CHAPTER 3

RESULTS

This study on generation of transgenic *P. berghei* parasites expressing *Pv*DHFR-TS for *in vivo* antifolate screening was divided into three parts. The first part focused on construction of transfection plasmids. The work included the construction of coding region of entire *pvdhfr-ts* either wild type or double mutant (SP21) genes flanked by *pbdhfr-ts* flanking regions which functioned as promoter and terminator of the transfection plasmids and used as sites for homologous recombination into the genome of *P. berghei*. Following the double crossover into the genome of *P. berghei*, the *pbdhfr-ts* endogenous gene was replaced with *pvdhfr-ts* construct. For drug selection after *P. berghei* transfection, SP21 conferring pyrimethamine resistance can be used as a drug selectable marker directly, whereas wild type *pvdhfr-ts* gene needed to be sub-cloned into another transfection plasmid which contains positive-negative drug selectable markers. A fusion of positive (*hdhfr*)-negative (*yfcu*) selectable marker gene was used to select pyrimethamine-resistant parasite after transfection, and 5-FC was subsequently used to treat the transfected parasites that contain *yfcu*. After 5-FC treatment, the drug selectable marker cassette was removed leaving the wild type *pvdhfr-ts* gene intact in the integrated locus.

The second part was transfection of *P. berghei* parasite to integrate the plasmid construct into the genome and replace the endogenous *pbdhfr-ts* gene. Linearized transfection plasmid was introduced to purified mature schizonts by electroporation using Amaxa Nucleofector device and pre-set U-033 program. In addition, protocols for small-scale *in vitro* culture from 20 µl of mouse tail blood and purification of mature schizonts sufficient for a single transfection experiment were also developed. Drug selection was performed after 24 hr post-injection with pyrimethamine prepared in drinking water. Integration events and replacement of *pbdhfr-ts* were confirmed by plasmid rescue, specific PCR, Southern blot, and RT-PCR. Transfected parasites were subsequently cloned by limiting dilution technique.

The third part was the evaluation of the transgenic parasites as *in vivo* models for primary screening of the inhibitors against *Pv*DHFR-TS. The 4-day suppressive test was used to test the transgenic models with standard inhibitors including pyrimethamine, chloroquine and artesunate.

3.1 Construction of transfection plasmids for *P. vivax* dihydrofolate reductase-thymidylate synthase expression in *P. berghei* parasites

3.1.1 Design of transfection plasmids for introduction of *P. vivax* dihydrofolate reductase-thymidylate synthase gene into *P. berghei* parasites

In order to introduce *pvdhfr-ts* gene into the genome of *P. berghei*, the targeting plasmids were designed to have two homologous sequences for double crossover recombination to replace the endogenous *pbdhfr-ts* gene. For homologous sequences, 5' and 3'UTR of *pbdhfr-ts* were used. The pyrimethamine-resistant marker of *tgdhfr-ts* in the plasmid pL0017 was substituted by *pvdhfr-ts* either wild

type or double mutant SP21 genes via *Age*I and *Nhe*I restriction sites. In the case of *pvdhfr-ts* double mutant SP21 that by itself confers pyrimethamine resistance, the plasmid could be used directly for transfection and selection with pyrimethamine. For the wild type gene of *pvdhfr-ts* which is sensitive to pyrimethamine, the plasmid needed further modification to contain drug selectable markers. Therefore, the *pbdhfr-ts* wild type expression cassette was sub-cloned into the plasmid pL0035 that contains an expression cassette of a fusion gene of the positive (*hdhfr*) and negative selectable markers (*yfcu*), via the restriction sites of *Hind*III and *Afl*II. After transfection, pyrimethamine was used to select resistant parasites that contained *hdhfr* gene. In order to remove the positive-negative selectable cassette, 5-fluorocytosine (5-FC) was used.

3.1.2 Construction of transfection plasmids

3.1.2.1 Construction of transfection plasmid pL35pvDHFR-TS

Wild type *pvdhfr-ts* gene was PCR amplified from the plasmid pETpvDHFR-TS as template (a gift from Dr. Ubolsree Leartsakulpanich at Protein-Ligand Engineering and Molecular Biology Laboratory, BIOTEC, NSTDA) using the primers Sense-*Pv*DT-*Age*I and Antisense-*Pv*DT-*Nhe*I (**Table 2-1**). *Age*I and *Nhe*I restriction sites were incorporated into the 5' and 3' end of the resulting PCR product, respectively. The amplification reaction was set up in a total volume of 25 µl, containing 10 ng of pETpvDHFR-TS, 0.8 µM of each primer, 400 µM of dNTPs, 1x Pfu buffer, 1µg/ml of BSA, 1 U of *Taq* DNA polymerase (Promega), and 1 U of *Pfu* DNA polymerase (Promega). Temperature cycling parameters used for PCR reaction were as follows: (1 cycle) 95^{o}C for 2 min, (30 cycles) 95^{o}C for 45 sec, 55^{o}C for 45

sec, 60°C for 2 min, (last cycle) 95°C for 45 sec, 55°C for 45 sec, and 60°C for 7 min. The expected 1.8-kb PCR product was cleaned up using QIAquick Gel/PCR Extraction Kit (Qiagen). The purified PCR product was double digested with *Age*I and *Nhe*I, and re-purified using the same extraction kit to remove small DNA fragments after digestion with these enzymes (**Figure 3-1**).

The plasmid pL0017 was prepared from QIAprep Spin Miniprep Kit and double-digested with *Age*I and *Nhe*I restriction enzymes. The digested plasmid was subjected to agarose gel electrophoresis and the DNA band of ~9.2 kb was excised and purified using QIAquick Gel/PCR Extraction Kit. The purified digested plasmid was subsequently ligated to the *Age*I/*Nhe*I-digested wild type *pvdhfr-ts*. The ligation reaction included 100 ng of *Age*I/*Nhe*I-digested wild type *pvdhfr-ts*, 50 ng of purified *Age*I/*Nhe*I-digested pL0017 plasmid, 400 U of T4 DNA Ligase (New England Biolabs, NEB), and 1x ligation buffer in a total volume of 20 µl. After incubation at 14°C overnight, the ligation sample was used to transform into *E. coli* DH5α competent cells by heat shock protocol (**Section 2.2.6.2**). The transformed sample was plated onto LB agar plate containing 100 µg/ml ampicillin and incubated at 37°C overnight. Colonies were randomly picked, the plasmid DNA was prepared by alkaline lysis method and then analyzed by restriction digestion followed by analysis on agarose gel electrophoresis. The desired clone was verified for the inserted *pvdhfr-ts* sequence by automated DNA sequencing (BioService Unit/Biodesign) using *Pv*DTF and *Pv*DTR primers (**Table 2-1**). The recombinant plasmid containing full-length gene of wild type *pvdhfr-ts* was designated '**pL17pvDHFR-TS'** (**Figure 3-1** and **3-4**).

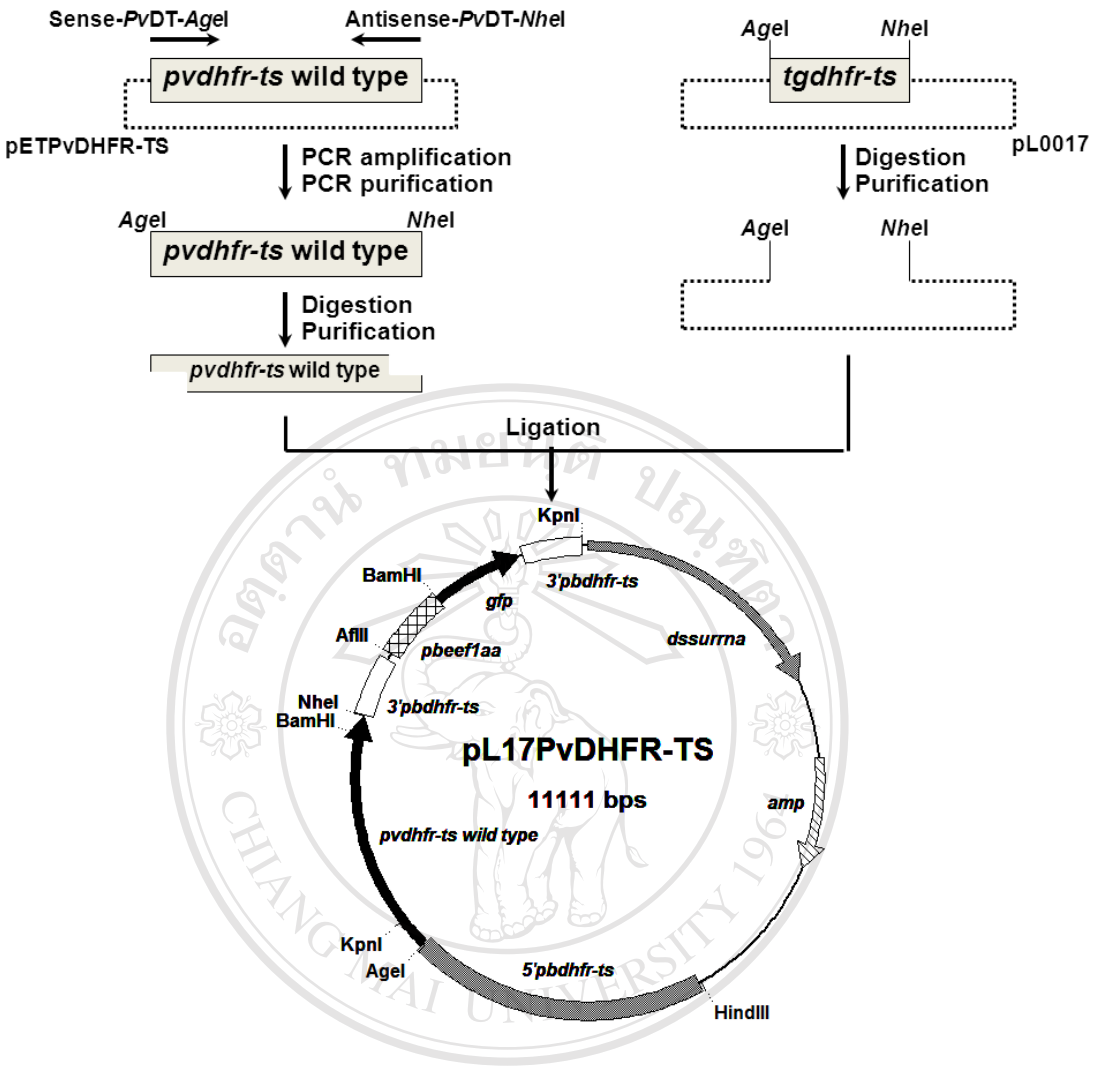


Figure 3-1 Construction of pL17pvDHFR-TS plasmid. Wild type *pvdhfr-ts* gene was PCR amplified and cloned into *Age*I and *Nhe*I restriction sites of plasmid pL0017 replacing *tgdhfr-ts* locus. Wild type *pvdhfr-ts* gene was flanked with 5' and 3' UTR of *pbdhfr-ts* flanking regions which serve as target regions for integration into *P. berghei* genome.

For construction of transfection plasmid harboring wild type *pvdhfr-ts* and an expression cassette of the fusion gene of positive (*hdhfr*) and negative selectable markers (*yfcu*), the plasmid pL0035 plasmid was prepared by QIAprep Spin Miniprep Kit and double-digested with *Hind*III and *Afl*II restriction enzymes. The digested plasmid was run on agarose gel electrophoresis and the DNA band of ~5.4 kb was purified using QIAquick Gel/PCR Extraction Kit. Then, *Hind*III and *Afl*II restriction enzymes were used to digest the plasmid pL17pvDHFR-TS and analyzed on agarose gel electrophoresis. The DNA band of ~4.7 kb was purified using QIAquick Gel/PCR Extraction Kit. The 5.4-kb DNA backbone fragment (50 ng) was subsequently ligated to 4.7-kb insert DNA fragment (500 ng). After incubation at 14^{o}C overnight, the ligation sample was transformed into *E. coli* DH5α competent cells by heat shock method (**Section 2.2.6.2**). The transformed sample was plated onto LB agar plate containing 100 µg/ml ampicillin and incubated at 37^{o}C overnight. Colonies were randomly picked, the plasmid DNA was prepared by alkaline lysis method and then analyzed by restriction digestion followed by analysis on agarose gel electrophoresis. The recombinant plasmid containing full-length gene of wild type *pvdhfr-ts* and an expression cassette of the fusion gene of positive (*hdhfr*) and negative selectable markers (*yfcu*) was designated '**pL35pvDHFR-TS'** (**Figure 3-2** and **3-5**).

