular marker is shown in lane M.

```
clone1          TGGATCCATGATGGAACAAGTCTGCGACGTTTTCGATATTTATGCCATATGTGCATGTTG 393
clone2          TGGATCCATGATGGAACAAGTCTGCGACGTTTTCGATATTTATGCCATATGTGCATGTTG 418
Pfdhfr          -------ATGATGGAACAAGTCTGCGACGTTTTCGATATTTATGCCATATGTGCATGTTG 53
                       **************************************************

clone1          TAAGGTTGAAAGCAAAAATGAGGGGAAAAAAAAATGAGGTTTTTAATAACTACACATTTAG 453
clone2          TAAGGTTGAAAGCAAAAATGAGGGGAAAAAAAAATGAGGTTTTTAATAACTACACATTTAG 478
Pfdhfr          TAAGGTTGAAAGCAAAAATGAGGGGAAAAAAAAATGAGGTTTTTAATAACTACACATTTAG 113
                ************************************************************

clone1          AGGTCTAGGAAATAAAGGAGTATTACCATGGAAATGTAATTCCCTAGATATGAAATATTT 513
clone2          AGGTCTAGGAAATAAAGGAGTATTACCATGGAAATGTAATTCCCTAGATATGAAATATTT 538
Pfdhfr          AGGTCTAGGAAATAAAGGAGTATTACCATGGAAATGTAATTCCCTAGATATGAAATATTT 173
                ************************************************************

clone1          TTGTGCAGTTACAACATATGTGAATGAATCAAAATATGAAAAATTGAAATATAAGAGATG 573
clone2          TTGTGCAGTTACAACATATGTGAATGAATCAAAATATGAAAAATTGAAATATAAGAGATG 598
Pfdhfr          TTGTGCAGTTACAACATATGTGAATGAATCAAAATATGAAAAATTGAAATATAAGAGATG 233
                ************************************************************

clone1          TAAATATTTAAACAAAGAAACTGTGGATAATGTAAATGATATGCCTAATTCTAAAAAATT 633
clone2          TAAATATTTAAACAAAGAAACTGTGGATAATGTAAATGATATGCCTAATTCTAAAAAATT 658
Pfdhfr          TAAATATTTAAACAAAGAAACTGTGGATAATGTAAATGATATGCCTAATTCTAAAAAATT 293
                 ***********************************************************

                                        108
clone1          ACAAAATGTTGTAGTTATGGGAAGAACAAACTGGGAAAGCATTCCAAAAAAATTTAAACC 693
clone2          ACAAAATGTTGTAGTTATGGGAAGAACAAACTGGGAAAGCATTCCAAAAAAATTTAAACC 718
Pfdhfr          ACAAAATGTTGTAGTTATGGGAAGAACAAGCTGGGAAAGCATTCCAAAAAAATTTAAACC 353
                ****************************** ***************************

clone1          TTTAAGCAATAGGATAAATGTTATATTGTCTAGAACCTTAAAAAAAGAAGATTTTGATGA 753
clone2          TTTAAGCAATAGGATAAATGTTATATTGTCTAGAACCTTAAAAAAAGAAGATTTTGATGA 778
Pfdhfr          TTTAAGCAATAGGATAAATGTTATATTGTCTAGAACCTTAAAAAAAGAAGATTTTGATGA 413
                ************************************************************

clone1          AGATGTTTATATCATTAACAAAGTTGAAGATCTAATAGTTTTACTTGGGAAATTAAATTA 813
clone2          AGATGTTTATATCATTAACAAAGTTGAAGATCTAATAGTTTTACTTGGGAAATTAAATTA 838
Pfdhfr          AGATGTTTATATCATTAACAAAGTTGAAGATCTAATAGTTTTACTTGGGAAATTAAATTA 473
                ************************************************************

clone1          CTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAGAAATT 873
clone2          CTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAGAAATT 898
Pfdhfr          CTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAGAAATT 533
                ************************************************************

                                                                196
clone1          AATAAAAAAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTTTTTCC 933
clone2          AATAAAAAAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTCTTTCC 958
Pfdhfr          AATAAAAAAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTTTTTCC 593
                ************************************************************ *****
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Figure 3.20 Sequence alignment of two *Pfdhfr* variations from parasite transfected with random mutant *Pfdhfr* library (wild-type *Pfdhfr* as template). The variations were aligned with wild-type *Pfdhfr* sequence using ClustalW program. Clone 1 represents S108N mutation and clone 2 represents S108N+F196F mutations. Codons with base substitution are boxed. The amino acid positions are indicated above the aligned DNA sequences.

3.6 Construction of *Pfdhfr* random mutant library using single mutant *Pfdhfr* as a template

A random mutant library of *Pfdhfr* was constructed by error-prone PCR by using plasmid containing single mutant *Pfdhfr* (S108N) as a template. The *Pfdhfr* random mutation was carried out using GoTaq®Flexi DNA polymerase which lacks proof- reading property, therefore the accumulation of mis-incorporation of nucleotides was allowed during DNA amplification cycles. Randomly mutated *Pfdhfr* PCR product was digested with *Bam*HI and *Afl*II and served as insert for the ligation reaction (Figure 3.21). Plasmid pY005 was also digested with *Bam*HI and *Afl*II to remove *Pfdhfr*. The 6.9 kb fragment of the digested pY005 is approximately, the band size is shown in (Figure 3.21) served as backbone for ligation reaction. The ligation product was transformed into *E. coli* DH5α by heat shock method and the transformed cells were plated on LB selective agar containing 100 µg/ml ampicillin. After overnight incubation at 37°C, colonies containing plasmid harboring random mutant *Pfdhfr* were obtained. The clones were verified by randomly picking colonies from the agar plates for PCR amplification. The positive variant clones were selected for plasmid purification. The *Pfdhfr* gene in the plasmids was subjected to sequenced determination by DNA sequencing using *Afl*II primer. The representative variations of *Pfdhfr* mutant obtain at this stage are shown in Table 3.3. Approximately 8,300 colonies were collected from each transformation of ligation product.

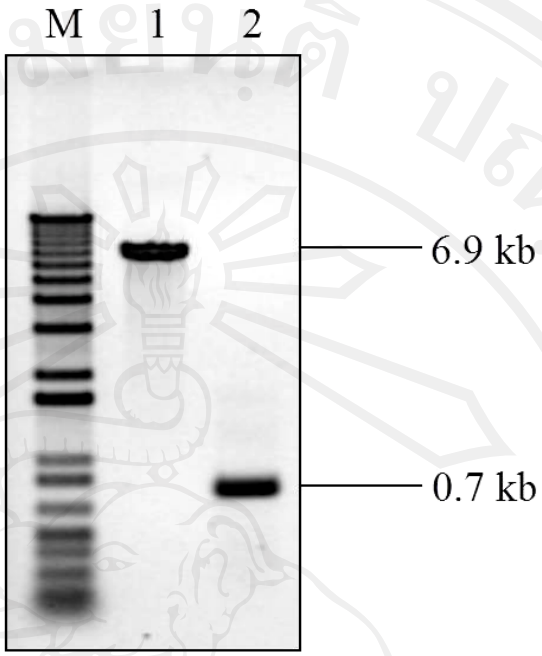


Figure 3.21 Agarose gel-electrophoresis of digested pY005 plasmid backbone (lane 1) and random mutant *Pfdhfr* 0.7 kb PCR product (S108N template (lane 2)), both digested with *Bam*HI and *Afl*II. The 1 kb plus molecular marker is shown in lane M.

Table 3.3 Variation of *Pfdhfr* mutation constructed in bacteria compared with *Pfdhfr* S108N template. The amino acids are shown in alphabetical symbol, whereas the codon that codes for the amino acids is shown in the brackets.

Clone	Amino acid position	Base substitution conferring change in amino acid	
		Template (S108N)	Generated mutant
1	45	A (GTA)	A (GCA)
	197	F (TTT)	S (TCT)
	226	Y (TAT)	Y (TAC)
2	108	S108N	-
3	56	K (AAA)	stop (TAA)
	135	D (GAT)	G (GGT)
	148	D (GAT)	N (AAT)
4	108	S108N	-
5	74	K(AAA)	stop (TAA)
	151	V (GTT)	I (ATT)
6	58	F (TTT)	I (ATT)
	108	S108N	
	121	N (AAT)	H (GAU)
	176	K (AAG)	R (AGG)

Table 3.3 Variation of *Pfdhfr* mutation constructed in bacteria compared with *Pfdhfr* S108N template. The amino acids are shown in alphabetical symbol, whereas the codon that code for the amino acids is shown in the brackets (continued).

Clone	Amino acid Position	Base substitution conferring change in amino acid	
		Template (S108N)	Generated mutant
7	108	S108N	-
	145	K (AAA)	R (AGA)
8	65	V (GTG)	A (GCG)
	108	S108N	
9	16	A (GCA)	P (CCA)
	46	L (TTA)	F (TTC)
	51	N (AAT)	I (ATT)
	62	T (ACA)	P (CCA)
	63	T (ACA)	P (CCT)
	115	L (AAA)	R (AGA)
10	181	L (AAA)	R (AGA)
11	81	L (TTA)	L (CTA)
12	67	E (GAA)	G (GGA)
	132	L (AAA)	L (AAG)

3.7 Transfection and selection of transfected resistant mutant parasite from *Pfdhfr* S108N random libraries

An approximately 8,300 colonies were pooled and plasmid DNA containing random DNA library was prepared. After plasmid extraction and precipitation to small volume, 20 µg of circular construct that contain different *Pfdhfr* mutants were transfected to *P. berghei* parasites. The transfected parasites were then injected into tail vein of 2 mice, before they were selected with pyrimethamine at 0.25 mg/kg (ED_{95}) injected intraperitoneally everyday. The genomic DNA of resistant parasite was extracted from mouse tail blood on alternate day. The parsitemia of transfected drug resistant *P. berghei* parasite was monitored daily as shown in Figure 3.22. On day 15 post transfection, the heart blood was collected from the mouse and extracted for genomic DNA before it was verified for *Pfdhfr* by PCR using FBamHI and RAflII primers as shown in figure 3.22. In addition, the extracted genomic DNA of transfected parasite was transformed into *E. coli* DH5α. Colonies were picked for overnight culture before they were extracted for plasmid extraction. The extracted plasmids were subjected to sequence determination by DNA sequencing. The sequence was aligned with wild-type *Pfdhfr* sequence as shown in Figures 3.24 and 3.25. The codon variation that codes for the amino acids are shown in Table 3.4. The variations of *Pfdhfr* mutation compared with S108N template are summarized in Table 3.5.