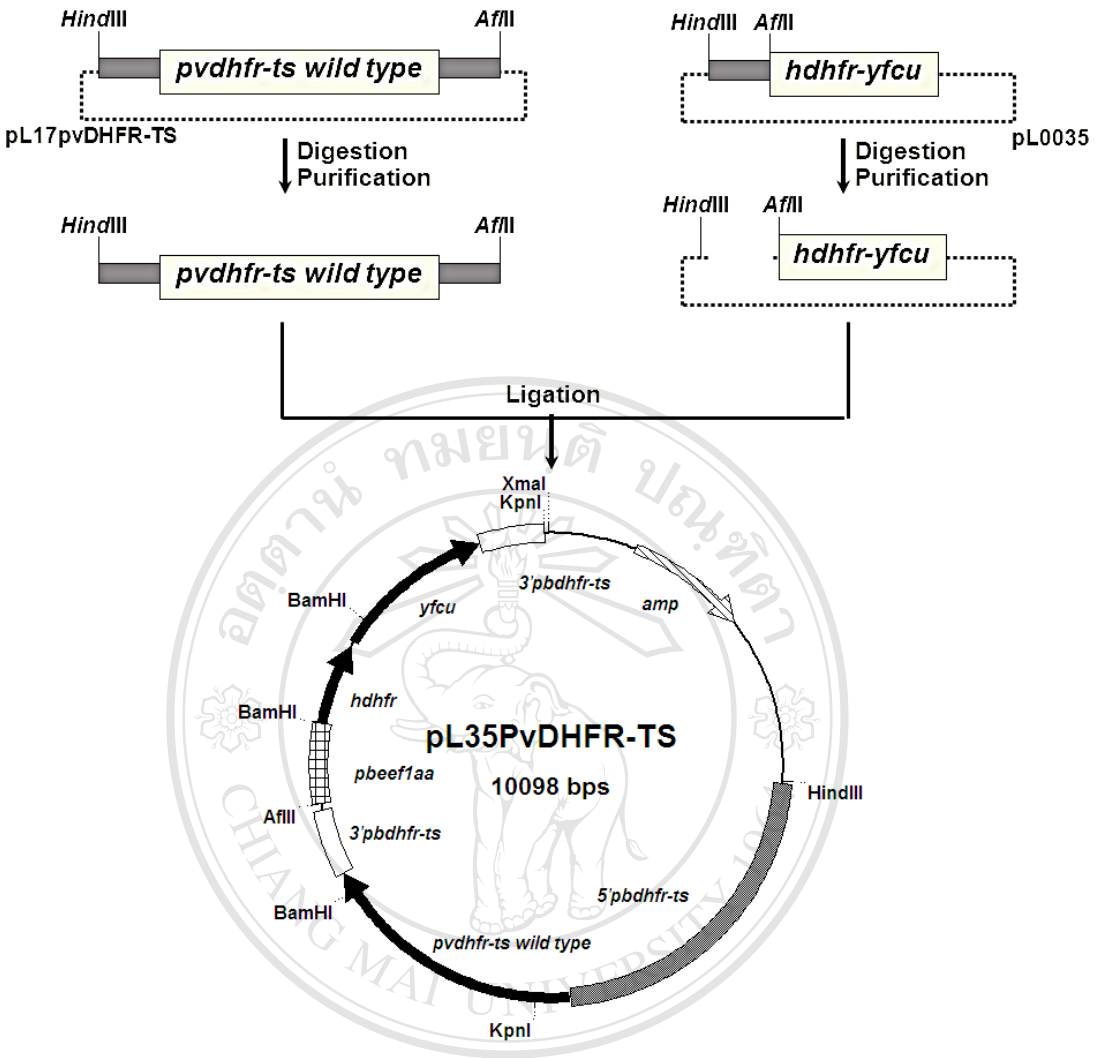


Figure 3-2 Construction of pL35pvDHFR-TS plasmid. The *Hind*III/*Afl*II digested fragment from plasmid pL17pvDHFR-TS was sub-cloned into *Hind*III and *Afl*II restriction sites of plasmid pL0035 to obtain pL35pvDHFR-TS. The *hdhfr* was used as a positive selectable marker after transfection for selection of transgenic parasites. In addition, *yfcu* was used as a negative selectable marker after treatment with 5-FC to remove drug selection cassette by reversible recombination of *3'pbdhfr-ts* locus.

3.1.2.2 Construction of transfection plasmid pL17pvSP21

A similar protocol was adopted for construction of transfection plasmid harbouring *pvdhfr-ts* double mutant (SP21) gene. The SP21 gene was PCR amplified using Sense-*Pv*DT-*Age*I/Antisense-*Pv*DT-*Nhe*I specific primers (**Table 2-1**) and pETpvSP21 as template (a gift from Dr. Ubolsree Leartsakulpanich, BIOTEC, NSTDA). After purification by QIAquick Gel/PCR Extraction Kit, the PCR product was digested with *Age*I and *Nhe*I restriction enzymes, purified and subsequently cloned into *Age*I/*Nhe*I-digested pL0017 plasmid as describe above (**Section 3.1.2.1**). After incubation at 14^{o}C overnight, the ligation sample was used to transform into *E. coli* DH5α competent cells by heat shock method (**Section 2.2.6.2**). The transformed sample was plated onto LB agar plate containing 100 µg/ml ampicillin and incubated at 37^{o}C overnight. Colonies were randomly picked, the plasmid DNA was prepared by alkaline lysis method and then analyzed by restriction digestion followed by analysis on agarose gel electrophoresis. The desired clone was verified for the inserted *pvdhfr-ts* sequence by automated DNA sequencing using PvDTF/PvDTR primers (**Table 2-1**). The recombinant plasmid containing full-length gene of *pvdhfr-ts* double mutant (SP21) was designated '**pL17pvSP21**' (**Figure 3-3** and **3-4**).

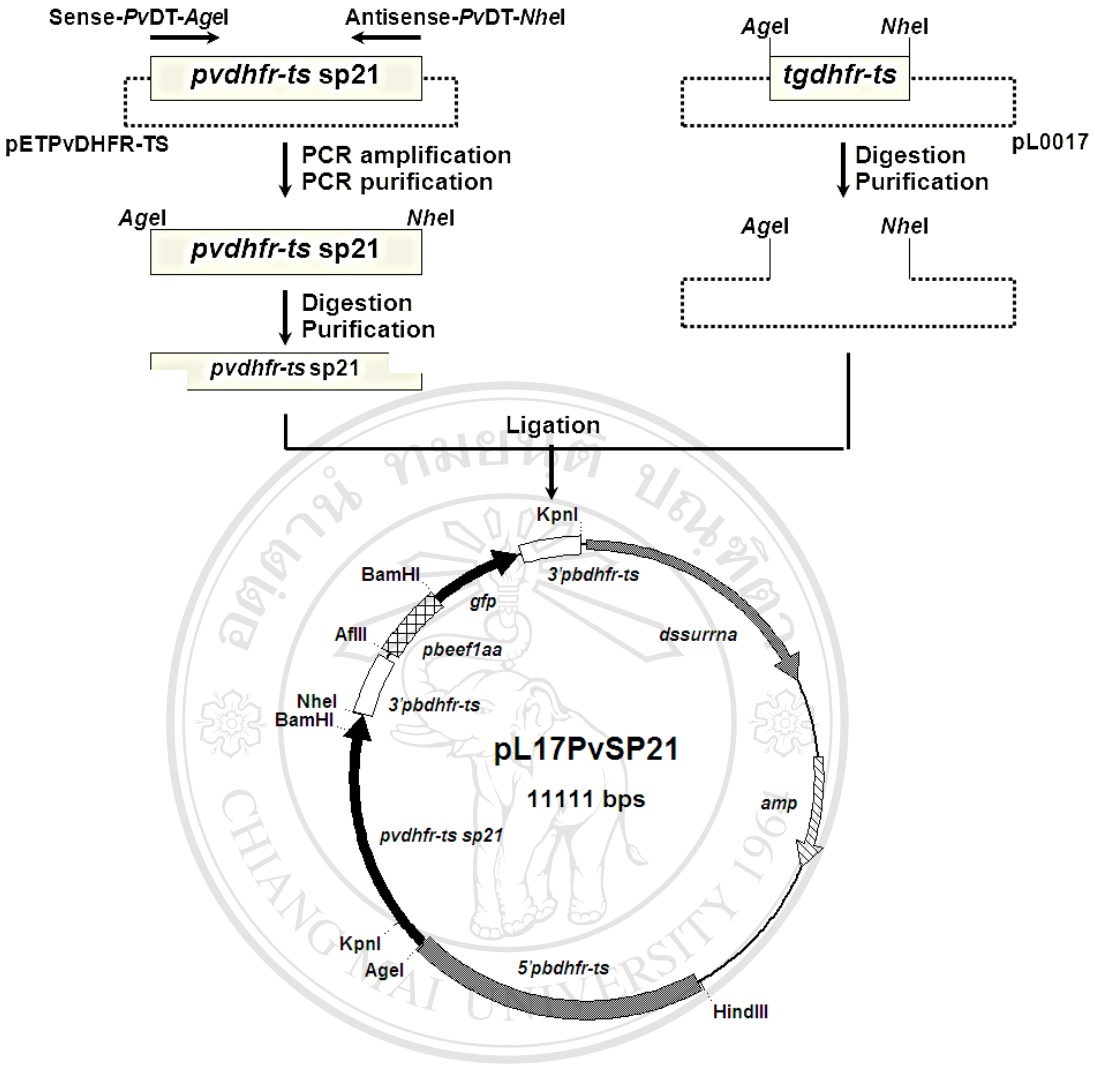


Figure 3-3 Construction of pL17pvSP21 plasmid. The double mutant (S58R/S117N; SP21) *pvdhfr-ts* was PCR amplified and cloned into *Age*I and *Nhe*I restriction sites of plasmid pL0017, replacing *tgdhfr-ts* locus. This mutant gene was flanked with 5' and 3' UTR of *pbdhfr-ts* flanking regions which serve as target regions for integration into *P. berghei* genome. This pyrimethamine-resistant *pvdhfr-ts* double mutant SP21 gene was also used directly as a positive selectable marker for selection of transgenic parasites. The plasmid was linearized with *Hind*III and *Eco*RV restriction enzymes before transfection.

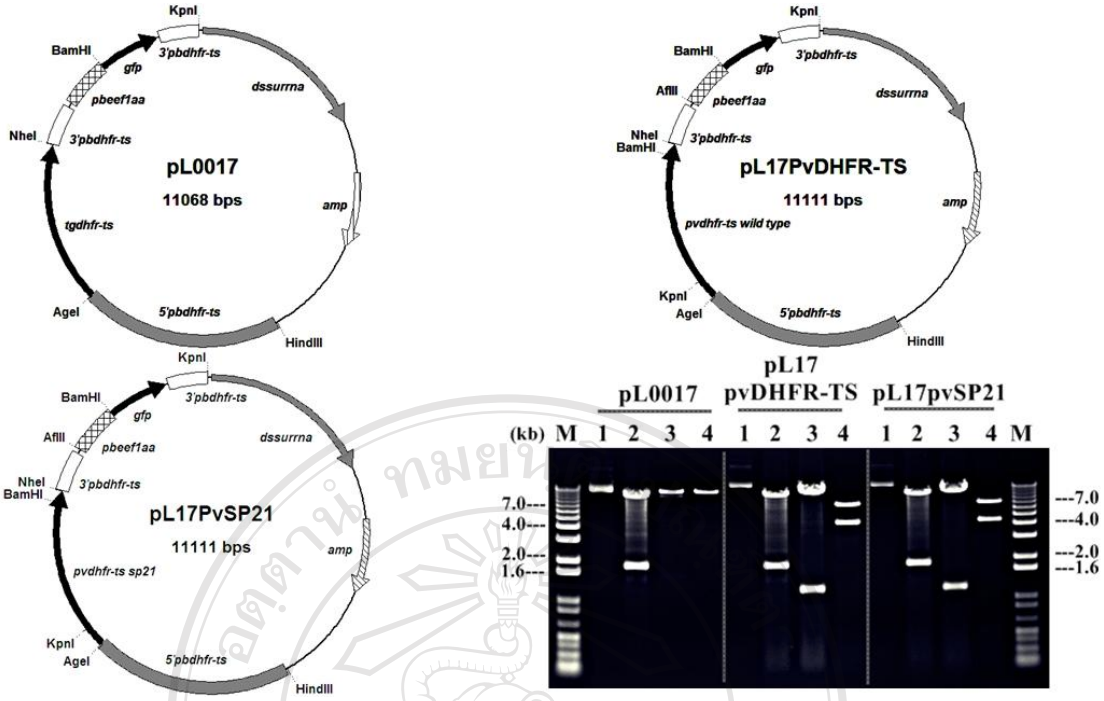


Figure 3-4 Agarose gel electrophoresis of restriction digested pL17pvDHFR-TS and pL17pvSP21 transfection plasmids. Digested plasmids were analysed on 0.8% agarose gel, stained with ethidium bromide, and visualized by UV transilluminator.

Lane M = 1kb plus DNA ladder (standard DNA size marker)

Lane 1 = Undigested plasmids

Lane 2 = *Age*I/*Nhe*I-digested plasmids

Lane 3 = *Bam*HI-digested plasmids

Lane 4 = *Kpn*I-digested plasmids

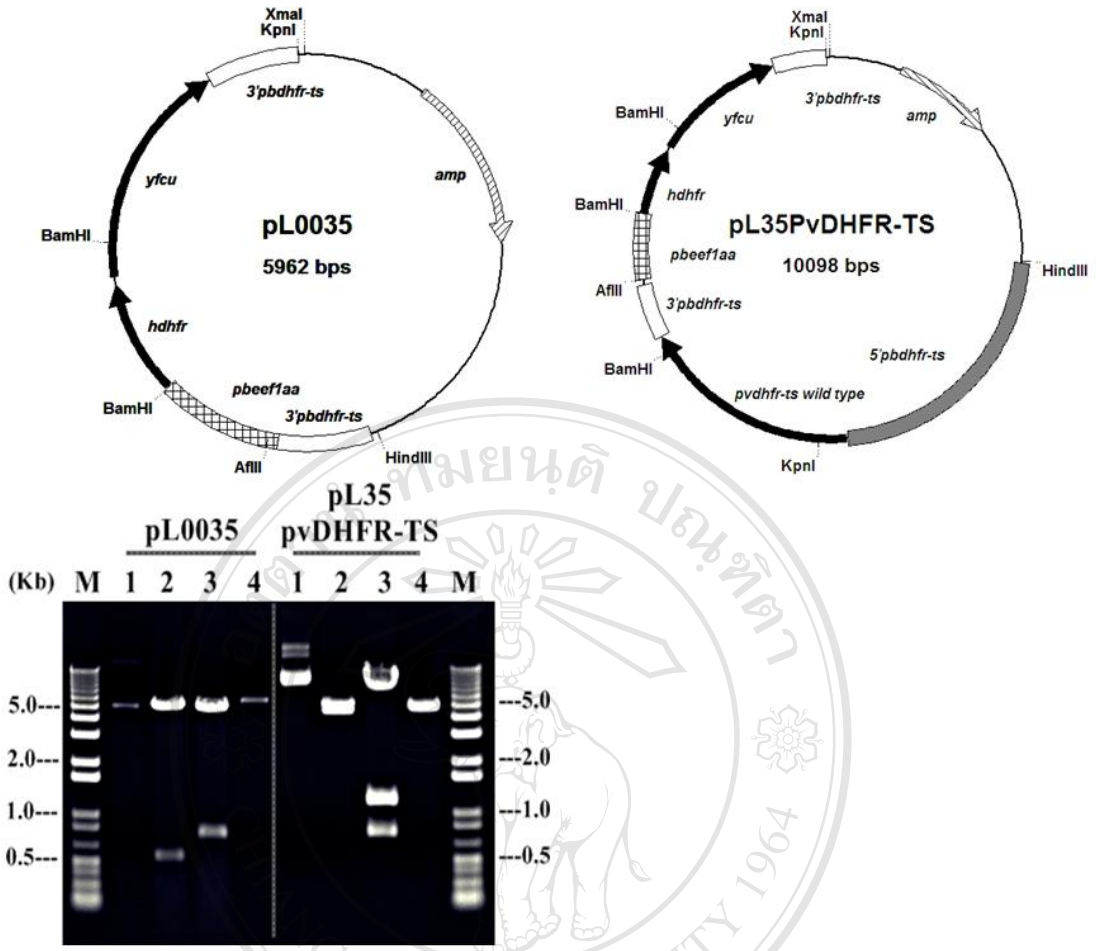


Figure 3-5 Agarose gel electrophoresis of restriction digested pL35pvDHFR-TS transfection plasmid.

Lane M = 1kb plus DNA ladder (standard DNA size marker)

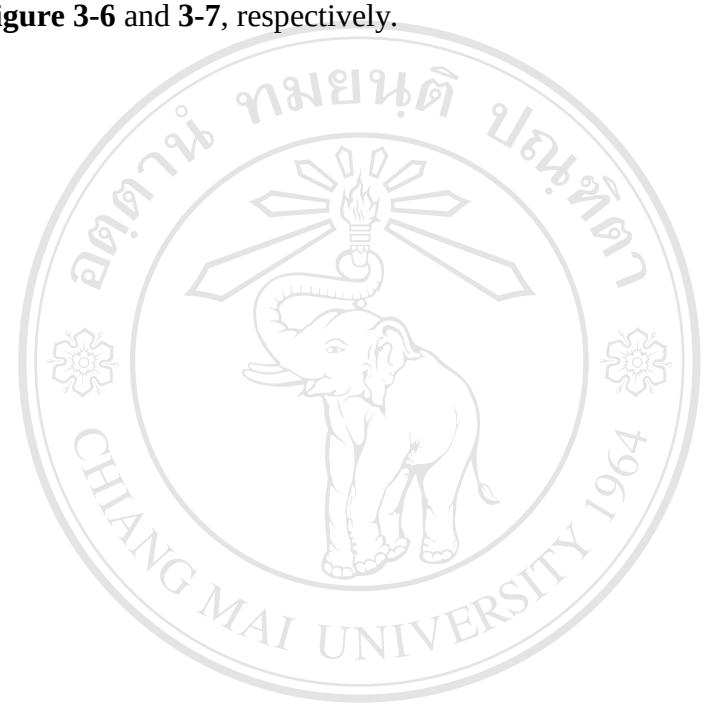
Lane 1 = Undigested plasmids

Lane 2 = *Hind*III/*Afl*II-digested plasmids

Lane 3 = *Bam*HI-digested plasmids

Lane 4 = *Kpn*I-digested plasmids

DNA sequences of the selected clones were confirmed by DNA sequencing at BioService Unit, BIOTEC, NSTDA or at Biodesign, Thailand. The sequencing results showed that the transfection plasmids pL35pvDHFR-TS and pL17pvSP21 contained the full length genes of *pvdhfr-ts* wild type and double mutant SP21, respectively. The nucleotide and amino acid sequences of *Pv*DHFR-TS are shown in **Figure 3-6** and **3-7**, respectively.



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                    *        20         *        40         *
pvdhfr-ts : ATGGAGGACCTTTCAGATGTATTTGACATTTACGCCATCTGCGCATGCTG :   50
pvsp21    : ATGGAGGACCTTTCAGATGTATTTGACATTTACGCCATCTGCGCATGCTG :   50
            ATGGAGGACCTTTCAGATGTATTTGACATTTACGCCATCTGCGCATGCTG

                   60         *        80         *       100
pvdhfr-ts : CAAGGTCGCCCCCACCAGTGAAGGGACAAAGAATGAACCGTTCAGCCCGC :  100
pvsp21    : CAAGGTCGCCCCCACCAGTGAAGGGACAAAGAATGAACCGTTCAGCCCGC :  100
            CAAGGTCGCCCCCACCAGTGAAGGGACAAAGAATGAACCGTTCAGCCCGC

                    *       120         *       140         *
pvdhfr-ts : GGACCTTTAGGGGTCTGGGCAATAAGGGGACTCTCCCATGGAAATGCAAC :  150
pvsp21    : GGACCTTTAGGGGTCTGGGCAATAAGGGGACTCTCCCATGGAAATGCAAC :  150
            GGACCTTTAGGGGTCTGGGCAATAAGGGGACTCTCCCATGGAAATGCAAC

                   160         *       180         *       200
pvdhfr-ts : TCCGTCGATATGAAGTACTTCAGCTCGGTGACGACCTACGTGGATGAGTC :  200
pvsp21    : TCCGTCGATATGAAGTACTTCAGGTCGGTGACGACCTACGTGGATGAGTC :  200
            TCCGTCGATATGAAGTACTTCAG TCGGTGACGACCTACGTGGATGAGTC

                    *       220         *       240         *
pvdhfr-ts : AAAGTATGAGAAGCTAAAGTGGAAGAGGGAGAGGTACCTACGAATGGAAG :  250
pvsp21    : AAAGTATGAGAAGCTAAAGTGGAAGAGGGAGAGGTACCTACGAATGGAAG :  250
            AAAGTATGAGAAGCTAAAGTGGAAGAGGGAGAGGTACCTACGAATGGAAG

                   260         *       280         *       300
pvdhfr-ts : CCTCACAGGGGGGGGGTGACAACACAAGCGGTGGTGACAACGCCGACAAG :  300
pvsp21    : CCTCACAGGGGGGGGGTGACAACACAAGCGGTGGTGACAACGCCGACAAG :  300
            CCTCACAGGGGGGGGGTGACAACACAAGCGGTGGTGACAACGCCGACAAG

                    *       320         *       340         *
pvdhfr-ts : CTGCAAAACGTCGTGGTCATGGGGAGAAGCAACTGGGAGAGCATCCCCAG :  350
pvsp21    : CTGCAAAACGTCGTGGTCATGGGGAGAAGCAACTGGGAGAGCATCCCCAA :  350
            CTGCAAAACGTCGTGGTCATGGGGAGAAGCAACTGGGAGAGCATCCCCA

                   360         *       380         *       400
pvdhfr-ts : CCAGTACAAGCCGCTCCCAAACAGAATCAACGTCGTGCTTTCCAAGACGC :  400
pvsp21    : CCAGTACAAGCCGCTCCCAAACAGAATCAACGTCGTGCTTTCCAAGACGC :  400
            CCAGTACAAGCCGCTCCCAAACAGAATCAACGTCGTGCTTTCCAAGACGC

                    *       420         *       440         *
pvdhfr-ts : TAACAAAGGAAGACGTGAAGGAAAAGGTCTTCATAATTGACAGCATAGAT :  450
pvsp21    : TAACAAAGGAAGACGTGAAGGAAAAGGTCTTCATAATTGACAGCATAGAT :  450
            TAACAAAGGAAGACGTGAAGGAAAAGGTCTTCATAATTGACAGCATAGAT

                   460         *       480         *       500
pvdhfr-ts : GACCTACTGCTGCTCTTAAAGAAGCTGAAGTACTACAAATGCTTCATCAT :  500
pvsp21    : GACCTACTGCTGCTCTTAAAGAAGCTGAAGTACTACAAATGCTTCATCAT :  500
            GACCTACTGCTGCTCTTAAAGAAGCTGAAGTACTACAAATGCTTCATCAT

                    *       520         *       540         *
pvdhfr-ts : TGGGGGAGCACAAGTTTATAGGGAATGCCTAAGTAGAAACTTAATCAAGC :  550
pvsp21    : TGGGGGAGCACAAGTTTATAGGGAATGCCTAAGTAGAAACTTAATCAAGC :  550
            TGGGGGAGCACAAGTTTATAGGGAATGCCTAAGTAGAAACTTAATCAAGC
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                560         *       580         *       600
pvdhfr-ts : AGATCTACTTCACGAGGATCAACGGCGCCTACCCGTGTGACGTCTTCTTC :  600
pvsp21    : AGATCTACTTCACGAGGATCAACGGCGCCTACCCGTGTGACGTCTTCTTC :  600
            AGATCTACTTCACGAGGATCAACGGCGCCTACCCGTGTGACGTCTTCTTC

                  *       620         *       640         *
pvdhfr-ts : CCCGAGTTTGACGAAAGCCAGTTTCGGGTGACGTCCGTCAGTGAGGTGTA :  650
pvsp21    : CCCGAGTTTGACGAAAGCCAGTTTCGGGTGACGTCCGTCAGTGAGGTGTA :  650
            CCCGAGTTTGACGAAAGCCAGTTTCGGGTGACGTCCGTCAGTGAGGTGTA

                660         *       680         *       700
pvdhfr-ts : CAACAGCAAGGGCACCACTCTGGACTTTTTGGTTTACAGCAAGGTGGGGG :  700
pvsp21    : CAACAGCAAGGGCACCACTCTGGACTTTTTGGTTTACAGCAAGGTGGGGG :  700
            CAACAGCAAGGGCACCACTCTGGACTTTTTGGTTTACAGCAAGGTGGGGG

                  *       720         *       740         *
pvdhfr-ts : GGGGAGTTGACGGGGGCGCCTCCAACGGGAGCACTGCGGACAGCGCTTCG :  750
pvsp21    : GGGGAGTTGACGGGGGCGCCTCCAACGGGAGCACTGCGGACAGCGCTTCG :  750
            GGGGAGTTGACGGGGGCGCCTCCAACGGGAGCACTGCGGACAGCGCTTCG

                760         *       780         *       800
pvdhfr-ts : GAGAACTGCAATGCGTTGAACTGCAATGCGCCGAAATGTAGCGCCCCGAA :  800
pvsp21    : GAGAACTGCAATGCGTTGAACTGCAATGCGCCGAAATGTAGCGCCCCGAA :  800
            GAGAACTGCAATGCGTTGAACTGCAATGCGCCGAAATGTAGCGCCCCGAA

                  *       820         *       840         *
pvdhfr-ts : CTGCCGCTCCCCCAATGGGGGGACCGCACAGCAGGGCGAATGGGGAAAGG :  850
pvsp21    : CTGCCGCTCCCCCAATGGGGGGACCGCACAGCAGGGCGAATGGGGAAAGG :  850
            CTGCCGCTCCCCCAATGGGGGGACCGCACAGCAGGGCGAATGGGGAAAGG

                860         *       880         *       900
pvdhfr-ts : GCCCCGCCGCGTGCCCGTGGCAGAAGAACAACGCCGAGGCAGAGGAAGAC :  900
pvsp21    : GCCCCGCCGCGTGCCCGTGGCAGAAGAACAACGCCGAGGCAGAGGAAGAC :  900
            GCCCCGCCGCGTGCCCGTGGCAGAAGAACAACGCCGAGGCAGAGGAAGAC

                  *       920         *       940         *
pvdhfr-ts : GACCTCGTGTACTTCAGCTTTAACAACAAAGTGGGGGGAGAAAAACCCGGA :  950
pvsp21    : GACCTCGTGTACTTCAGCTTTAACAACAAAGTGGGGGGAGAAAAACCCGGA :  950
            GACCTCGTGTACTTCAGCTTTAACAACAAAGTGGGGGGAGAAAAACCCGGA

                960         *       980         *      1000
pvdhfr-ts : ACACCTGCAAGAATTTAAAATTTACAACAGCCTGAAGATTAAGCAGCACC : 1000
pvsp21    : ACACCTGCAAGAATTTAAAATTTACAACAGCCTGAAGATTAAGCAGCACC : 1000
            ACACCTGCAAGAATTTAAAATTTACAACAGCCTGAAGATTAAGCAGCACC

                  *      1020         *      1040         *
pvdhfr-ts : CAGAGTACCAATACCTAGGCATCATATACGACATCATCATGAATGGAAAC : 1050
pvsp21    : CAGAGTACCAATACCTAGGCATCATATACGACATCATCATGAATGGAAAC : 1050
            CAGAGTACCAATACCTAGGCATCATATACGACATCATCATGAATGGAAAC

               1060         *      1080         *      1100
pvdhfr-ts : AAACAAGGAGACAGAACAGGTGTGGGAGTGATGAGCAAATTTGGCTACAT : 1100
pvsp21    : AAACAAGGAGACAGAACAGGTGTGGGAGTGATGAGCAAATTTGGCTACAT : 1100
            AAACAAGGAGACAGAACAGGTGTGGGAGTGATGAGCAAATTTGGCTACAT
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                    *      1120         *      1140         *
pvdhfr-ts : GATGAAATTTAATTTAAGTGAATACTTCCCCCTATTAACAACAAAGAAGT : 1150
pvsp21    : GATGAAATTTAATTTAAGTGAATACTTCCCCCTATTAACAACAAAGAAGT : 1150
            GATGAAATTTAATTTAAGTGAATACTTCCCCCTATTAACAACAAAGAAGT

                  1160         *      1180         *      1200
pvdhfr-ts : TATTTTTGAGGGGAATAATTGAGGAACTGCTTTGGTTCATTCGAGGAGAA : 1200
pvsp21    : TATTTTTGAGGGGAATAATTGAGGAACTGCTTTGGTTCATTCGAGGAGAA : 1200
            TATTTTTGAGGGGAATAATTGAGGAACTGCTTTGGTTCATTCGAGGAGAA

                    *      1220         *      1240         *
pvdhfr-ts : ACAAACGGAAACACTTTGTTAAATAAAAACGTGAGGATATGGGAAGCAAA : 1250
pvsp21    : ACAAACGGAAACACTTTGTTAAATAAAAACGTGAGGATATGGGAAGCAAA : 1250
            ACAAACGGAAACACTTTGTTAAATAAAAACGTGAGGATATGGGAAGCAAA

                  1260         *      1280         *      1300
pvdhfr-ts : TGGAACGAGGGAGTTCCTCGACAACAGGAAATTATTCCACAGAGAAGTGA : 1300
pvsp21    : TGGAACGAGGGAGTTCCTCGACAACAGGAAATTATTCCACAGAGAAGTGA : 1300
            TGGAACGAGGGAGTTCCTCGACAACAGGAAATTATTCCACAGAGAAGTGA

                    *      1320         *      1340         *
pvdhfr-ts : ATGACTTGGGGCCCATTTATGGCTTCCAGTGGAGACACTTCGGTGCTGAA : 1350
pvsp21    : ATGACTTGGGGCCCATTTATGGCTTCCAGTGGAGACACTTCGGTGCTGAA : 1350
            ATGACTTGGGGCCCATTTATGGCTTCCAGTGGAGACACTTCGGTGCTGAA

                  1360         *      1380         *      1400
pvdhfr-ts : TACACAAATATGCATGACAATTACGAAGATAAAGGTGTAGACCAATTAAA : 1400
pvsp21    : TACACAAATATGCATGACAATTACGAAGATAAAGGTGTAGACCAATTAAA : 1400
            TACACAAATATGCATGACAATTACGAAGATAAAGGTGTAGACCAATTAAA