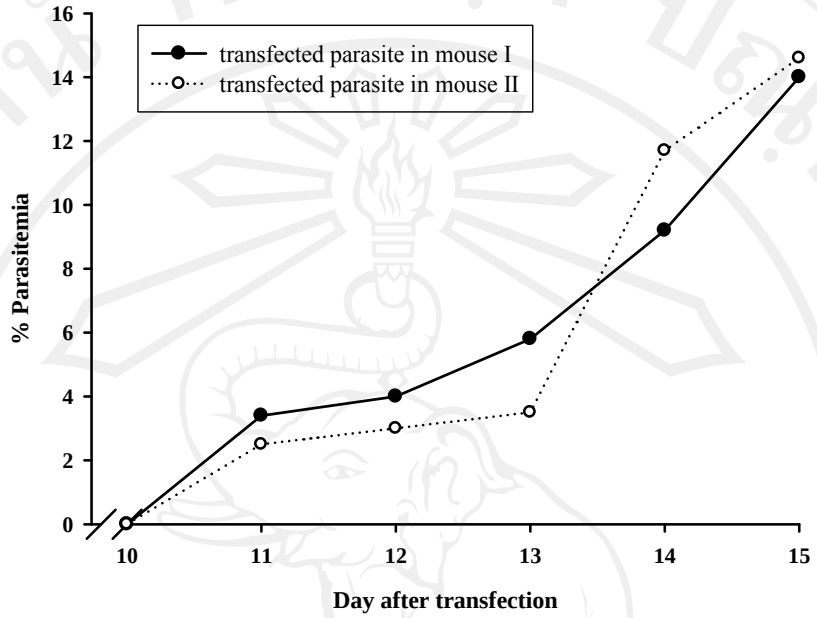


Figure 3.22 Development of transfected *P. berghei* parasite harbouring random mutant *Pfdhfr* library (S108N template) in two mice. Genomic DNA from transfected parasite was extracted on days 11, 13 and 15 after transfection. Filled and opened circles represent parasitemia of transfected parasite in mouse I and mouse II, respectively.

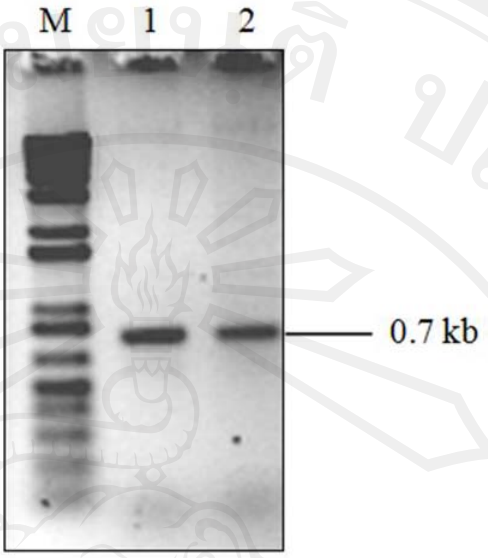
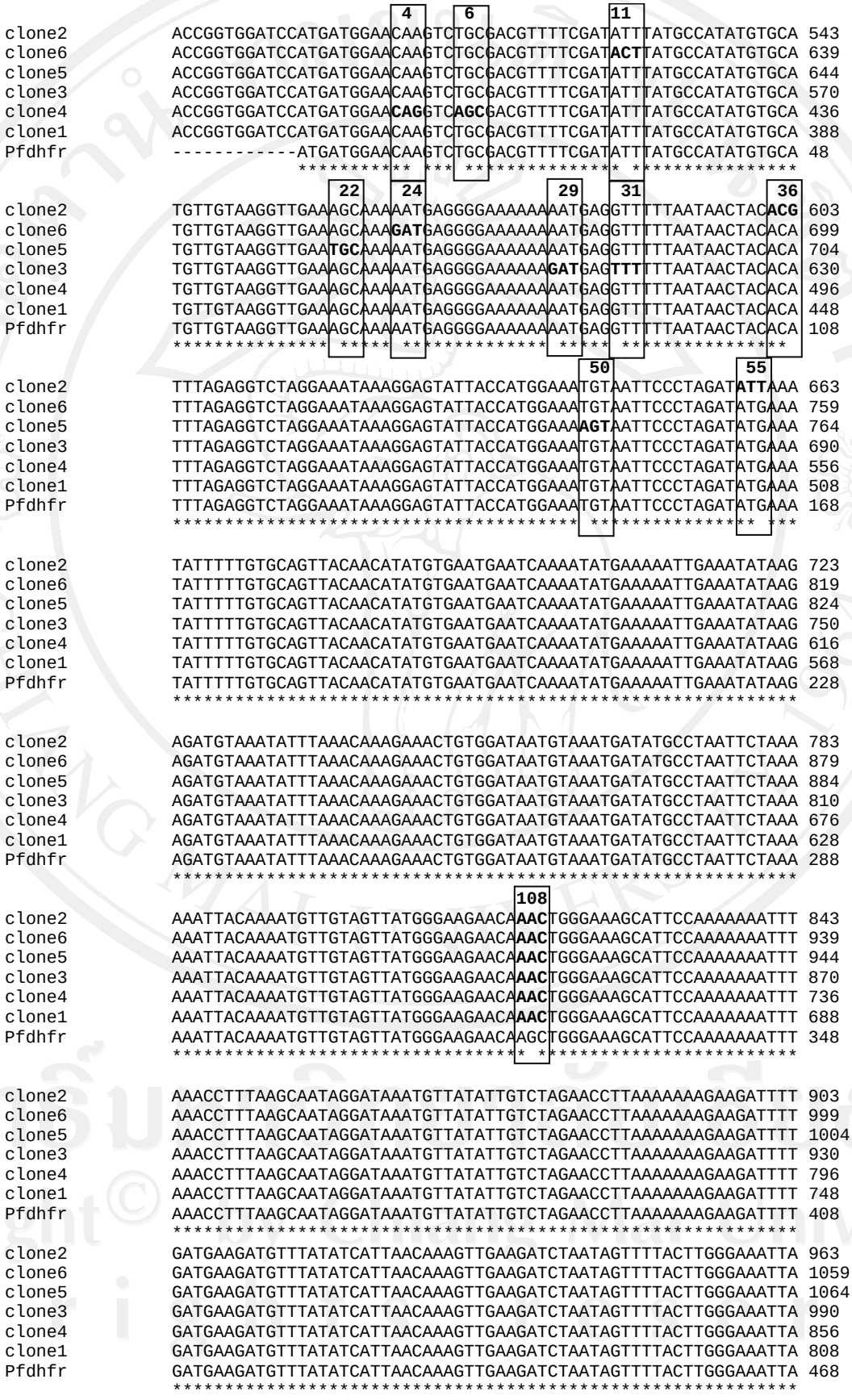


Figure 3.23 Agarose gel-electrophoresis of PCR product of 0.7 kb from genomic DNA of parasites transfected with mutant *Pfdhfr* library (S108N template) in mouse I and mouse II (lane 1 and lane 2, respectively). The 1 kb plus molecular marker is shown in lane M.


4
6
11
22
24
29
31
36
50
55
108

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clone2          AATTACTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAG 1023
clone6          AATTACTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAG 1119
clone5          AATTACTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAG 1124
clone3          AATTACTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAG 1050
clone4          AATTACTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAG 916
clone1          AATTACTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAG 868
Pfdhfr          AATTACTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAG 528
                ************************************************************

                                                  189
clone2          AAATTAATAAAAAAAATATATTTTACTAGAATAAATTGTACATATGAATGTGATGTATTT 1083
clone6          AAATTAATAAAAAAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTT 1179
clone5          AAATTAATAAAAAAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTT 1184
clone3          AAATTAATAAAAAAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTT 1110
clone4          AAATTAATAAAAAAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTT 976
clone1          AAATTAATAAAAAAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTT 928
Pfdhfr          AAATTAATAAAAAAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTT 588
                ************************************ ************************

clone2          TTTCCAGAAATAAATGAAAATGAGTATCAAATTATTTCTGTTAGCGATGTATATACTAGT 1143
clone6          TTTCCAGAAATAAATGAAAATGAGTATCAAATTATTTCTGTTAGCGATGTATATACTAGT 1239
clone5          TTTCCAGAAATAAATGAAAATGAGTATCAAATTATTTCTGTTAGCGATGTATATACTAGT 1244
clone3          TTTCCAGAAATAAATGAAAATGAGTATCAAATTATTTCTGTTAGCGATGTATATACTAGT 1170
clone4          TTTCCAGAAATAAATGAAAATGAGTATCAAATTATTTCTGTTAGCGATGTATATACTAGT 1036
clone1          TTTCCAGAAATAAATGAAAATGAGTATCAAATTATTTCTGTTAGCGATGTATATACTAGT 988
Pfdhfr          TTTCCAGAAATAAATGAAAATGAGTATCAAATTATTTCTGTTAGCGATGTATATACTAGT 648
                ************************************************************

clone2          AACGAATACAACT-TTGGATTTTATCATTTATAAGAAAACGAATAA--TAAAATGTTAAA 1200
clone6          AACGAATACAACTATTGGATTTTATCATTTATAAGAAAACGAATAAATAAAAATGTGAAA 1299
clone5          AACGAATACAACTATTGGATTTTATCATTTATAAGAAAACGAATAA--TAAAATGTTAAA 1302
clone3          AACGAATACAAC-ATTGGATTTTATCATTTATAAGAAAACGAATAA--TAAAATGTTAAA 1227
clone4          AAC-AATACAAC-ATTGGATTTTATCATTTATAAGAAAACGAATAA--TAAAATGTTAAA 1092