                    *      1420         *      1440         *
pvdhfr-ts : AAATGTTATTCATTTAATAAAAAATGAACCAACAAGTAGGAGAATTATTT : 1450
pvsp21    : AAATGTTATTCATTTAATAAAAAATGAACCAACAAGTAGGAGAATTATTT : 1450
            AAATGTTATTCATTTAATAAAAAATGAACCAACAAGTAGGAGAATTATTT

                  1460         *      1480         *      1500
pvdhfr-ts : TGTGTGCATGGAATGTAAAAGATTTGGATCAAATGGCTTTACCTCCTTGT : 1500
pvsp21    : TGTGTGCATGGAATGTAAAAGATTTGGATCAAATGGCTTTACCTCCTTGT : 1500
            TGTGTGCATGGAATGTAAAAGATTTGGATCAAATGGCTTTACCTCCTTGT

                    *      1520         *      1540         *
pvdhfr-ts : CATATTTTATGCCAATTTTACGTTTTCGATGGGAAACTATCATGCATTAT : 1550
pvsp21    : CATATTTTATGCCAATTTTACGTTTTCGATGGGAAACTATCATGCATTAT : 1550
            CATATTTTATGCCAATTTTACGTTTTCGATGGGAAACTATCATGCATTAT

                  1560         *      1580         *      1600
pvdhfr-ts : GTACCAGAGGTCTTGTGACTTGGGTCTTGGGGTCCCCTTCAACATCGCTT : 1600
pvsp21    : GTACCAGAGGTCTTGTGACTTGGGTCTTGGGGTCCCCTTCAACATCGCTT : 1600
            GTACCAGAGGTCTTGTGACTTGGGTCTTGGGGTCCCCTTCAACATCGCTT

                    *      1620         *      1640         *
pvdhfr-ts : CGTATTCCATATTCACACACATGATTGCGCAGGTGTGCAATTTGCAGCCT : 1650
pvsp21    : CGTATTCCATATTCACACACATGATTGCGCAGGTGTGCAATTTGCAGCCT : 1650
            CGTATTCCATATTCACACACATGATTGCGCAGGTGTGCAATTTGCAGCCT
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                 1660         *      1680         *      1700
pvdhfr-ts : GCACAGTTCATACACATTTTGGGCAACGCGCACGTCTACAACAACCATGT : 1700
pvsp21    : GCACAGTTCATACACATTTTGGGCAACGCGCACGTCTACAACAACCATGT : 1700
            GCACAGTTCATACACATTTTGGGCAACGCGCACGTCTACAACAACCATGT

                    *      1720         *      1740         *
pvdhfr-ts : TGACAGCTTGAAAGTGCAGCTGAACAGGATCCCCTACCCGTTCCCAACGC : 1750
pvsp21    : TGACAGCTTGAAAGTGCAGCTGAACAGGATCCCCTACCCGTTCCCAACGC : 1750
            TGACAGCTTGAAAGTGCAGCTGAACAGGATCCCCTACCCGTTCCCAACGC

                 1760         *      1780         *      1800
pvdhfr-ts : TTAAACTGAACCCGGAGGTGAAGAACATTGAGGATTTTACCATTTCGGAT : 1800
pvsp21    : TTAAACTGAACCCGGAGGTGAAGAACATTGAGGATTTTACCATTTCGGAT : 1800
            TTAAACTGAACCCGGAGGTGAAGAACATTGAGGATTTTACCATTTCGGAT

                    *      1820         *      1840         *
pvdhfr-ts : TTCACAATAGAGAATTACGTGCACCACGATAAAATAACCATGGAGATGGC : 1850
pvsp21    : TTCACAATAGAGAATTACGTGCACCACGATAAAATAACCATGGAGATGGC : 1850
            TTCACAATAGAGAATTACGTGCACCACGATAAAATAACCATGGAGATGGC


pvdhfr-ts : CGCCTAA : 1857
pvsp21    : CGCCTAA : 1857
            CGCCTAA
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Figure 3-6 Nucleotide sequences of *pvdhfr-ts* wild type and double mutant (SP21). Nucleotide sequences indicated an open reading frame of 1857 bp of *pvdhfr-ts* genes. Black box indicates a complete alignment of two sequences. Grey boxes indicate nucleotide mutations.

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                    *        20         *        40         *
PvDHFR-TS : MEDLSDVFDIYAICACCKVAPTSEGTKNEPFSPRTFRGLGNKGTLPWKCN :  50
PvSP21    : MEDLSDVFDIYAICACCKVAPTSEGTKNEPFSPRTFRGLGNKGTLPWKCN :  50
            MEDLSDVFDIYAICACCKVAPTSEGTKNEPFSPRTFRGLGNKGTLPWKCN

                   60         *        80         *       100
PvDHFR-TS : SVDMKYFSSVTTYVDESKYEKLKWKRERYLRMEASQGGGDNTSGGDNADK : 100
PvSP21    : SVDMKYFRSVTTYVDESKYEKLKWKRERYLRMEASQGGGDNTSGGDNADK : 100
            SVDMKYF SVTTYVDESKYEKLKWKRERYLRMEASQGGGDNTSGGDNADK

                    *       120         *       140         *
PvDHFR-TS : LQNVVVMGRSNWESIPSQYKPLPNRINVVLSKTLTKEDVKEKVFIIDSID : 150
PvSP21    : LQNVVVMGRSNWESIPNQYKPLPNRINVVLSKTLTKEDVKEKVFIIDSID : 150
            LQNVVVMGRSNWESIP QYKPLPNRINVVLSKTLTKEDVKEKVFIIDSID

                   160         *       180         *       200
PvDHFR-TS : DLLLLLKKLKYYKCFIIGGAQVYRECLSRNLIKQIYFTRINGAYPCDVFF : 200
PvSP21    : DLLLLLKKLKYYKCFIIGGAQVYRECLSRNLIKQIYFTRINGAYPCDVFF : 200
            DLLLLLKKLKYYKCFIIGGAQVYRECLSRNLIKQIYFTRINGAYPCDVFF

                    *       220         *       240         *
PvDHFR-TS : PEFDESQFRVTSVSEVYNSKGTTLDFLVYSKVGGGVDGGASNGSTADSAS : 250
PvSP21    : PEFDESQFRVTSVSEVYNSKGTTLDFLVYSKVGGGVDGGASNGSTADSAS : 250
            PEFDESQFRVTSVSEVYNSKGTTLDFLVYSKVGGGVDGGASNGSTADSAS

                   260         *       280         *       300
PvDHFR-TS : ENCNALNCNAPKCSAPNCRSPNGGTAQQGEWGKGPAACPWQKNNAEAEED : 300
PvSP21    : ENCNALNCNAPKCSAPNCRSPNGGTAQQGEWGKGPAACPWQKNNAEAEED : 300
            ENCNALNCNAPKCSAPNCRSPNGGTAQQGEWGKGPAACPWQKNNAEAEED

                    *       320         *       340         *
PvDHFR-TS : DLVYFSFNNKVGEKNPEHLQEFKIYNSLKIKQHPEYQYLGIIYDIIMNGN : 350
PvSP21    : DLVYFSFNNKVGEKNPEHLQEFKIYNSLKIKQHPEYQYLGIIYDIIMNGN : 350
            DLVYFSFNNKVGEKNPEHLQEFKIYNSLKIKQHPEYQYLGIIYDIIMNGN

                   360         *       380         *       400
PvDHFR-TS : KQGDRTGVGVMSKFGYMMKFNLSEYFPLLTTKKLFLRGIIEELLWFIRGE : 400
PvSP21    : KQGDRTGVGVMSKFGYMMKFNLSEYFPLLTTKKLFLRGIIEELLWFIRGE : 400
            KQGDRTGVGVMSKFGYMMKFNLSEYFPLLTTKKLFLRGIIEELLWFIRGE

                    *       420         *       440         *
PvDHFR-TS : TNGNTLLNKNVRIWEANGTREFLDNRKLFHREVNDLGPIYGFQWRHFGAE : 450
PvSP21    : TNGNTLLNKNVRIWEANGTREFLDNRKLFHREVNDLGPIYGFQWRHFGAE : 450
            TNGNTLLNKNVRIWEANGTREFLDNRKLFHREVNDLGPIYGFQWRHFGAE

                   460         *       480         *       500
PvDHFR-TS : YTNMHDNYEDKGVDQLKNVIHLIKNEPTSRRIILCAWNVKDLDQMALPPC : 500
PvSP21    : YTNMHDNYEDKGVDQLKNVIHLIKNEPTSRRIILCAWNVKDLDQMALPPC : 500
            YTNMHDNYEDKGVDQLKNVIHLIKNEPTSRRIILCAWNVKDLDQMALPPC

                    *       520         *       540         *
PvDHFR-TS : HILCQFYVFDGKLSCIMYQRSCDLGLGVPFNIASYSIFTHMIAQVCNLQP : 550
PvSP21    : HILCQFYVFDGKLSCIMYQRSCDLGLGVPFNIASYSIFTHMIAQVCNLQP : 550
            HILCQFYVFDGKLSCIMYQRSCDLGLGVPFNIASYSIFTHMIAQVCNLQP
```

```
                    560         *       580         *       600
PvDHFR-TS : AQFIHILGNAHVYNNHVDSLKVQLNRIPYPFPTLKLNPEVKNIEDFTISD : 600
PvSP21    : AQFIHILGNAHVYNNHVDSLKVQLNRIPYPFPTLKLNPEVKNIEDFTISD : 600
            AQFIHILGNAHVYNNHVDSLKVQLNRIPYPFPTLKLNPEVKNIEDFTISD