clone1          AAC-AATACAAC-ATTGGATTTTATCATTTATAAGAAAACGAATAA--TAAAATGTTAAA 1044
Pfdhfr          AAC-AATACAAC-ATTGGATTTTATCATTTATAAGAAAACGAATAA--TAAAATGTTAAA 704
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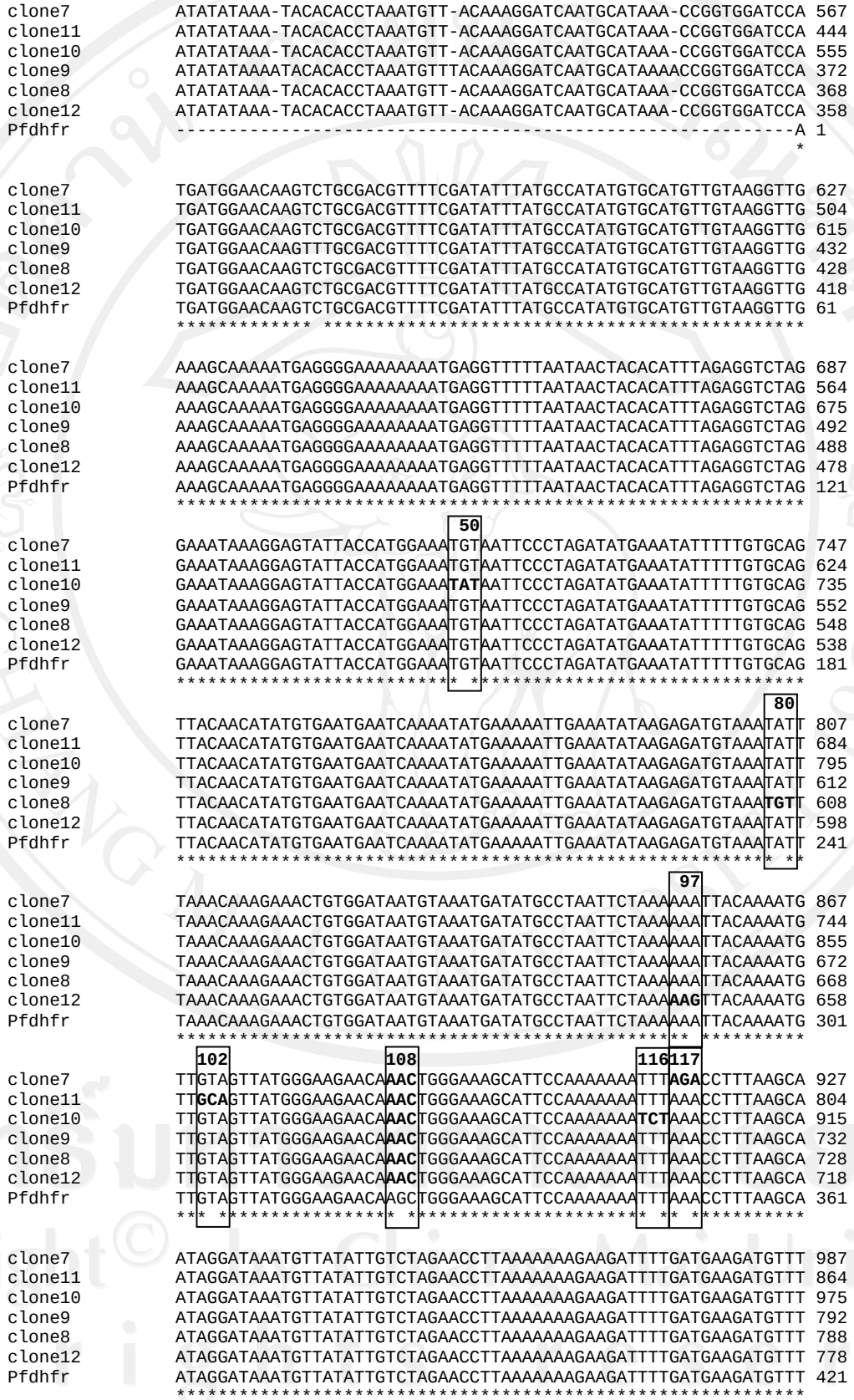
Figure 3.24 Sequence alignment of *Pfdhfr* variations (clones 1-6) from parasite transfected with random mutant *Pfdhfr* library (S108N template). The variations were aligned with wild-type *Pfdhfr* sequence using ClustalW program. Codons with base substitution are boxed. The amino acid positions are indicated above the aligned DNA sequences.



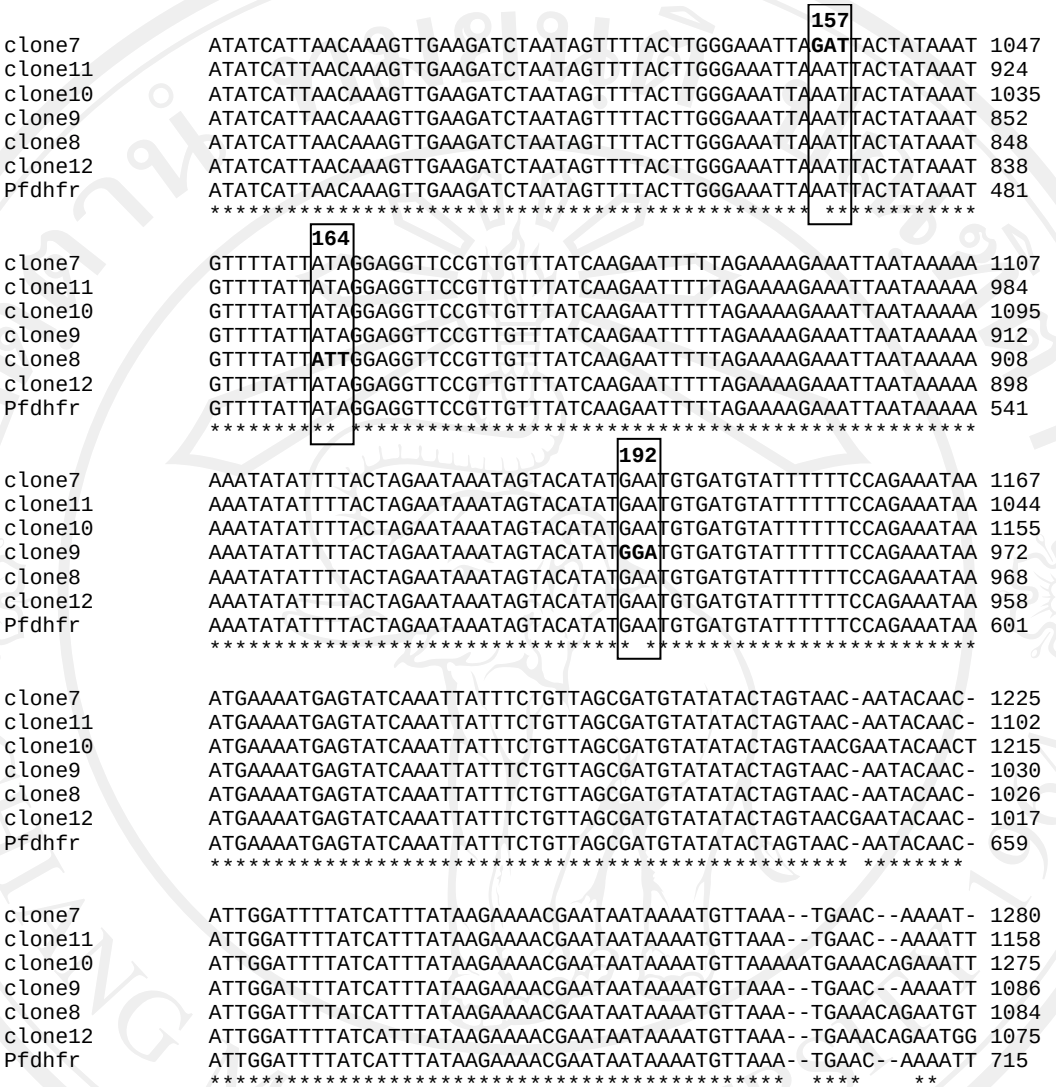


Figure 3.25 Sequence alignment of *Pfdhfr* variations (clones 7-12) from parasite transfected with random mutant *Pfdhfr* library (S108N template). The variations were aligned with wild-type *Pfdhfr* sequence using ClustalW program. Codons with base substitution are boxed. The amino acid positions are indicated above the aligned DNA sequences.

Table 3.4 Variation of *Pfdhfr* mutations recovered from transfected pyrimethamine-resistant parasites compared with *Pfdhfr* S108N template after transfection to *P. berghei* parasite and selection with 0.25 mg/kg of pyrimethamine. The amino acids are shown in alphabetical symbol, whereas the codon that codes for the amino acids is shown in brackets.

Clone	Amino acid Position	Base substitution conferring change in amino acid	
		Template (S108N)	Generated mutant
1	108	S108N	-
2	36	T (ACA)	T (ACG)
	55	M (ATG)	I (ATT)
	108	S108N	-
	189	S (AGT)	C (TGT)
3	29	N (AAT)	F (GAT)
	31	L (GTT)	F (TTT)
	108	S108N	
4	4	Q (CAA)	Q (CAG)
	6	C (TGC)	S (AGC)
	108	S108N	-
5	22	S (AGC)	C (TGC)
	50	C (TGT)	S (AGT)
	108	S108N	-
6	11	I (ATT)	T(ACT)
	24	N (AAT)	D (GAT)
	108	S108N	-

Table 3.4 Variation of *Pfdhfr* mutations recovered from transfected pyrimethamine-resistant parasites compared with *Pfdhfr* S108N template after transfection to *P. berghei* parasite and selection with 0.25 mg/kg of pyrimethamine. The amino acids are shown in alphabetical symbol, whereas the codon that codes for the amino acids is shown in brackets (continued).

Clone	Amino acid Position	Base substitution conferring change in amino acid	
		Template (S108N)	Generated mutant
7	108	S108N	-
	117	K (AAA)	R (AGA)
	157	N (AAT)	D (GAT)
8	80	Y (TAT)	C (TGT)
	108	S108N	-
	164	I (ATA)	I (ATT)
9	108	S108N	-
	192	E (GAA)	G (GGA)
10	50	C (TGT)	Y (TAT)
	108	S108N	
	116	F (TTT)	S (TCT)
11	102	V (GTA)	A (GCA)
	108	S108N	-
12	97	K (AAA)	K (AAG)
	108	S108N	-

Table 3.5 Summary of the variation of *Pfdhfr* mutation compared with S108N template after transfection to *P. berghei* parasite and selection with 0.25 mg/kg pyrimethamine.

Clone #	Amino acid #																			
	4	6	11	22	24	29	31	36	50	55	80	97	102	108	116	117	157	164	189	192
Template	Q	C	I	S	N	N	V	T	C	M	Y	K	V	N	F	K	N	I	S	E
1														N						
2								*T*		**I**				N					**C**	
3						F	F							N						
4	*Q*	S												N						
5				C					S					N						
6			T		D									N						
7														N		R	D			
8											C			N				*I*		
9														N						G
10									**Y**					N	**S**					
11													A	N						
12												*K*		N						

Note: Silent mutations are in italic. Mutations of interest are in bold.