                      *
PvDHFR-TS : FTIENYVHHDKITMEMAA : 618
PvSP21    : FTIENYVHHDKITMEMAA : 618
            FTIENYVHHDKITMEMAA
```

Figure 3-7 Amino acid sequences of PvDHFR-TS wild type and double mutant (SP21). Amino acid sequences indicated an encoding of 618 amino acids of PvDHFR-TS. Black box indicates a complete alignment of two sequences. Grey boxes indicate amino acid mutations at positions 58 and 117.

3.2 Preparation of linearized plasmid for transfection

The transfection plasmids were purified from overnight culture of *E. coli* harboring the plasmid using the QIAGEN Kit as described in the Materials and Methods section. The purified plasmid DNA (5 µg) was then digested overnight with the appropriate restriction enzymes. For pL35pvDHFR-TS, digestion was performed with *Hind*III and *Sma*I restriction enzymes. Expected fragments were 2.6 kb and 7.4 kb as shown in **Figure 3-8**. In addition, *Hind*III and *Afl*II were used to digest the plasmid pL17pvSP21 to obtain fragments of 4.7 kb and 7.0 kb (**Figure 3-8**). The DNA fragments of 7.4 kb from digested pL35pvDHF-TS and 4.7 kb from digested pL17pvSP21 plasmids were used for transfection and integration into the parasite genome. Traces of undigested plasmids or presence of the plasmid backbone do not impede the recovery of the correct integration events (Menard & Janse, 1997). Therefore, the digested plasmids were directly precipitated with isopropanol followed

by washing with 70% ethanol. The DNA pellets were then resuspended with 10 µl of TE buffer and kept at -20^{o}C for use in transfection.

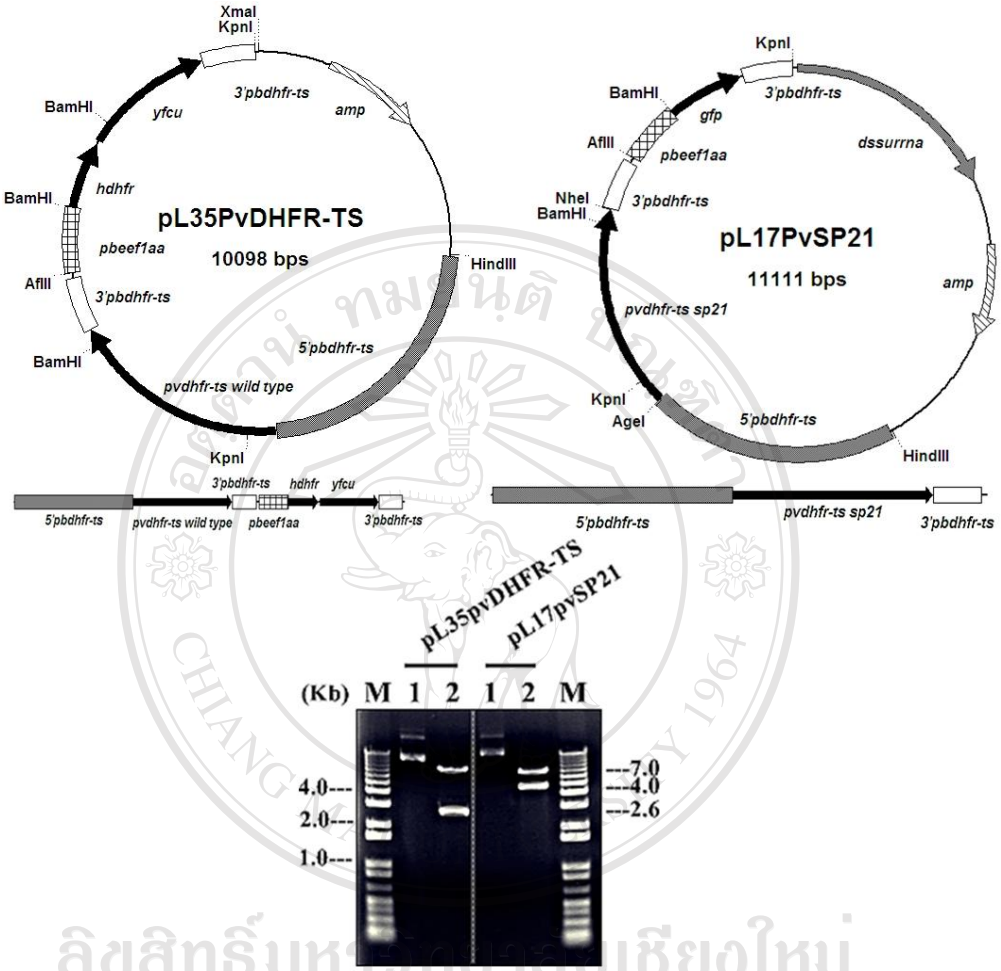


Figure 3-8 Agarose gel electrophoresis of linearized transfection plasmids. pL35pvDHFR-TS plasmid was digested with *Hind*III and *Sma*I to obtain 2.6 kb and 7.4 kb fragments. For pL17pvSP21, *Hind*III and *Afl*II restriction enzymes were used to obtain 4.7 kb and 7.0 kb fragments. The digested plasmids were analysed on 0.8% agarose gel, stained with ethidium bromide and visualized by UV transilluminator.

Lane M = 1kb plus DNA ladder (standard DNA size marker)

Lane 1 = Undigested plasmids

Lane 2 = *Hind*III/*Sma*I-digested plasmids

3.3 Re-evaluation of parental *P. berghei* (PbGFP507m6cl1) for use in transfection

Transgenic *P. berghei* line 507m6cl1 (PbGFP507m6cl1) was used as starting parental parasite for generation of transgenic parasites in this study. It expresses GFP (mutant 3) and was generated in the reference clone of the ANKA strain cl15cy1 (Janse *et al*, 2006b). It contains the *gfp* stably integrated as a single copy gene by double cross-over recombination into the *230p* locus and the reporter gene is under control of the constitutive *eef1αa* promoter and does not contain a drug selectable marker gene. This parasite line was selected by flow cytometric sorting based on expression of GFP, directly after transfection of parasites, as described by Janse et al (Janse *et al*, 2006a; Janse *et al*, 2006b).

Although this transgenic parasite line has been shown to be sensitive to pyrimethamine and constitutively expresses GFP throughout the complete life cycle, re-evaluation on the phenotypes of this parasite, including visualization of GFP under fluorescence microscope and flow cytometric analysis, the sensitivity of this line to pyrimethamine, examination of blood stage development *in vivo*, and survival rate of infected mice, was performed before using it in this study. In addition, examination of point mutations at target *dhfr* gene was also performed.

3.3.1 Visualization of PbGFP507m6cl1 parasites during blood stage development

To demonstrate expression of GFP, a systematic evaluation of GFP-expression by fluorescence microscopy during blood stage development was performed. The parasite DNA in nucleus was stained with DNA-specific dye Hoechst 33258. Analysis of blood stage parasites confirmed the expression of GFP by the parasites (**Figure 3-9**).

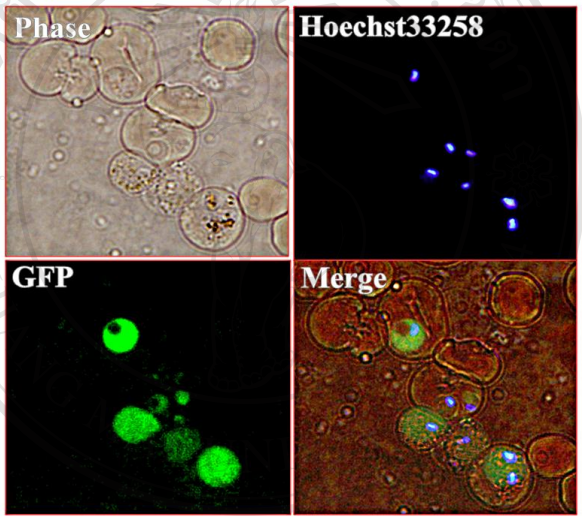


Figure 3-9 Visualization of GFP-expressing of PbGFP507m6cl1 parasite during the blood stage development. The parasites were counter-stained with the DNA-specific fluorescent dye Hoechst 33258.

3.3.2 Flow cytometric analysis of PbGFP507m6cl1 parasite

GFP-fluorescence intensity of blood stage parasites was examined using flow cytometry. In **Figure 3-10**, typical dot plots showing fluorescence intensity (FL1 detector at 530/30 nm) versus granularity (side scatter, SS) of samples containing uninfected erythrocytes (A) and PbGFP507m6cl1 infected erythrocytes (B) were shown. In our study, the infected erythrocytes could be separated from the uninfected cells based on the GFP-fluorescence intensity. Flow cytometric analysis could be used to detect tail blood from mice infected with *P. berghei* with parasitemia ranging from 0.1 to 50%. A good agreement between flow cytometric counting of infected cells and manual microscopic counting of Giemsa-stained blood films was observed (**Figure 3-11**).

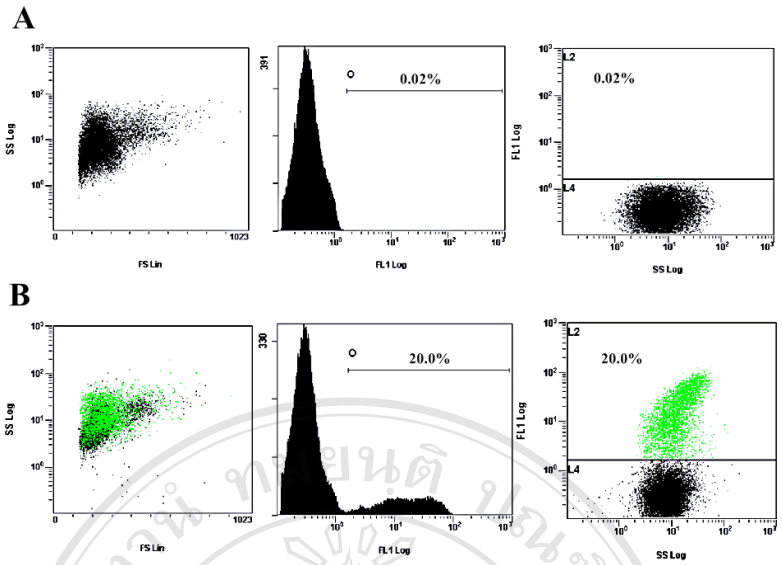


Figure 3-10 Analysis of GFP-fluorescence intensity by flow cytometry of live blood stages of PbGFP507m6cl1 parasites. Dot plot represented the populations of blood cells showing fluorescence intensity (FL1) versus granularity (Safeukui *et al*, 2004) of blood samples containing uninfected cells (**A**) and infected cells with high parasitemia (**B**). The samples were obtained from infected mouse with an asynchronous infection.

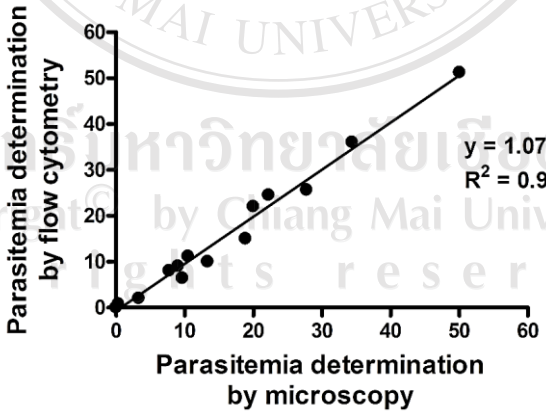


Figure 3-11 Correlation of flow cytometric and microscopic methods for determination of parasitemia. Blood samples from infected mice with a parasitemia between 0.1 and 50% were subjected to measurement of GFP-expressing parasites using both automatic flow cytometric and manual counting of infected erythrocytes on Giemsa stained blood films.

3.3.3 Observation of PbGFP507m6cl1 blood stage development *in vivo*

Development of parasitemia was measured daily from tail blood of mouse infected with $6x10^6$ parasitized erythrocytes of either wild type *P. berghei* ANKA (WT PbANKA) or PbGFP507m6cl1 parasites by microscopic examination or flow cytometric analysis as previously described in Materials and Methods section. As shown in **Figure 3-12**, the asexual multiplication rate *in vivo*, during of asexual cycle (22-24 hr), of PbGFP507m6cl1 parasite was similar to that of WT PbANKA. It was observed that PbGFP507m6cl1 parasite could grow a little faster than WT PbANKA and reached 10% parasitemia on day 5 after infection but not significant. The growth of WT PbANKA parasite could later on catch up. However there were not significant differences on the growth rate between the two parasite lines.

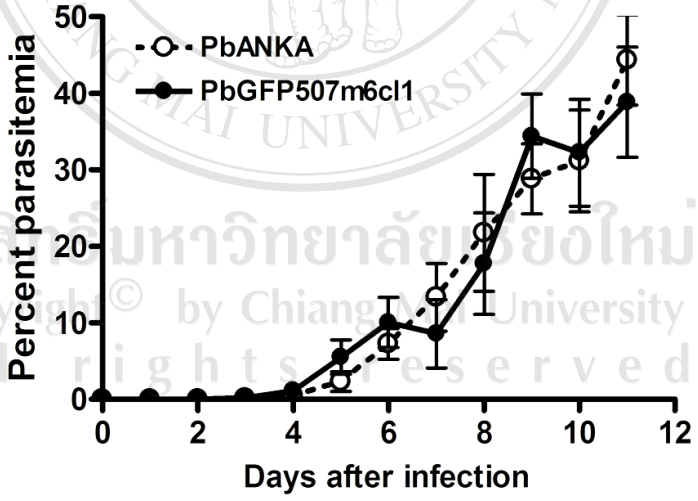


Figure 3-12 Proliferation of wild type *P. berghei* ANKA and PbGFP507m6cl1 parasites in mice. The course of parasitemia was determined in groups of 5 mice infected with $6x10^6$ parasites over 12 days of infection. Each point represented the mean parasitemia. Bars represented standard errors of the mean.

3.3.4 Observation of virulence of PbGFP507m6cl1 parasite

In order to determine virulence of PbGFP507m6cl1 parasite, group of 5 BALB/C mice were infected with $6x10^6$ parasitized erythrocytes by i.p. injection and survival of infected mice were then monitored daily. As shown in **Figure 3-13**, with respect to the mortality, a rate of 100% death was observed at day 12 post-infection. There was not significant difference on the virulence of PbGFP507m6cl1 compared with WT PbANKA parasite.

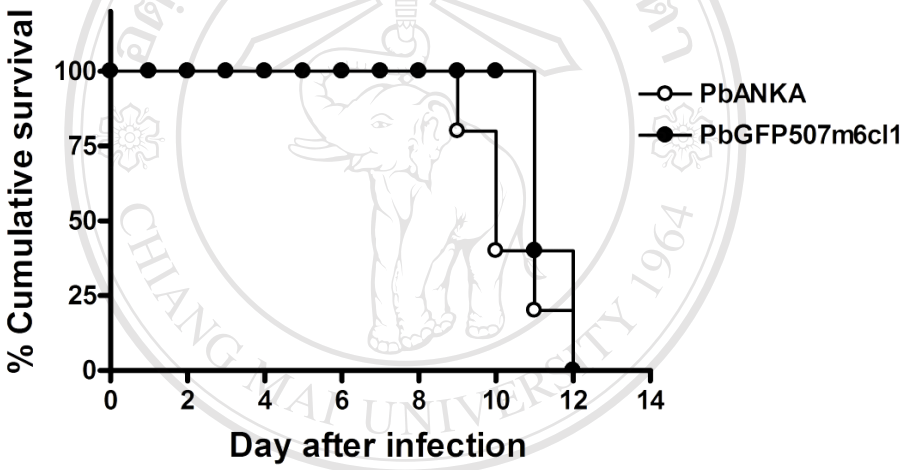


Figure 3-13 Observation of virulence of wild type *P. berghei* ANKA and PbGFP507m6cl1 in mice. Groups of 5 mice were infected with $6x10^6$ infected erythrocytes of WT PbANKA and PbGFP507m6cl1 parasites. The survival of infected mice was monitored daily until death.

3.3.5 Determination of susceptibility of blood stage PbGFP507m6cl1 to pyrimethamine

The ED_{50} values of pyrimethamine against two parasite lines, WT PbANKA and PbGFP507m6cl1, were determined using standard 4-day suppressive test. The parasitemia was measured either by microscopic examination or flow cytometric analysis as previously described in Materials and Methods section. The parasitemia counts were plotted against the logarithm of pyrimethamine concentration. The drug response curve was fit by non-linear regression (sigmoidal dose-response/variable slope equation) to yield ED_{50} values using GraphPad Prism software. As shown in **Figure 3-14**, ED_{50} values for WT PbANKA and PbGFP507m6cl1 were 0.7 and 0.6 mg/kg, respectively. These results were consistent with their ED_{50} values reported in previous studies (Snyder *et al*, 2007).

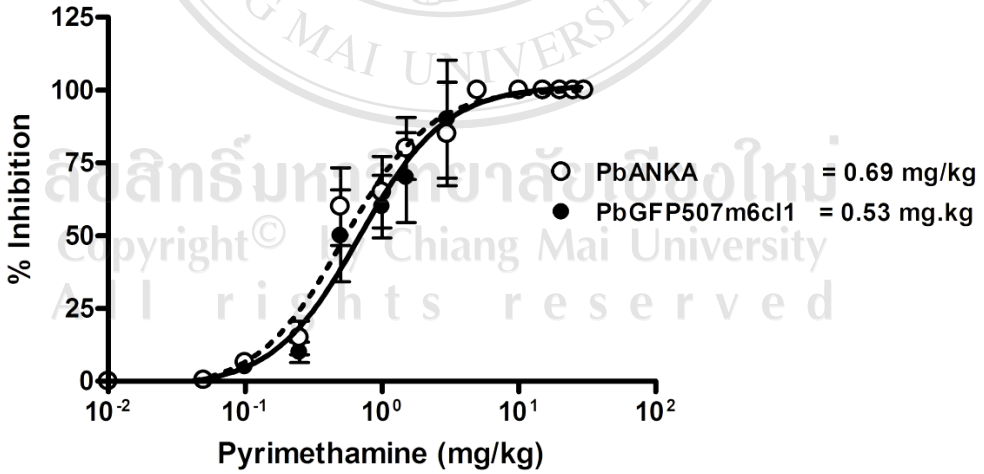


Figure 3-14 Susceptibility of blood stages WT PbANKA and PbGFP507m6cl1 parasites to pyrimethamine. Calculated percent parasitemia was plotted against drug concentration as the means ± SEM from 3 individual experiments. ED_{50}: 50% effective dose.

3.3.6 Re-confirmation of the *dhfr-ts* sequence of PbGFP507m6cl1

The *dhfr*-ts gene was amplified by PCR from genomic DNA of PbGFP507m6cl1 parasite, and analysed for its sequence. As expected, there was no mutation, at the positions responsible for pyrimethamine resistance, in *dhfr* locus of pyrimethamine-sensitive strain PbGFP507m6cl1 (**Figure 3-15**).

Taking all the results together, PbGFP507m6cl1 parasite was confirmed to be the correct drug-sensitive strain. Therefore, it was further used as parental parasite strain to generate transgenic *P. berghei* parasite model in this study.