3.8 Generation of transgenic *P. berghei* stably expressing *Pf*DHFR S108N single mutant (*PbPf*S108N parasite)

S108N mutation in *Pf*DHFR enzyme is the first single mutation that confers resistance to antifolate drugs. In this study, transgenic *P. berghei* parasite stably expressing *Pf*DHFR S108N single mutant was generated. The strategy for replacing *Pbdhfr* with *Pfdhfr* S108N gene is shown in Figure 3.28. The correct double crossover integration of targeting constructs was investigated by PCR analysis on genomic DNA using different pairs of primer set. The 5'UTR integration was determined by using forward primer 5'PbINF (A1) and reverse primer RAflII (C2). The 3'UTR integration was determined by using forward primer FBAMHI (C1) and reverse primer 3'PbINTR (A2). The PCR products of 4.0 kb and 3.0 kb band sizes confirmed 5'UTR and 3'UTR integration, respectively as shown in Figure 3.29 (lanes 1, 5). The presence of *Pfdhfr-ts* in the transgenic *PbPf*S108N parasite was analyzed by PCR using forward primer FBamHI (C1) and reverse primer RAflII (C2). The 0.7 kb band size of *Pfdhfr* was detected as shown in Figure 3.30, lane 1. The transgenic *PbPf*S108N was verified not to harbor *Pbdhfr-ts* by using forward primer PbDTF (B1) and forward primer PbDTR (B2), which are primers specific for *Pbdhfr-ts* gene. The *Pbdhfr-ts* was not detected in the transgenic *PbPf*S108N parasite as shown in Figure 3.31 (lane 1). The correct integration of targeting construct in *PbPf*S108N was further confirmed by Southern blot analysis using *Pfdhfr* probe as shown in Figure 3.33 (lane 1)

3.9 Generation of transgenic *P. berghei* stably expressing *Pf*DHFR M55I+S108N+S189C triple mutant (*PbPf*DHFR3m1 parasite)

The triple mutant *Pfdhfr*3m1 (M55I+S108N+S189C) was identified in section 3.7 as pyrimethamine resistant mutant from *P. berghei* episomally transfected with random library of *Pfdhfr* S108N. The amino acid position 55 is located around the active site region of *Pf*DHFR-TS. The model of double mutant *Pf*DHFR-TS (C59R+S108N; PfDHFR2M) complex with pyrimethamine and the positions of M55 and S189 are shown in Figure 3.26. It is possible that such mutations might decrease pyrimethamine sensitivity to parasites. In order to study the drug susceptibility of *PbPf*DHFR3m1 mutant parasite stably expressing *Pf*DHFR3m1 mutant was generated. The strategy for replacing endogenous *Pbdhfr-ts* with *Pfdhfr-ts*3m1 is shown in Figure 3.28. After transgenic parasite was obtained, the correct integrations were investigated by PCR analysis on genomic DNA using different pairs of primer set. The 5'UTR integration was determined by using forward primer 5'PbINF (A1) and reverse primer RAflII (C2). The 3'UTR integration was determined by using forward primer FBAMHI (C1) and reverse primer 3'PbINTR (A2). The PCR products of 4.0 kb and 3.0 kb band sizes confirmed 5'UTR and 3'UTR integrations, respectively as shown in Figure 3.29 (lanes 2, 6). The sequence of *Pfdhfr* in the transgenic *PbPf*DHFR3m1 parasite was analyzed by PCR using forward primer FBamHI (C1) and reverse primer RAflII (C2). The 0.7 kb band size of *Pfdhfr* is shown in Figure 3.30 (lane 2). The transgenic *PbPf*DHFR3m1 was also verified for the presence of *Pbdhfr-ts* by using forward primer PbDTF (B1) and reverse primer PbDTR (B2). The *Pbdhfr-ts* was not detected in the transgenic parasite

*PbPf*DHFR3m2 as shown in Figure 3.31 (lane 2). The correct integration of *Pfdhfr-ts*3m1 in *PbPf*DHFR3m1 was further confirmed by Southern blot analysis using *Pfdhfr* probe as shown in Figure 3.33 (lane 2).

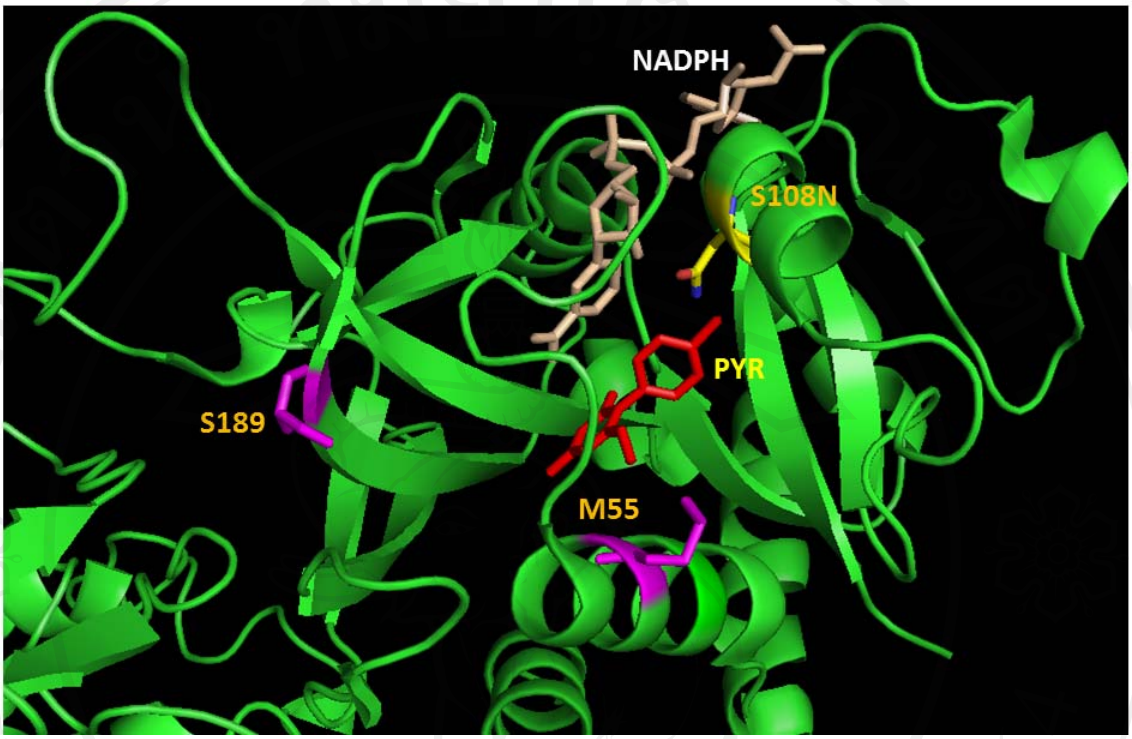


Figure 3.26 The model of double mutant *Pf*DHFR-TS (C59R+S108N; PfDHFR2M) in complexed with pyrimethamine. The positions of interest mutation, M55 and S189 are shown (pink amino acids). Figure was generated with PyMOL program (Haynes et al., 2005).

3.10 Generation of transgenic *P. berghei* stably expressing *Pf*DHFR C50Y+S108N+F116S triple mutant (*PbPf*DHFR3m2 parasite)

The triple mutant *Pfdhfr*3m2 (C50Y+S108N+F116S) was identified in section 3.7 as pyrimethamine resistant mutant from *P. berghei* episomally transfected with random library of *Pfdhfr* S108N. The amino acid position 50 and 116 are located around the active site region of *Pf*DHFR-TS. The model of double mutant *Pf*DHFR-TS (C59R+S108N; PfDHFR2M) complex with pyrimethamine and the positions of C50 and F116 are shown in Figure 3.27. It is possible that such mutations might decrease pyrimethamine sensitivity to parasites. In order to, study the drug susceptibility of *PbPf*DHFR3m2 mutant parasite stably expressing *Pf*DHFR3m2 mutant was generated. The strategy for replacing endogenous *Pbdhfr-ts* with *Pfdhfr-ts*3m2 event is shown in Figure 3.28. After transgenic parasite was obtained, the correct integrations were investigated by PCR analysis on genomic DNA using different pairs of primer set. The 5'UTR integration was determined by using forward primer 5'PbINF (A1) and reverse primer RAflII (C2). The 3'UTR integration was determined by using forward primer FBAMHI (C1) and reverse primer 3'PbINTR (A2). The PCR products of 4.0 kb and 3.0 kb band sizes confirmed 5'UTR and 3'UTR integrations, respectively as shown in Figure 3.29 (lanes 3, 7). The sequence of *Pfdhfr* in the transgenic *PbPf*DHFR3m2 parasite was analyzed by PCR using forward primer FBamHI (C1) and reverse primer RAflII (C2). The 0.7 kb band size of *Pfdhfr* is shown in Figure 3.30, lane 3. The transgenic *PbPf*DHFR3m2 was also verified for the presence of *Pbdhfr-ts* by using forward primer PbDTF (B1) and reverse primer PbDTR (B2). The *Pbdhfr-ts* was not detected in the transgenic parasite

PbPfDHFR3m2 as shown in Figure 3.31 (lane 3). The correct integration of *Pf*DHFR3m2 in *PbPf*DHFR3m1 was further confirmed by southern blot analysis using *Pfdhfr* probe as shown in Figure 3.32 (lane 3).