```
   1 M  E  D  L  S  E  T  F  D  I  Y  A  I  C  A  C  C  K  V  L  N  D  D  E  K
   1 ATGGAAGACTTATCTGAAACATTCGATATATATGCAATATGTGCATGTTGTAAAGTTCTGAACGATGATGAAAAG
  26 V  R  C  F  N  N  K  T  F  K  G  I  G  N  A  G  V  L  P  W  K  C  N  L  I
  76 GTTAGATGCTTTAATAATAAAACGTTTAAGGGAATTGGAAATGCGGGGGTGTTACCTTGGAAATGTAATTTAATC
  51 D  M  K  Y  F  S  S  V  T  S  Y  I  N  E  N  N  Y  I  R  L  K  W  K  R  D
 151 GATATGAAATATTTTAGTTCTGTAACATCATATATAAATGAAAATAATTATATAAGATTGAAATGGAAAAGAGAT
  76 K  Y  M  E  K  H  N  L  K  N  N  V  E  L  N  T  N  I  I  S  S  T  N  N  L
 226 AAATATATGGAAAAACATAATTTAAAAAATAATGTAGAACTAAATACTAATATAATTTCTTCAACTAATAATTTA
 101 Q  N  I  V  V  M  G  K  K  S  W  E  S  I  P  K  K  F  K  P  L  Q  N  R  I
 301 CAAAATATTGTAGTAATGGGAAAAAAAAGTTGGGAAAGTATTCCCAAAAAATTTAAACCTTTACAAAATCGAATA
 126 N  I  I  L  S  R  T  L  K  K  E  D  I  V  N  E  N  N  N  N  E  N  N  N  V  I
 376 AACATTATTTTGTCTAGAACTTTGAAAAAAGAAGATATTGTAAACGAAAATAATAATGAAAATAATAATGTTATT
 151 I  I  K  S  V  D  D  L  F  P  I  L  K  C  T  K  Y  Y  K  C  F  I  I  G  G
 451 ATAATTAAAAGTGTAGATGATTTATTTCCTATTTTAAAATGCACAAAATATTACAAATGTTTTATTATAGGGGGT
 176 S  S  V  Y  K  E  F  L  D  R  N  L  I  K  K  I  Y  F  T  R  I  N  N  S  Y
 526 TCGTCTGTTTATAAAGAATTTTTAGATCGTAATTTAATAAAAAAAATATATTTTACAAGAATAAATAATTCTTAT
 201 N  C  D  V  L  F  P  E  I  N  E  N  L  F  K  I  T  S  I  S  D  V  Y  N  S
 601 AATTGTGATGTTTTATTCCCAGAAATAAACGAAAATTTGTTTAAAATAACTTCAATAAGTGATGTATATAATAGT
 226 N  N  T  T  L  D  F  I  I  Y  S  K  T  K  E  I  N  P  N  E  E  V  P  N  N
 676 AACAACACAACTTTAGATTTTATAATTTATAGTAAGACAAAAGAAATAAATCCAAATGAGGAAGTACCTAATAAT
 251 T  F  L  G  V  C  D  E  Q  N  K  A  F  D  D  E  D  D  Y  T  Y  F  S  F  N
 751 ACATTTTTAGGTGTATGTGATGAACAAAATAAAGCCTTTGATGATGAAGACGATTATACATATTTCAGTTTCAAT
 276 K  N  K  E  N  I  K  K  N  S  E  H  A  H  N  F  K  I  Y  N  S  I  K  Y  K
 826 AAAAATAAAGAAAATATTAAAAAAAAATTCTGAACATGCTCATAATTTTAAAATATATAATAGCATAAAATATAAA
 301 N  H  H  E  Y  Q  Y  L  N  I  I  Y  D  M  I  M  H  G  N  N  Q  D  D  R  T
 901 AATCATCATGAATATCAATATTTAAATATTATATATGATATGATAATGCATGGAAATAACCAAGATGATAGAACA
 326 G  G  V  L  S  K  F  G  Y  M  M  K  F  N  L  N  E  Y  F  P  L  L  T  T  K
 976 GGTGGTGTGTTAAGTAAATTTGGATATATGATGAAATTTAATTTAAATGAATATTTTCCATTATTAACAACAAAA
 351 K  L  F  I  R  G  I  I  E  E  L  L  W  F  I  R  G  E  T  N  G  N  T  L  L
1051 AAATTATTTATAAGAGGTATTATCGAAGAATTATTGTGGTTTATAAGAGGGGAAACAAATGGAAATACTTTGTTA
 376 E  K  N  V  R  I  W  E  A  N  G  T  R  E  F  L  D  R  K  L  F  H  R  E  V
1126 GAAAAAAACGTAAGAATATGGGAAGCTAATGGAACAAGGGAATTTTTAGATAGAAAATTATTTCATAGAGAAGTT
 401 N  D  L  G  P  I  Y  G  F  Q  W  R  H  F  G  A  E  Y  T  D  M  H  D  N  Y
1201 AATGATCTCGGTCCAATTTATGGATTTCAATGGAGGCATTTTGGTGCTGAATATACAGATATGCATGATAATTAT
 426 K  D  K  G  V  D  Q  L  K  N  I  I  N  L  I  K  N  D  P  T  C  R  R  I  I
1276 AAAGACAAAGGAGTTGATCAATTAAAAAATATTATAAATTTAATTAAAAATGATCCTACTTGTAGACGAATTATT
 451 L  C  A  W  N  V  K  D  L  D  Q  M  A  L  P  P  C  H  I  L  C  Q  F  Y  V
1351 TTGTGTGCATGGAATGTAAAAGATTTAGATCAAATGGCATTACCTCCTTGTCATATTTTATGTCAATTTTATGTT
 476 F  D  G  K  L  S  C  I  M  Y  Q  R  S  C  D  L  G  L  G  V  P  F  N  I  A
1426 TTTGACGGAAAATTATCATGTATTATGTATCAAAGATCTTGTGATTTAGGGCTTGGGGTTCCATTCAATATTGCT
 501 S  Y  S  I  F  T  Y  M  I  A  Q  V  C  N  L  Q  A  A  E  F  I  H  V  L  G
1501 TCCTATTCTATATTTACATATATGATAGCACAAGTATGTAACTTACAGGCAGCTGAATTTATACATGTATTGGGT
 526 N  A  H  V  Y  N  N  H  I  E  S  L  K  I  Q  L  N  R  T  P  Y  P  F  P  T
1576 AATGCTCATGTTTATAATAATCATATTGAAAGCTTAAAGATTCAGTTAAATAGAACTCCTTACCCTTTTCCTACT
 551 L  K  L  N  P  D  I  K  N  I  E  D  F  T  I  S  D  F  T  V  Q  N  Y  V  H
1651 CTTAAATTAAATCCTGACATTAAAAATATCGAGGATTTTACAATTTCTGATTTTACTGTTCAAAATTATGTTCAT
 576 H  D  K  I  N  M  D  M  A  A  *
1726 CACGATAAAATAAATATGGATATGGCAGCTTAA
```


Figure 3-15 Nucleotides and the deduced amino acid sequences of PbDHFR-TS. Nucleotide sequence and the deduced amino acid sequence indicated an open reading frame of 1,758 bp encoding 585 amino acid of *pbdhfr-ts* gene. The symbol (*) indicated a stop codon. The protein sequence was translated using the Sequence Manipulation Suit (DNA analysis and go to translate) available on http://www.bioinformatics.org/sms/.

3.4 Generation of transgenic *P. berghei* expressing *P. vivax* dihydrofolate reductase-thymidylate synthase enzyme

3.4.1 Standard short-term *in vitro* culture of *P. berghei* and purification of schizonts

Following overnight culture of blood stage parasites, Giemsa stained thin blood smear was made and examined for morphology of schizonts under a light microscope. As shown in **Figure 3-16**, viable schizonts were distinguished by the presence of 10-14 merozoites within one erythrocyte and one cluster of pigment (hemozoin). When 70-80% of the parasites in culture have matured into schizont stage, it is therefore suitable for proceeding to the step of purification for schizonts.

It was observed that the infected mice were needed to be kept on a normal day-night light cycle. *Plasmodium berghei* has a 22-24 hr asexual blood stage cycle which is partly synchronized in mice with the normal day-night light regime (Janse *et al*, 2006c). In these mice, the rupture of schizonts and invasion of erythrocytes mainly occur between 02.00 and 04.00 every 24 hr. As a result, most parasites were at the ring or young trophozoite stage when the infected blood was collected between 13.00 and 15.00. Moreover, a temperature between 36-37^{o}C is optimal to collect viable, mature schizonts after overnight culture at the time of 09.00-11.00. In addition, it was also found that shaking condition in order to keep the cell in suspension was a major rate-limiting step to obtain mature healthy schizonts for transfection (Allen & Kirk, 2010).

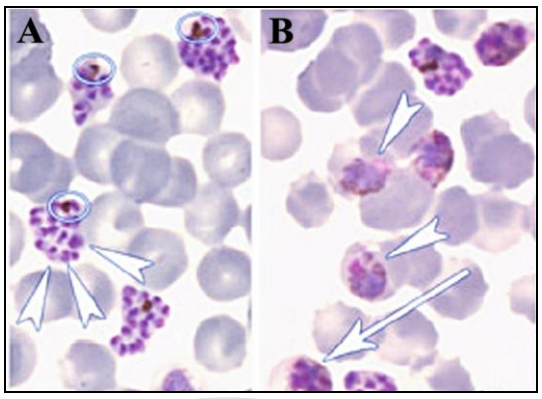


Figure 3-16 Images of cultured *P. berghei* schizonts in Giemsa stained thin blood smear. (**A)** Fully mature schizonts. Viable schizonts were recognized by the presence of 10-14 merozoites (arrowheads) and one dot of clustered malaria pigment (circled) within an erythrocyte. (**B**) Most cultures contain not only mature schizonts but also immature schizonts (white arrow) that are still in the process of merozoite formation and single nucleated gametocytes (arrowheads).

For use in transfection experiments, mature schizonts were separated from uninfected erythrocytes and other blood stages parasites in the culture using density gradient separation technique. Nycodenz® solution was used instead of Percoll® solution in this study. Percoll® solution was used by many researchers to separate different stages of *Plasmodium* parasites (Deharo *et al*, 1996; Krungkrai *et al*, 1993; Munderloh & Kurtti, 1987; Nillni *et al*, 1981; Russmann *et al*, 1982; Zhang *et al*, 1993). It was reported that Percoll® solution could affect the viability of parasites, while Nycodenz® solution does not (Menard & Janse, 1997; Thathy & Menard, 2002)

Nycodenz® solution was freshly prepared in a concentration of 60% with 1xPBS. For purification of schizonts, the parasite culture was layered on top of

the 60% Nycodenz® solution. After centrifugation at 250 x *g* for 20 min without brake setting, mature schizonts were collected and washed once with culture medium. **Figure 3-17** shows the Nycodenz®-purified *P. berghei* schizonts in Giemsa stained thin blood film. Fully mature schizonts contain 10-14 merozoites and free merozoites from schizonts that rupture during purification was observed (**Figure 3-17a**). Some immature schizonts and leucocytes were able to be seperated by Nycodenz® gradient solution (**Figure 3-17b**).

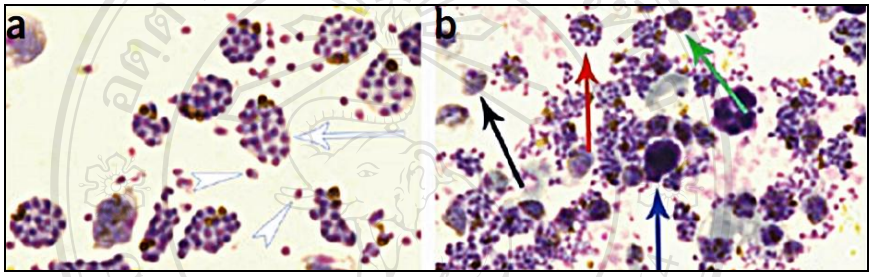


Figure 3-17 Images of Nycodenz®-purified *P. berghei* schizonts in Giemsa stained thin blood smear. (**a**) Fully mature schizonts containing 10-14 merozoites (white arrow) and free merozoites (arrowheads) from schizonts that ruptured during purification. (**b**) An overview of Nycodenz®-purified cells from *P. berghei* schizont culture showing the presence of mature schizonts (red arrow) and leucocytes (blue arrow), gametocytes (black arrow) and immature schizonts (green arrow).

3.4.2 Small-scale *in vitro* culture of *P. berghei* and purification of schizonts

The minimum volume of complete culture media required for overnight small-scale culture to obtain mature schizonts was determined. As shown in Figure 3-18a and 3-18b, the overnight small-scale culture volume of 500 µl gave

the highest proportion of mature schizonts at approximately 88% as compared with other culture volumes of 100 μl (~76%), 300 μl (~82%) and 1 ml (~73%). The proportion of mature schizonts obtained from a 500 μl culture was thus comparable with ~90% obtainable from the standard culture protocol. Although the proportion of mature schizonts obtained from the 100 μl or 300 μl culture volumes were similar to the 500 μl culture, the smaller culture volumes did not look as healthy as those obtained from 500 μl and 1 ml cultures as observed under a microscope (data not shown). Healthy schizonts were recognized by the presence of more than 10 merozoites within the erythrocyte, intact host membrane and visible malaria pigment.

In order to determine the optimal small-scale purification protocol to efficiently separate mature schizonts from other parasite stages, the volume of reagents needed to perform density gradient separation was investigated. As shown in Figure 3-18c and 3-18d, the separation protocol layering 1000 μl of cell suspension on top of 500 μl of 60% Nycodenz/PBS solution gave the highest yield of mature schizonts at ~86% with mature trophozoites/young schizonts at ~14%, without contamination of ring stages or non-infected erythrocytes. The result is comparable with the standard large-scale separation protocol that typically obtains >90% mature schizonts and <10% mature trophozoites/young schizonts. The small proportion of mature trophozoites/young schizonts does not interfere with transfection (Menard & Janse, 1997; Waters *et al*, 1997).

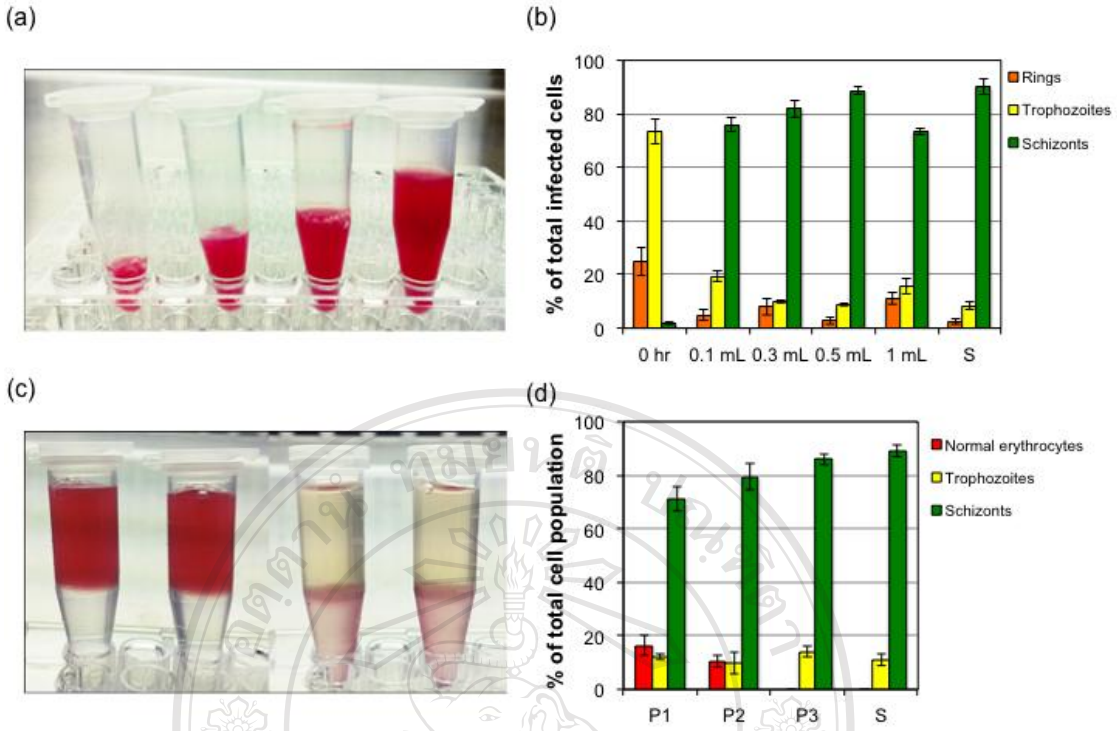


Figure 3-18 Small-scale culture and purification of schizonts from *P. berghei* in a 1.5-ml microcentrifuge tube. (a) Setup of *P. berghei* culture in 1.5-ml microcentrifuge tubes. Twenty microliters of infected blood at 10% parasitemia were inoculated in different volumes of complete RPMI-1640 medium of 100 µl, 300 µl, 500 µl and 1 ml. (b) Percentage of infected cells from each culture tube after culture for 20 h at 37°C, 5% CO_2, 5% O_2, 90% N_2 with continuous shaking. (c) Images of small-scale purification of mature schizonts in 1.5-ml microcentrifuge tubes, before and after centrifugation. (d) Percentage of cell population obtained from using different density gradient separation protocols. The volume of 60% Nycodenz/PBS solution: cell suspension in P1, P2 and P3 are 150 : 300, 250 : 500 and 500 : 1000 µl, respectively. S: standard large-scale protocol. Error bars indicate the standard error of the mean (SEM) obtained from 3 independent experiments.

A comparison of protocols for culture and purification of mature *P. berghei* schizonts is shown in Table 3-1. Since only a small volume of infected blood is needed for the proposed small-scale protocol, it is possible to obtain sufficient infected blood from a clipped tail, without animal sacrifice. The infected animal and parasites could thus be used for other studies. Although infected blood with 1-2% parasitemia is used in the standard protocol (Janse *et al*, 2006c; Thathy & Menard, 2002), a higher starting parasitemia of 5-10% is required for the small-scale culture protocol to obtain enough mature schizonts (at least $1x10^6$) for a single transfection experiment.

Table 3-1 Comparison between standard and small-scale protocols of reagent volume and material used for overnight culture and purification of *P. berghei* schizont

	Standard protocol	**Small-scale protocol**
Infected blood volume	1-2 mL	20 µL
Starting parasitemia	1-5%	5-10%
Container	Culture flask ($75cm^2$ growth area)	1.5-ml microcentrifuge tube
Culture media volume	50-100 mL	500 µL
60%Nycodenz/PBS : cell suspension volume	10 : 30 mL	500 : 1000 µL
Number of purified schizonts	10^7-10^9	10^6-10^7
Number of transfections	Up to 10	1

3.4.3 Generation of transgenic *P. berghei* stably expressing wild type *P. vivax* dihydrofolate reductase-thymidylate synthase enzyme

Mature schizonts of the reference GFP-expressing parasite line, PbGFP507m6cl1 were transfected with the linear form of pL35pvDHFR-TS plasmid by electroporation in order to introduce wild-type *pvdhfr-ts* gene replacing the endogenous *pbdhfr-ts* gene. For selection of *P. berghei* harboring wild-type *pvdhfr-ts*, a two-step drug selection procedure was performed. First, transgenic parasites with integrated wild-type *pvdhfr-ts* were selected with pyrimethamine. Subsequently, negative selection was performed to select for parasites which had excised the positive-negative drug selection cassette in order to obtain parasites that have only wild-type *pvdhfr-ts* gene integrated to the *P. berghei* genome (**Figure 3-19 and 3-20**). A single clone of the transgenic *P. berghei* parasite clones stably expressing wild-type *Pv*DHFR-TS (designated **PbGFPpvDHFR-TScl4**) was obtained by limiting dilution method. The correct integration of *pvdhfr-ts* gene replacing *pbdhfr-ts* was further confirmed by PCR (**Figure 3-22**) and Southern analysis (**Figure 3-23**).

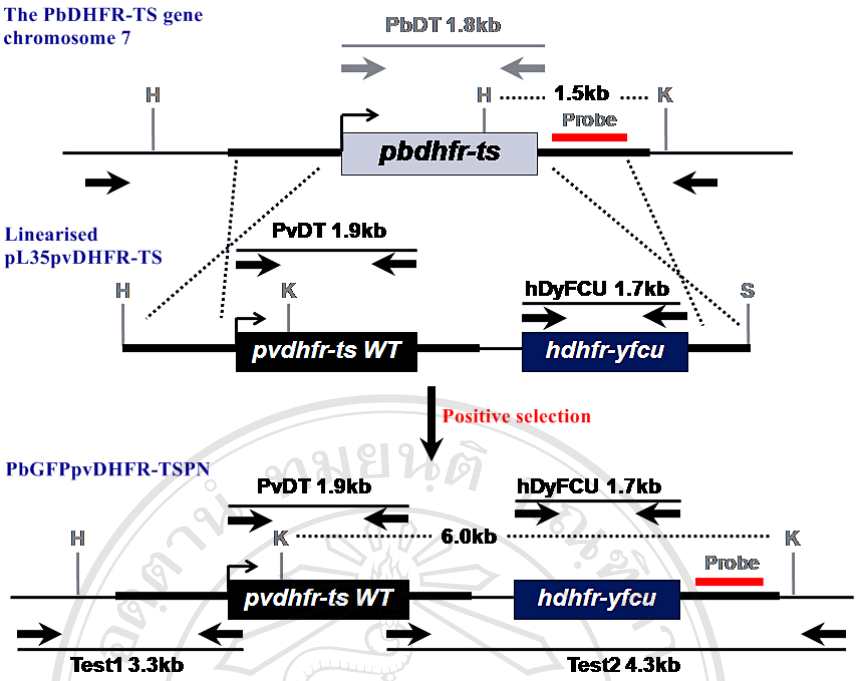


Figure 3-19 Generations of PbGFP507m6cl1 expressing PvDHFR-TS wild type and negative selectable marker. Diagrammatic representation of the *pbdhfr-ts* genomic locus, linearized pL35pvDHFR-TS replacement plasmid and the predicted integration event after positive selection with pyrimethamine (bottom). The linearized pL35pvDJFR-TS plasmid contained the *pvdhfr-ts* wild type selectable cassette flanked by 5' and 3' UTR regions of the *pbdhfr-ts* gene. This construct contains a fusion gene of the positive (*hdhfr*) and negative selectable marker (*yfcu*) under control of the *ef1α-a* promoter. Red lines represent the probe used in Southern blot analysis. Arrows represent primers used for PCR analysis of expressions. H, *Hind*III; K, *Kpn*I; S, *Sma*I.

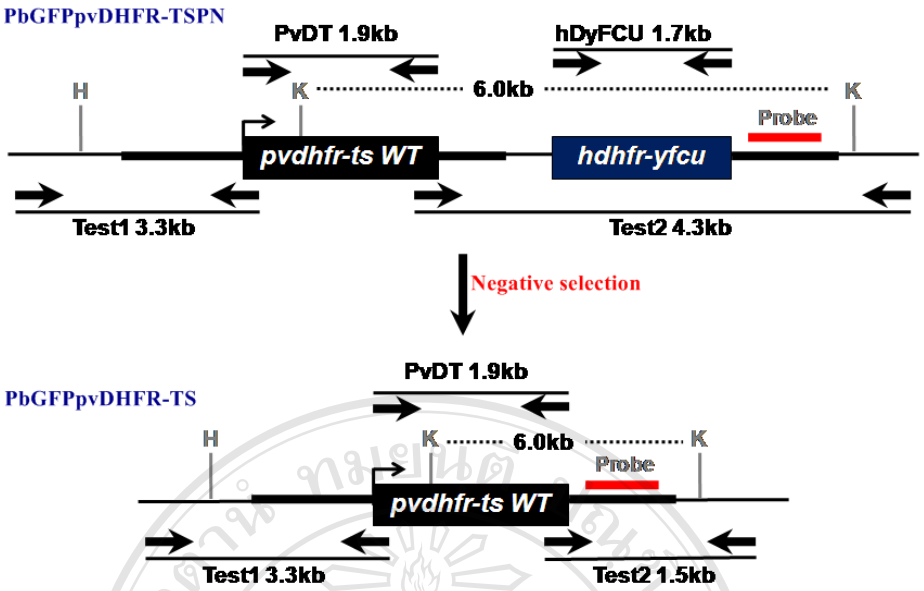


Figure 3-20 Generations of PbGFP507m6cl1 expressing PvDHFR-TS wild type. Diagrammatic representation of the integration event after positive selection with pyrimethamine, and predicted integration locus after negative selection with 5-FC of the transfected parasites (bottom). The negative selectable marker cassette has been excised from the integrated construct by a recombination event between the two 3' UTR of *pbdhfr-ts* sequences. Red lines represent the probe used in Southern blot analysis. Arrows represent primers used for PCR analysis of expressions. H, *Hind*III; K, *Kpn*I.

3.4.4 Generation of transgenic *P. berghei* stably expressing mutant *P. vivax* dihydrofolate reductase-thymidylate synthase enzyme

Mature schizonts of the reference GFP-expressing parasite line, PbGFP507m6cl1 were transfected with the linear form of pL17pvSP21 plasmid by electroporation in order to introduce mutant *pvdhfr-ts* gene replacing the endogenous *pbdhfr-ts* gene (**Figure 3-21**). After transfection, transgenic parasites with integrated mutant *pvdhfr-ts* were selected with pyrimethamine (**Figure 3-21**). A single clone of

the transgenic *P. berghei* parasite clones stably expressing mutant *Pv*DHFR-TS SP21 (designated **PbGFPpvSP21cl2**) was obtained by limiting dilution method. The correct integration of pvdhfr-ts gene replacing pbdhfr-ts was further confirmed by PCR (**Figure 3-22**) and Southern analysis (**Figure 3-23**).

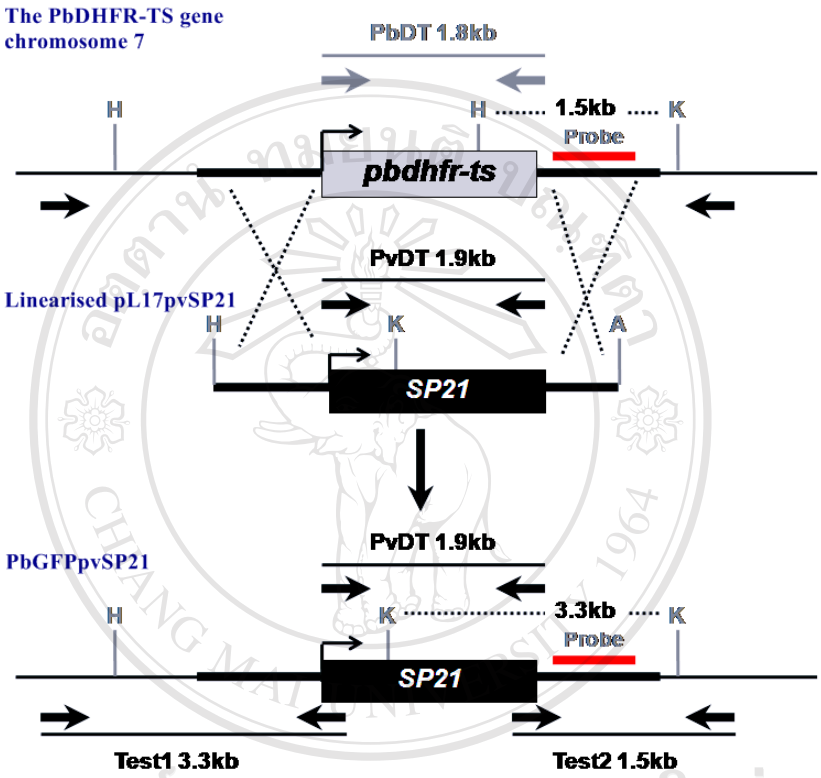