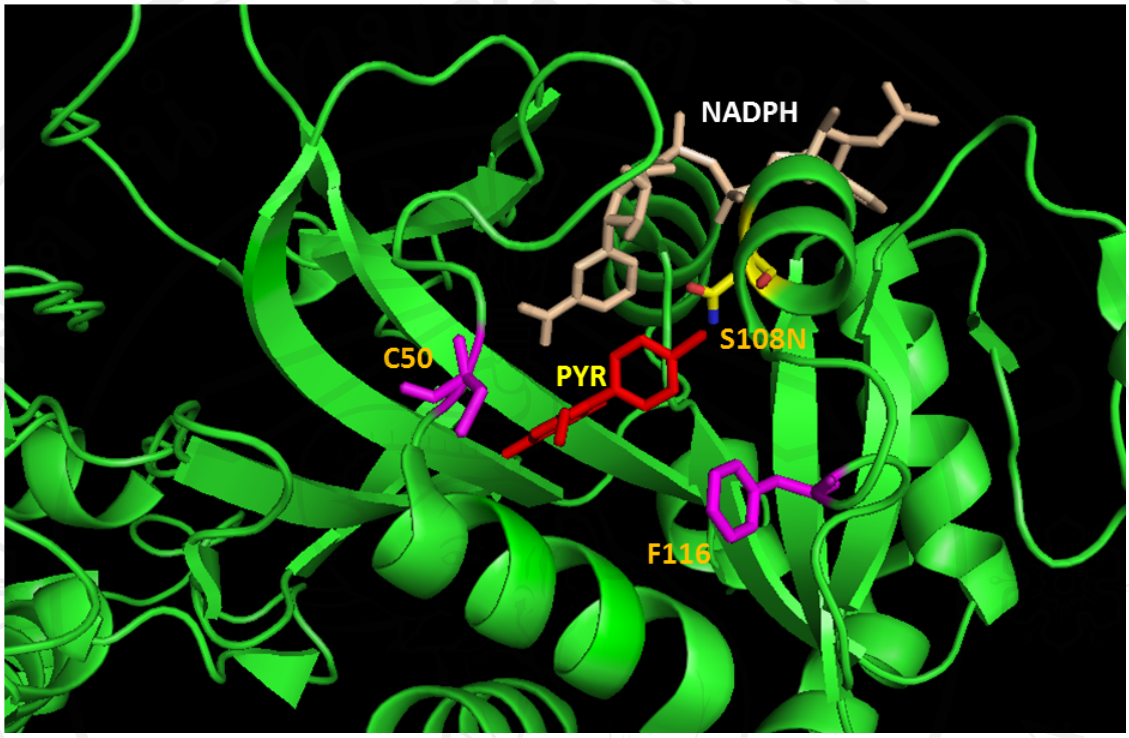


Figure 3.27 The model of double mutant *Pf*DHFR-TS (C59R+S108N; PfDHFR2M) in complexed with pyrimethamine. The positions of interest mutation, C50 and F116 are shown (pink amino acids). Figure was generated with PyMOL program (Haynes et al., 2005).

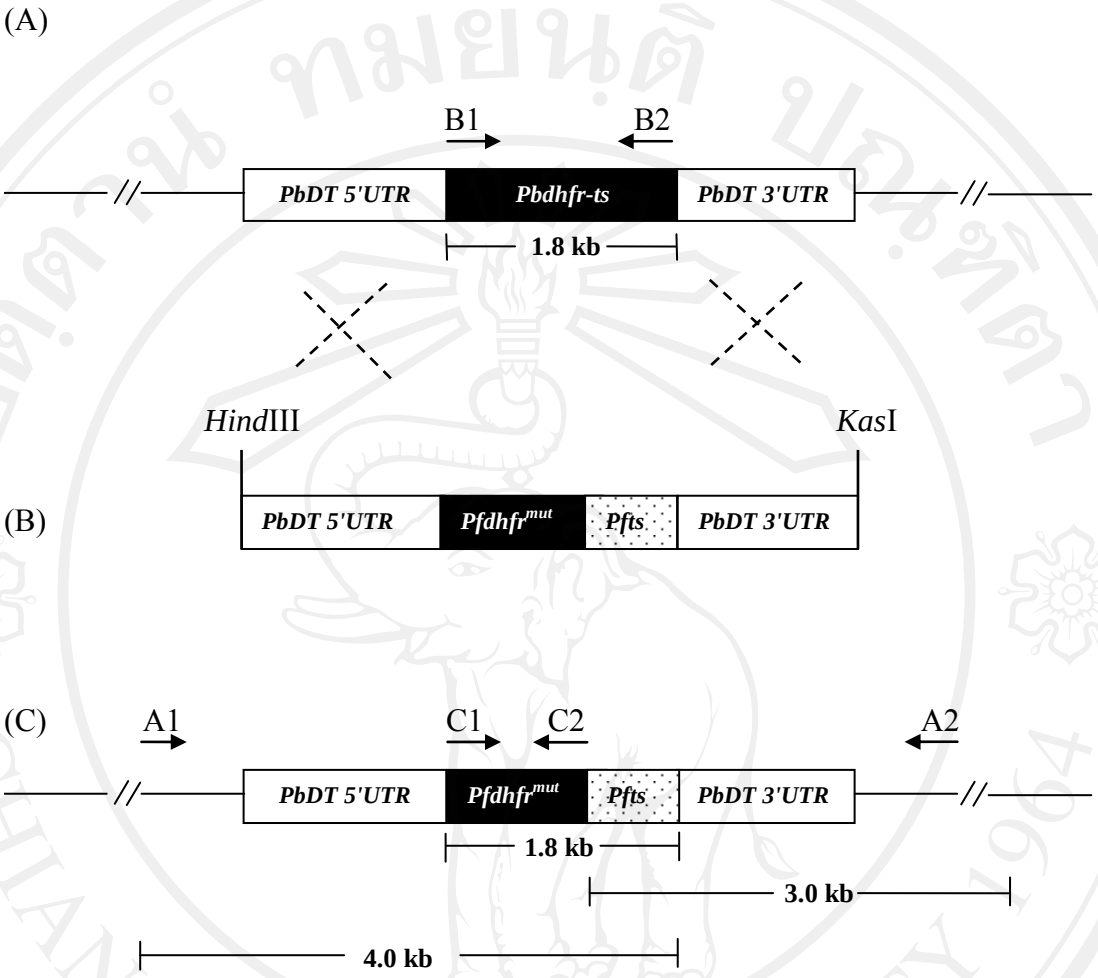


Figure 3.28 Replacement strategy of mutant *Pfdhfr-ts* into *Pbdhfr-ts* locus by double cross-over homologous recombination, (A) wild-type *P. berghei dhfr-ts* endogenous gene, (B) linearized plasmid containing mutant *Pfdhfr* digested with *Hind*III and *Kas*I enzyme, (C) correct integration of the construct contributed to the replacement of mutant *Pfdhfr*, the position of the primers used for PCR amplification are indicated by arrows. The expected band sizes are shown.

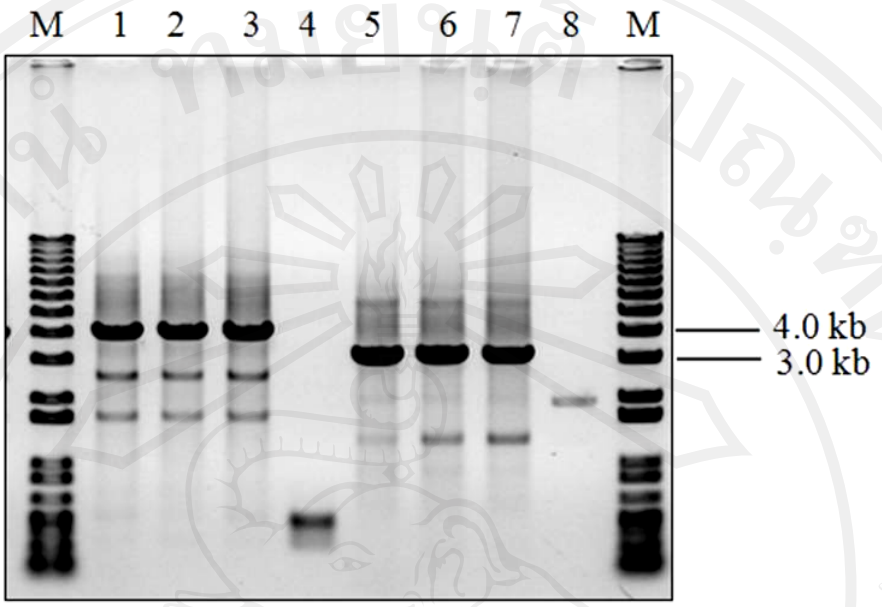


Figure 3.29 PCR analysis of 5' and 3'UTR integration of *Pfdhfr-ts* replacing endogenous *Pbdhfr-ts* locus on genomic DNA isolated from transgenic *P. berghei* parasites expressing *Pf*DHFR mutants. A 4.0 kb band indicating the 5'UTR integration was detected from the genomic DNA of *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2 transgenic parasites (lanes 1-3, respectively). A 3.0 kb band indicating the 3'UTR integration of the *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2 transgenic parasites was detected from (lanes 5-7, respectively). Genomic DNA of wild-type *P. berghei* was used as negative control (lanes 4 and 8). The 1 kb plus molecular markers are shown in lanes M.

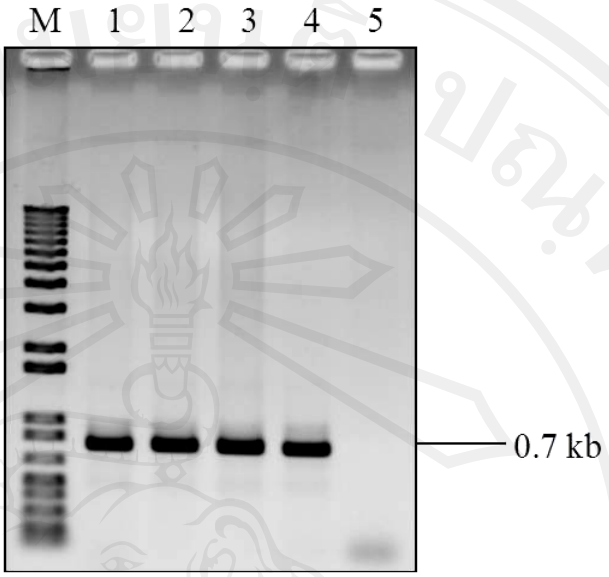


Figure 3.30 PCR analysis of *Pfdhfr* in genomic DNA isolated from transgenic parasites, *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2 (lanes 1-3, respectively). pY005 plasmid (lane 4) and *P. berghei* genomic DNA (lane 5) served as positive and negative control, respectively. The 1 kb plus molecular marker is shown in lane M.

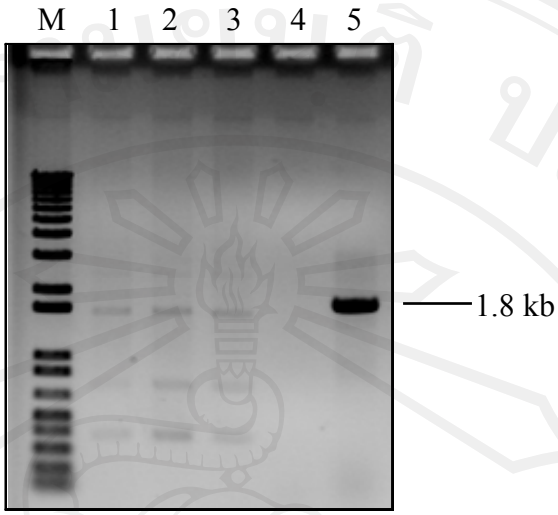


Figure 3.31 PCR analysis of *Pbdhfr-ts* in genomic DNA isolated from transgenic mutant parasites. *Pbdhfr-ts* was not detected in the transgenic parasites *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2.as shown in lanes 1-3, respectively. pY005 plasmid and *P. berghei* genomic DNA (lane 5) served as negative and positive control, (lanes 4 and 5), respectively. The 1 kb plus molecular marker is shown in lane M.

3.11 Southern blot analysis of transgenic *P. berghei* parasite stably expressing *Pf*DHFR mutants

The correct integration of *Pfdhfr-ts* gene in transgenic parasite was further verified by Southern blot analysis using *Pfdhfr* probe. The episomal plasmid contained pY005^{S108N} an *Eco*RI site in *Pfdhfr* gene whereas the transgenic *P. berghei* *Eco*RI site in endogenous gene of 5'UTR of *Pbdhfr-ts* and in the introduced *Pfdhfr* locus as shown in Figure 3.32. After digestion of the genomic DNA of transgenic mutant parasites with *Eco*RI enzyme, a 4.8 kb band size of DNA fragment could be detected as shown in Figure 3.33 while the pY005^{S108N} plasmid control shows a band of 7.5 kb. Genomic DNA of *P. berghei* parasite was used as the negative control.

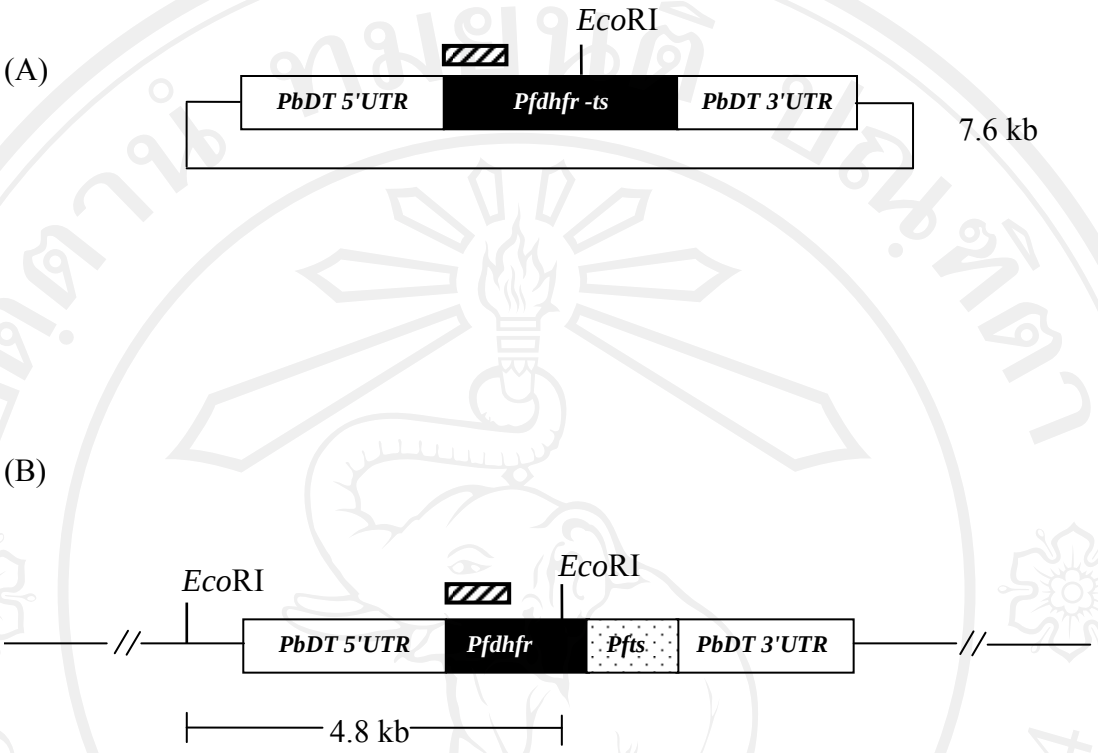


Figure 3.32 Restriction analysis pattern for Southern analysis of transgenic *P. berghei* parasites expressing *Pf*DHFR mutants. (A) the expected fragment size of pY005^{S108N} plasmid and (B) the expected fragment size of genomic DNA of transgenic parasites digested with *Eco*RI enzyme after probing with *Pfdhfr* probe (slash bar).

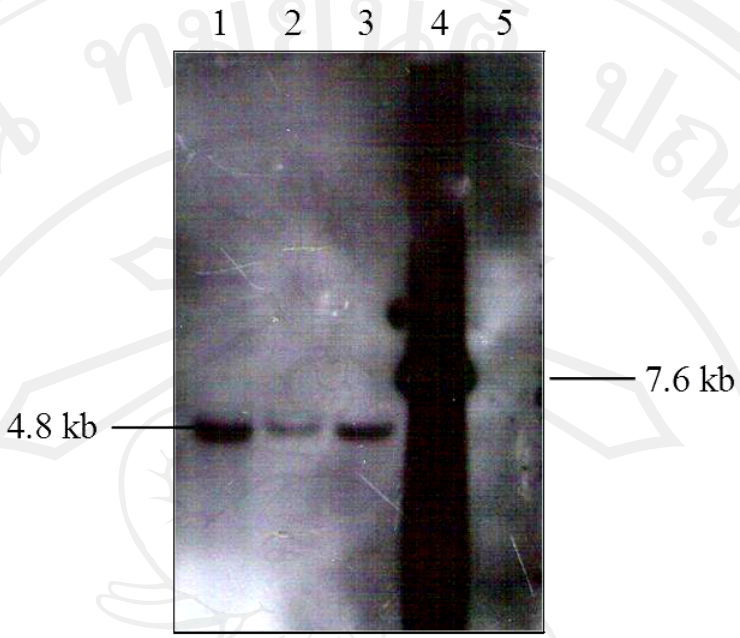


Figure 3.33 Southern blot analysis of transgenic *P. berghei* parasite expressing *Pf*DHFR mutant. Genomic DNA of transgenic mutant parasite (*PbPf*S108N = lane 1, *PbPf*DHFR3m1 = lane 2, *PbPf*DHFR3m2 = lane 3), pY005^{S108N} plasmid (lane 4) and *P. berghei* parasite genomic DNA (negative control, lane 5) were digested with *Eco*RI and the blot was probed with *Pfdhfr* probe.

3.12 Expression profile analysis of transgenic *P. berghei* expressing *Pf*DHFR mutants

The transgenic *P. berghei* parasites express *Pf*DHFR-TS under control of endogenous 5'UTR and 3'UTR of *Pbdhfr-ts.* The expression of the enzyme was verified by mRNA transcription using reverse-transcriptase polymerase chain reaction (RT-PCR). The mRNA was isolated from transgenic parasites, *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2. The cDNA of *Pfdhfr-ts*, *Pbdhfr-ts* and *P. berghei* alpha tubulin housekeeping gene from each transgenic parasite were derived by reverse transcription using gene specific primers. The cDNA were then used as template to amplify *Pfdhfr*, *Pbdhfr* and alpha tubulin gene. As shown in Figure 3.34, a 0.7 kb band corresponding to *Pfdhfr* was detected in all transgenic parasites, but not in *P. berghei* parasite. As shown in Figure 3.35, *Pbdhfr* was not detected in transgenic parasites but could be detected the wild-type in *P. berghei* parasite**.** A 0.85 kb band corresponding to *P. berghei* alpha tubulin cDNA were detected in all transgenic and wild-type *P. berghei* parasites while a 1.2 kb band corresponding to *P. berghei* alpha tubulin was detected in genomic DNA of all *P. berghei* as shown in Figure 3.36.

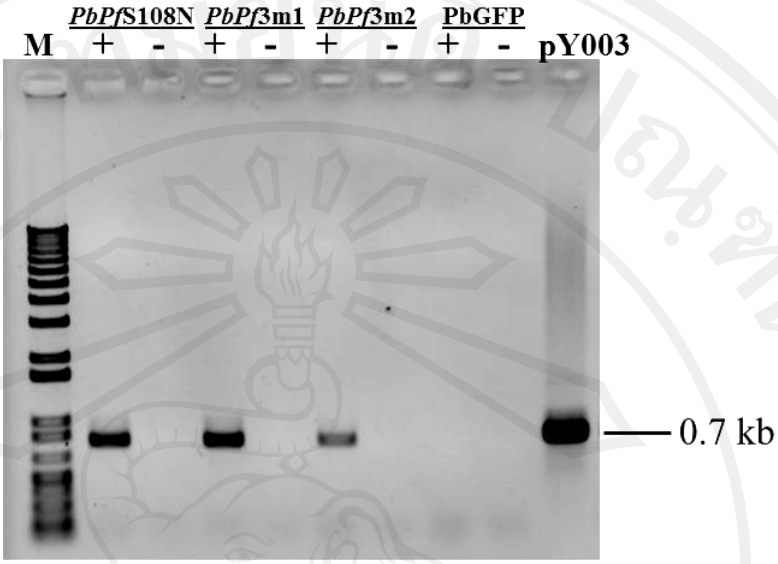


Figure 3.34 RT-PCR analysis of mutant *Pfdhfr* expression in transgenic *P. berghei*. RNA from transgenic *P. berghei* parasites expressing *Pf*DHFR mutants was reverse transcribed into cDNA and used as template for detection of *Pfdhfr* transcript. *PbPf*3m1: *PbPf*DHFR3m1, *PbPf*3m2: *PbPf*DHFR3m2. *P. berghei* cDNA derived from PbGFP was used as negative control. The reactions were performed with reverse transcription (+) and without reverse transcription (-). pY003 plasmid was used as positive control. The 1 kb plus molecular marker is shown in lane M.

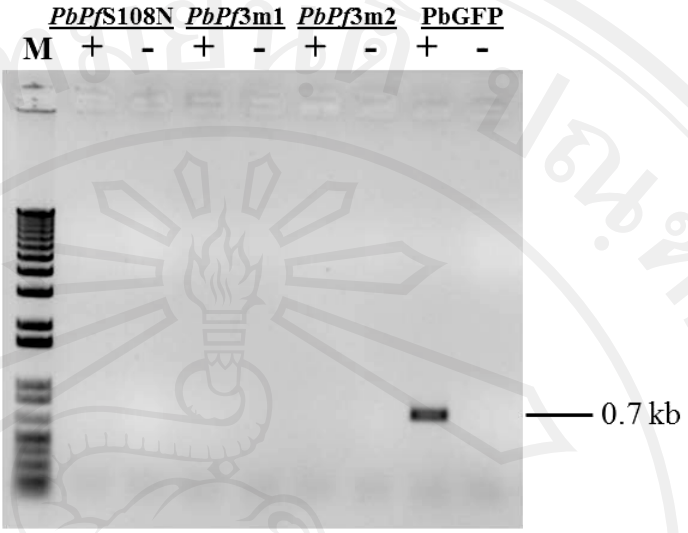


Figure 3.35 RT-PCR analysis of mutant *Pfdhfr* expression in transgenic *P. berghei*. RNA from transgenic *P. berghei* parasites expressing *Pf*DHFR mutant was reverse transcribed into cDNA and used as template for detection of *Pbdhfr* transcript. *PbPf*3m1: *PbPf*DHFR3m1, *PbPf*3m2: *PbPf*DHFR3m2. *P. berghei* cDNA derived from PbGFP was used as positive control. The reactions were performed with reverse transcription (+) and without reverse transcription (-). The 1 kb plus molecular marker is shown in lane M.

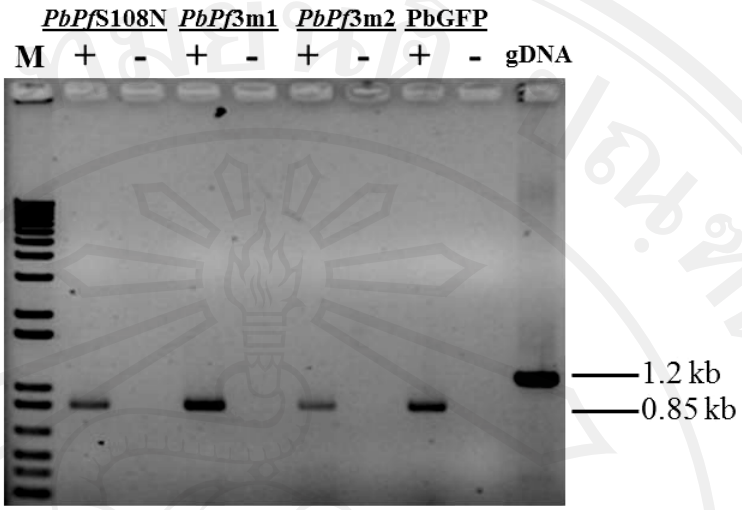


Figure 3.36 RT-PCR analysis of mutant *Pfdhfr* expression in transgenic *P. berghei*. RNA from transgenic *P. berghei* parasites expressing *Pf*DHFR mutants was reverse transcribed into cDNA and use as template for detection of *P. berghei* alpha tubulin gene *PbPf*3m1: *PbPf*DHFR3m1, *PbPf*3m2: *PbPf*DHFR3m2. *P. berghei* cDNA derived from PbGFP and PbGFP genomic DNA were used as positive controls. The reactions were performed with reverse transcription (+) and without reverse transcription (-). The 1 kb plus molecular marker is shown in lane M.

3.13 Determination of growth rate of transgenic *P. berghei* expressing *Pf*DHFR mutants

The growth rate of transgenic parasites, *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2 were compared with wild-type PbGFP parental parasite. Mice were infected with 1×10^7 parasites and the parasitemia was monitored for 4 days. The experiments were performed in three independent studies and the data represents mean values ± SD as shown in Table 3.6 and Figure 3.37. The growth rates of the four parasites were not significantly different.

Table 3.6 Growth profile of wild-type PbGFP parasite and transgenic *P. berghei* expressing *Pf*DHFR mutant in mice.

Parasite line	**Mean of % Parasitemia ± SD.**			
	Day 1	**Day 2**	**Day 3**	**Day 4**
PbGFP	0.31 ± 0.14	2.72 ± 0.36	7.66 ± 0.84	19.69 ± 2.23
***PbPf*S108N**	0.57 ± 0.13	2.05 ± 0.22	7.80 ± 1.06	15.98 ± 4.03
***PbPf*DHFR3m1**	0.71 ± 0.21	3.20 ± 0.59	9.49 ± 1.58	16.24 ± 0.40
***PbPf*DHFR3m2**	0.82 ± 0.12	3.04 ± 1.44	10.07 ± 2.67	19.44 ± 0.77

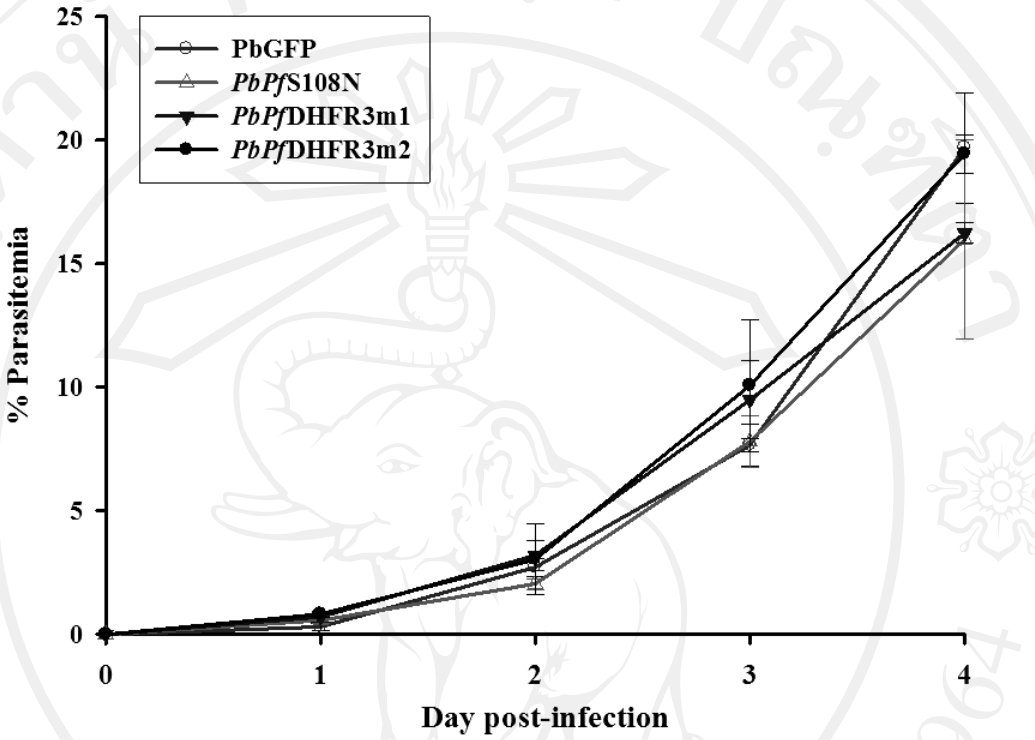


Figure 3.37 Growth curves of transgenic *P. berghei* expressing *Pf*DHFR mutants and wild-type PbGFP in mice. Data of wild-type PbGFP, *PbPf*S108N (S108N), *PbPf*DHFR3m1(M55I+S108N+S189C) and *PbPf*DHFR3m2 (C50Y+S108N+F116S) are represented as open circles, open triangles, filled triangles and filled circles, respectively. The experiments were performed in three independent studies and the data represents mean % parasitemia ± SD.

3.14 Determination of pyrimethamine sensitivity in transgenic *P. berghei* expressing single mutant *Pf*DHFRS108N parasite

The efficacy of pyrimethamine to inhibit *PbPf*S108N parasite was determined by 4-day suppressive test. The experimental mice were treated with different concentrations of pyrimethamine at 0.3 1, 3, 9, 18 mg/kg, respectively. The parasitemia of treated mice were determined by counting numbers of infected erythrocytes from Giemsa stained smear. The experiments were performed in three independent studies and the data represents mean values ± SD of percentage of growth inhibition as shown in Table 3.7. Figure 3.38 shows the dose-growth inhibition curve of pyrimethamine against transgenic *PbPf*S108N parasite depicting *in vivo* ED_{50} and ED_{90} as 1.08 mg/kg and 4.13 mg/kg, respectively.

Table 3.7 Inhibition of transgenic *PbPf*S108N parasite by pyrimethamine in mice.

Group	**Pyrimethamine (mg/kg)**	**% Growth inhibition**			**Mean of % inhibition ± SD.**
		Exp. I	Exp. II	Exp. III	
1	18.0	100	100	100	100 ± 0.00
2	9.0	96.74	100	96.35	97.70 ± 2.00
3	3.0	75.00	87.63	93.20	85.28 ± 9.33
4	1.0	47.73	56.54	31.66	45.31 ± 12.59
5	0.3	11.26	23.60	10.44	15.10 ± 4.26

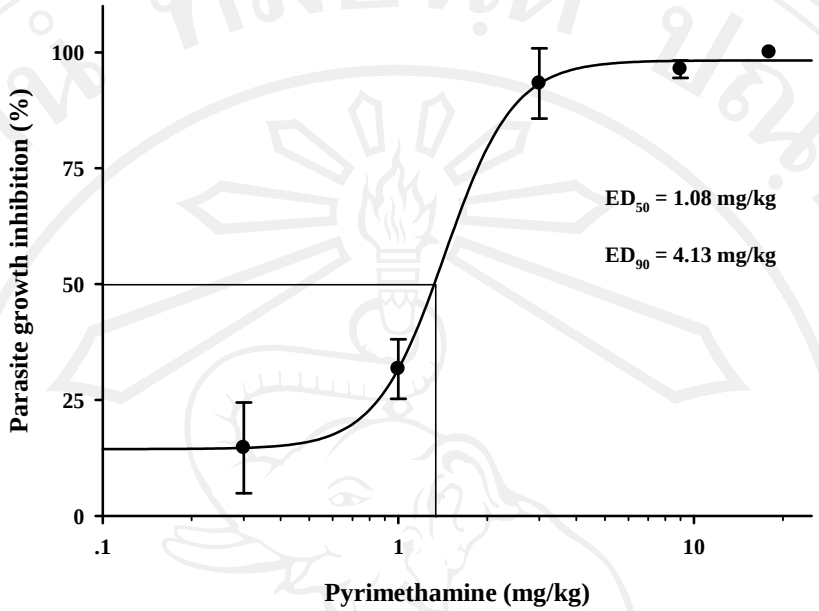


Figure 3.38 Dose-inhibition curve of pyrimethamine against transgenic *PbPf*S108N parasite. The experiments were performed in three independent studies and the data represents mean ± SD values of percentage of growth inhibition.

3.15 Determination of pyrimethamine sensitivity in transgenic *P. berghei* expressing triple mutant *PbPf*DHFR3m1 and *PbPf*DHFR3m2 parasite

The efficacy of pyrimethmaine to inhibit *PbPf*DHFR3m1 and *PbPf*DHFR3m2 parasite was determined by 4-day suppressive test. The experimental mice were treated with different concentration of pyrimethamine at 0.3, 1, 3, 9, 18 mg/kg. The parasitemia of treated mice were determined by counting numbers of infected erythrocytes on Giemsa stained smear. The experiments were performed in three independent studies and the data represents mean ± SD of percentage of growth inhibition values of *PbPf*DHFR3m1 and *PbPf*DHFR3m2 parasite as shown in Table 3.8 and Table 3.9, respectively. Figure 3.39 show the dose-growth inhibition curve of pyrimethamine against transgenic, *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2 parasites. The average ED_{50} values of pyrimethamine against *PbPf*DHFR3m1 and *PbPf*DHFR3m2 are 1.61 ± 0.70 and 1.07± 0.39 mg/kg, respectively.

Table 3.8 Percentage inhibition of transgenic *PbPf*DHFR3m1 parasites by pyrimethamine in mice.

Group	Pyrimethamine (mg/kg)	% Growth inhibition			Mean of % inhibition ± SD.
		Exp. I	Exp. II	Exp. III	
1	18.0	100	100	100	100 ± 0.00
2	9.0	90.66	96.22	100	95.63 ± 4.70
3	3.0	75.26	70.00	91.12	78.79 ± 10.99
4	1.0	15.71	38.50	51.89	35.37 ± 18.29
5	0.3	16.17	12.02	14.23	14.14 ± 2.00

Table 3.9 Inhibition of transgenic *PbPf*DHFR3m2 parasite by pyrimethamine in mice.

Group	Pyrimethamine (mg/kg)	% Growth inhibition			Mean of % inhibition ± SD.
		Exp. I	ExP. II	Exp. III	
1	18.0	100	100	100	100 ± 0.00
2	9.0	94.45	100	100	98.15 ± 3.20
3	3.0	71.13	76.52	80.00	75.88 ± 4.47
4	1.0	37.14	56.54	54.42	49.37 ± 10.64
5	0.3	13.53	18.70	21.31	17.85 ± 3.96

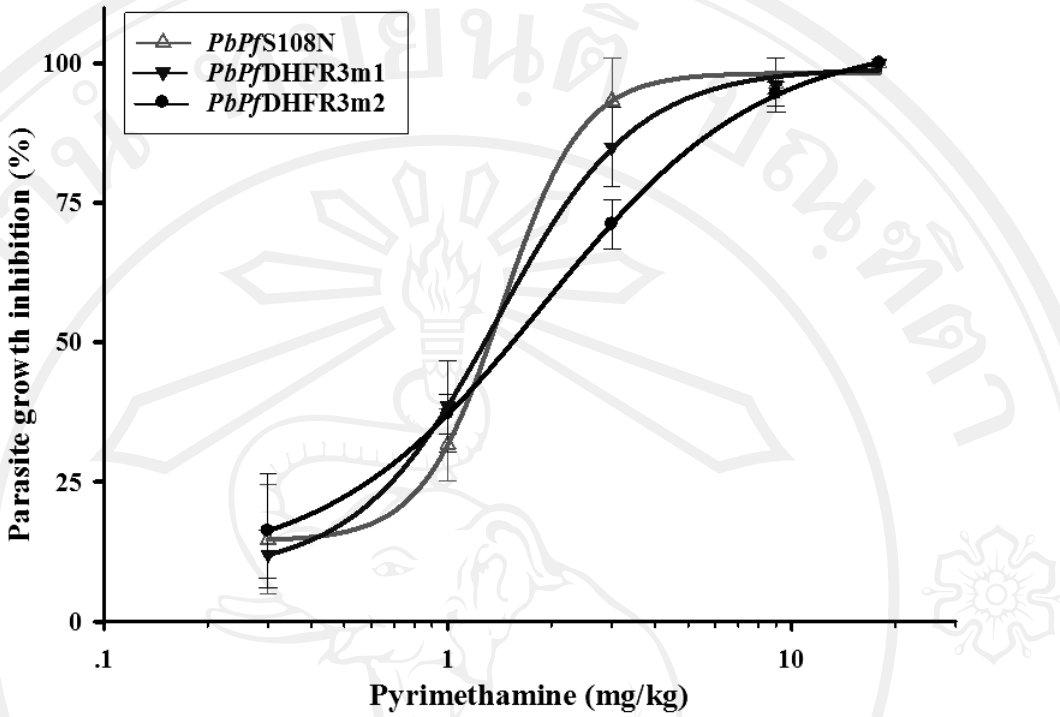


Figure 3.39 Pyrimethamine susceptibility profile of transgenic *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2 parasites. The experiments were performed in three independent studies and the data represents mean ± SD values. Data of *PbPf*S108N (S108N), *PbPf*DHFR3m1(M55I+S108N+S189C) and *PbPf*DHFR3m2 (C50Y+S108N+F116S) are represented as open triangles, filled triangles and filled circles, respectively. The average ED_{50} values of pyrimethamine against *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2 are 1.08 ± 0.23, 1.61 ± 0.70 and 1.07 ± 0.39 mg/kg, respectively.

3.16 Comparison of pyrimethamine sensitivity among transgenic parasites *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2

The averages ED_{50} of pyrimethamine against *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2 from three independent studies are 1.08, 1.61 and 1.07 mg/kg, respectively. There was no statistical significant difference in the ED_{50} at 95% confidence interval using the unpaired *t-test* to compare the means of each cloned parasite with the parent clone (*PbPf*S108N). *P*-values of *PbPf*DHFR3m1 and *PbPf*DHFR3m2 against *PbPf*S108N were 0.28 and 0.99, respectively as shown in Table 3.10. The inhibition profile of pyrimethamine against the parasites was similar with the original clone line (*PbPf*S108N). Mean percentage inhibition with standard deviation is shown in Tables 3.11 and 3.12.

Table 3.10 Comparison of ED_{50} of pyrimethamine against transgenic parasites *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2.

Parasite line	ED_{50} value (mg/kg)			Mean of ED_{50} ± SD.	*P.* value
	Exp. I	Exp. II	Exp. III		
***PbPf*S108N**	1.10	0.84	1.30	1.08 ± 0.23	-
***PbPf*DHFR3m1**	2.35	1.52	0.96	1.61 ± 0.70	0.28
***PbPf*DHFR3m2**	1.53	0.83	0.87	1.07 ± 0.39	0.99

Table 3.11 Inhibition profile of *PbPf*S108N and *PbPf*DHFR3m1 at different doses of pyrimethamine.

Group	**Pyrimethamine (mg/kg)**	**Mean of % inhibition ± SD.**		***P*. value**
		***PbPf*S108N**	***PbPf*DHFR3m1**	
1	18.0	100 ± 0.00	100 ± 0.00	1.00
2	9.0	97.7 ± 2.00	95.63 ± 4.70	0.78
3	3.0	85.28 ± 9.33	78.79 ± 10.99	0.38
4	1.0	45.31 ± 12.59	35.37 ± 18.29	0.19
5	0.3	15.10 ± 7.37	14.14 ± 2.07	0.90

Table 3.12 Inhibition profile of *PbPf*S108N and *PbPf*DHFR3m2 at different doses of pyrimethamine.

Group	**Pyrimethamine (mg/kg)**	**Mean of % inhibition ± SD.**		***P*. value**
		***PbPf*S108N**	***PbPf*DHFR3m2**	
1	18.0	100 ± 0.00	100 ± 0.00	1.00
2	9.0	97.7 ± 2.00	98.15 ± 3.20	0.94
3	3.0	85.28 ± 9.33	75.88 ± 4.47	0.11
4	1.0	45.31 ± 12.59	49.37 ± 10.64	0.47
5	0.3	15.10 ± 7.37	17.85 ± 3.96	0.63

3.17 Determination of pyrimethamine sensitivity in *PbPf*K1 parasite

The efficacy of pyrimethamine to inhibit transgenic *P. berghei* expressing double mutant *Pf*DHFR (C59R+S108N) (*PbPf*K1) parasite was determined by 4-day suppressive test. The experimental mice were treated with the different concentration of pyrimethamine at 3, 6, 12, 20 mg/kg. The parasitemia of treated mice were determined by counting numbers of infected erythrocytes from Giemsa stained smear. The data represents mean values of five mice in each group as shown in Table 3.13, Figure 3.43 shows the dose-growth inhibition curve of pyrimethamine against transgenic *PbPf*K1 parasite depicting *in vivo* ED_{50} as 10 mg/kg.

Table 3.13 Inhibition of transgenic *PbPf*K1 parasite (episomal form) by pyrimethamine in mice.

Group	Pyrimethamine (mg/kg)	Mean of % inhibition ± SD.
1	20.0	70.62 ± 2.93
2	12.0	56.01 ± 2.75
3	6.0	34.48 ± 10.66
4	3.0	24.92 ± 9.25

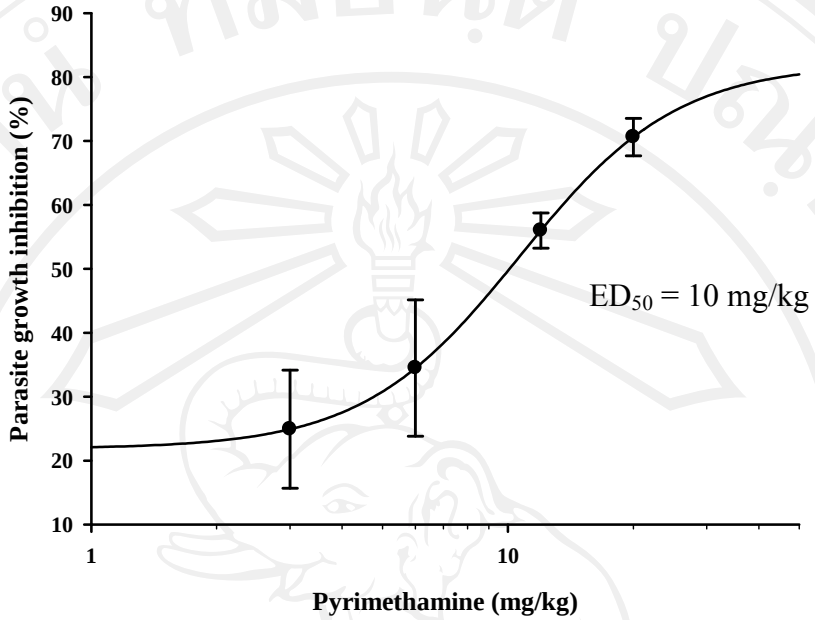


Figure 3.40 Dose-inhibition curve of pyrimethamine against transgenic *PbPf*K1 parasite (episomal form). The data represents mean values ± SD of percentage of growth inhibition for five animals per group.