Figure 3-21 Generation of PbGFP507m6cl1 parasite expressing PvDHFR-TS double mutation SP21. Diagrammatic representation of the *pbdhfr-ts* genomic locus, linearized pL17pvSP21 replacement plasmid, and the predicted integration event (bottom). The linearized pL17pvSP21 plasmid contained the *pvdhfr-ts* double mutant SP21 selectable cassette flanked by 5' and 3' UTR regions of the *pbdhfr-ts* gene. Red lines represent the probe used in Southern analysis. Arrows represent primers used for PCR analysis of expressions. H, *Hind*III; K, *Kpn*I; A, *Afl*II.

3.5 Genotypic analysis of transgenic *P. berghei* stably expressing *P. vivax* dihydrofolate reductase-thymidylate synthase enzyme

Genomic DNA of each clone was extracted as previously described in Materials and Methods section. The correct integration of the *pvdhfr-ts* gene replacing *pbdhfr-ts* gene was firstly verified by PCR using sets of specific primers (**Table 2-1**).

To verify the presence of *pvdhfr-ts* gene in the transgenic *P. berghei* parasite genome, 1.9 kb of *Pvdhfr-ts* fragment was amplified by *Pv*DT primers (**Figure 3-19, 3-20 and 3-21**). The products of 3.3 kb and 1.5 kb fragments amplified by integration-specific Test1 and Test2 primers, respectively, demonstrated the correct 5' and 3' integration of *pvdhfr-ts* gene into the *dhfr-ts* locus of *P. berghei* (**Figure 3-22A, B, lanes 5-6**). In the case of WT *Pv*DHFR-TS, 1.7 kb fragment of *hdhfr-yfcu* could be amplified by hDyFCU primers before negative selection (**Figure 3-22C**), while 3' integration fragment of this parasite showed 4.3 kb as a result of *hdhfr-yfcu* selectable marker cassette still contains in the genome. Following negative selection using 5-FC, the selectable marker cassette was excised from the genome through homologous recombination as shown in PCR product of 1.5 kb showing 3' integration event (**Figure 3-22B, lanes 5-6**) and the absence of *hdhfr-yfcu* fusion gene (**Figure 3-22C, lanes 5-6**). The endogenous *pbdhfr-ts* gene could only been amplified by specific PbDT primers in parental PbGFP507m6cl1 parasite showing that endogenous *pbdhfr-ts* gene has been replaced with respective *pvdhfr-ts* gene in the newly generated transgenic parasites (**Figure 3-22D**).

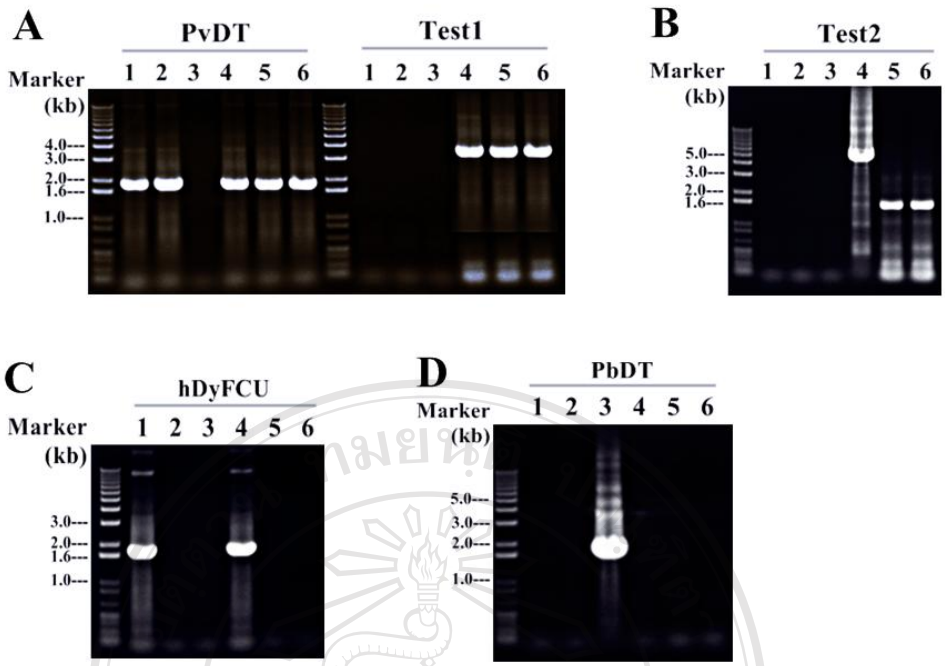


Figure 3-22 Correct integration of the expression constructs in the parasites as shown by PCR. (**A**) *Pv*DT, amplification of *pvdhfr-ts* (primers *Pv*DTF/*Pv*DTR); Test1, amplification of 5' integration in *pbdhfr-ts* locus (primers 5'*Pv*INTF/5'*Pv*INTR). (**B**) Test2, amplification of 3' integration in *pbdhfr-ts* locus (primers 3'*Pv*INTF/3'*Pv*INTR). (**C**) hDyFCU, amplification of positive-negative selectable marker gene (primers hDyFCUF/hDyFCUR). (**D**) *Pb*DT, amplification of *pbdhfr-ts* (primers *Pb*DTF/*Pb*DTR).

Lane 1 = pL35pvDHFR-TS plasmid control

Lane 2 = pL17pvSP21 plasmid control

Lane 3 = genomic DNA of PbGFP507m6cl1 transgenic parasite

Lane 4 = genomic DNA of PbGFPpvDHFR-TSPNcl1 transgenic parasite

Lane 5 = genomic DNA of PbGFPpvDHFR-TScl4 transgenic parasite

Lane 6 = genomic DNA of PbGFPpvSP21cl2 transgenic parasite

The transgenic parasite clones, termed PbGFPpvDHFR-TSPNcl1, PbGFPpvDHFR-TScl4 and PbGFPpvSP21cl2, were selected for Southern analysis. The genomic DNA of wild type and transgenic *P. berghei* parasites was digested with *Hind*III and *Kpn*I restriction enzymes and hybridized to 3'UTR *pbdhfr-ts* probe. Digestion of the genomic DNA with these restriction enzymes should generate a 1.5-kb fragment for the wild type *P. berghei* (PbGFP507m6cl1), but a 3.3-kb fragment for the transgenic parasites that hybridized to this probe. In addition, the presence of positive-negative selection cassette in PbGFPpvDHFR-TSPNcl1 parasite after positive selection with pyrimethamine showed a band corresponding to 6 kb. After negative selection with 5-FC and the positive-negative selection cassette had been excised, a 3.3 kb fragment of digested genomic DNA from this transgenic parasite could be detected by the 3'UTR *pbdhfr-ts* probe (**Figure 3-23**). These Southern results confirmed that all the transgenic parasites contained the correct integration of *pvdhfr-ts* gene replacing endogenous *pbdhfr-ts* in their genomes.

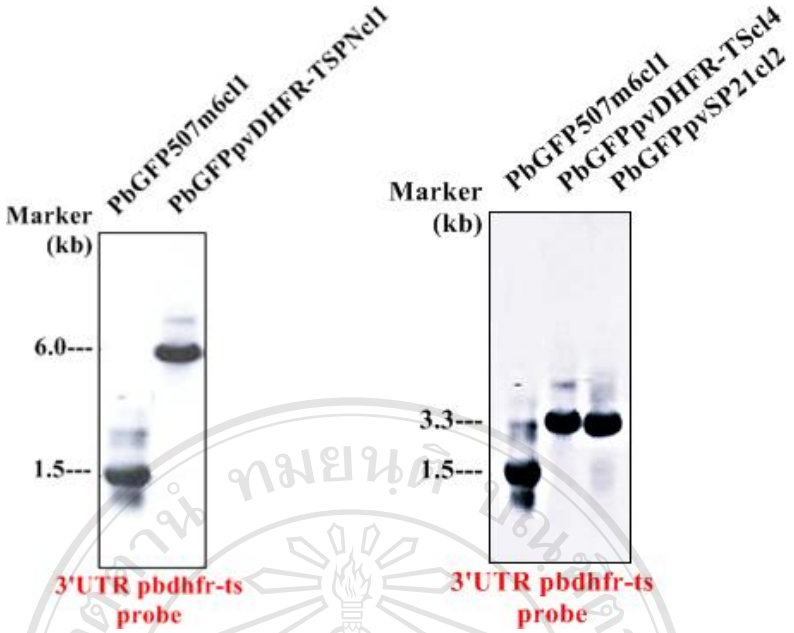
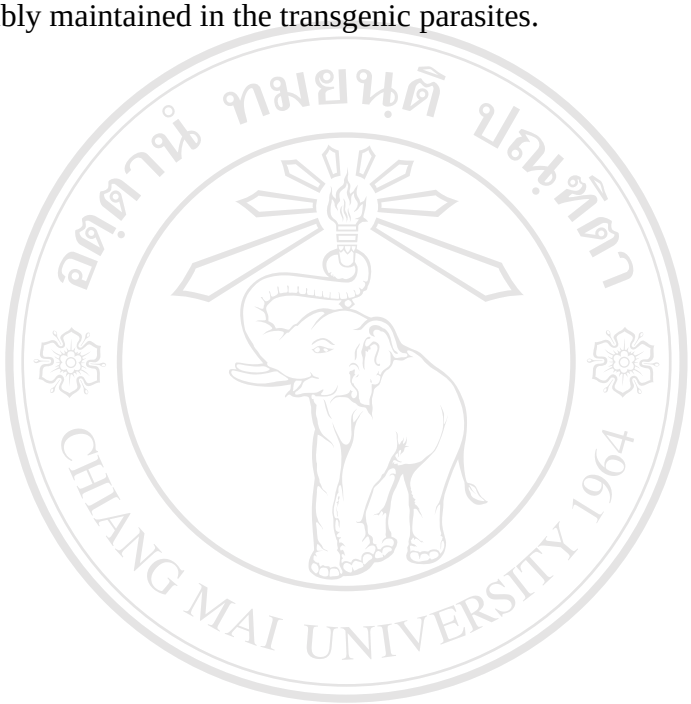


Figure 3-23 Southern analysis of correct integration of plasmids to the parasite genome. To demonstrate integration into the *pbdhfr-ts* locus, genomic DNA of PbGFP507m6cl1 and transgenic parasites were digested with *Hind*III and *Kpn*I, and hybridized to the 3'UTR *pbdhfr-ts* probe. In PbGFP507m6cl1 parasite, a 1.5 kb fragment hybridized to this probe whereas a transgenic parasite, a fragment of 6 kb was detected when the integration of plasmid containing positive-negative selection cassette occurred (PbGFPpvDHFR-TSPNcl1), and fragment of 3.3 kb (PbGFPpvDHFR-TScl4 and PbGFPpvSP21cl2) when the integration took place in the *pbdhfr-ts* locus without the positive-negative selection cassette.

In order to assess the stability of integrated *pvdhfr-ts* gene in the genome of transgenic parasites, all parasite clones were allowed to proliferate *in vivo* with or without drug pressure, and kept as cryopreservation stock in the liquid nitrogen for 2 years. Genomic DNA of the transgenic clones were prepared and subjected to PCR analysis for the introduced *pvdhfr-ts* gene. The PCR products of 1.5-kb,

corresponding to the integrated *dhfr-ts* locus, and 1.9-kb corresponding to *pvdhfr-ts* gene were amplified using the Test2 and *Pv*DT primers, respectively, from all of the templates of transgenic parasites collected at different time points. Only a 1.8-kb fragment was amplified from the template of wild type *P. berghei* parasites using the *Pb*DT primer (**Figure 3-24**). These results indicated that all the integrated *pvdhfr-ts* genes are stably maintained in the transgenic parasites.



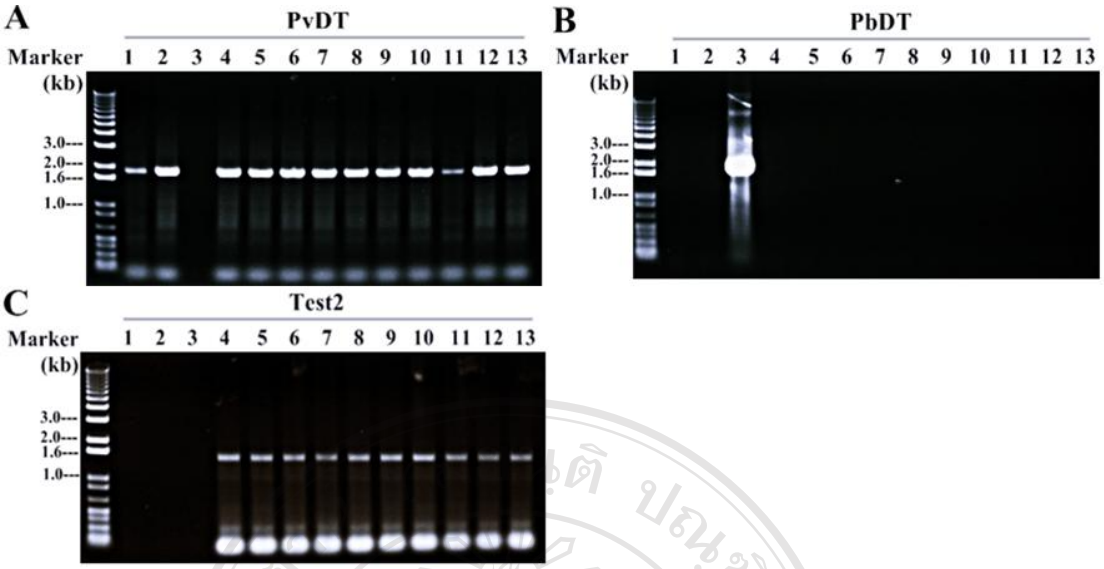


Figure 3-24 PCR analysis of the stability of the integrated locus in transgenic parasite clones. Genomic DNA was extracted from transgenic or PbGFP507m6cl1 control parasites at various times. The primer pair of *Pv*DTF/*Pv*DTR (**A**) was used to detect the *pvdhfr-ts* gene in the genome of parasite clones, while the primer pair of *Pb*DTF/*Pb*DTR (**B**) was used to amplify the native sequence of *pbdhfr-ts* in the wild type PbGFP507m6cl1 parasites used as control. The primer pair of 3'*Pv*INTF/3'*Pv*INTR (**C**) was used to detect the integrated fragment in the transgenic parasites.

Lane 1 = pL35pvDHFR-TS plasmid control

Lane 2 = pL17pvSP21 plasmid control

Lane 3 = genomic DNA of PbGFP507m6cl1 parental parasite

Lanes 4-8 = genomic DNA of PbGFPpvDHFR-TScl4 transgenic parasite that was extracted at various times (days 90, 180, 360, 540 and 2 years of cryopreservation stock, respectively)

Lanes 9-13 = genomic DNA of PbGFPpvSP21cl2 transgenic parasite that was extracted at various times (days 90, 180, 360, 540 and 2 years of cryopreservation stock, respectively)

3.6 Growth analysis of transgenic *P. berghei* parasites

Having confirmed that the *pbdhfr-ts* could be replaced with *pvdhfr-ts* genes in *P. berghei*, we examined the blood-stage phenotype of the transgenic parasite lines. After injecting mice with wild type PbGFP507m6cl1 parasites, parasitemia reached 3% at day 4 after infection. The same level of parasitemia was reached by parasites of the transgenic lines one or two days later. However, parasitemia observed in all mouse groups over a similar time frame showed no statistical differences (**Figure 3-25**). It was then concluded that the replacement of *pbdhfr-ts* with *pvdhfr-ts* alleles do not alter the growth rate of the transgenic parasites. Hence, *Pv*DHFR can function correctly when expressed in *P. berghei*.

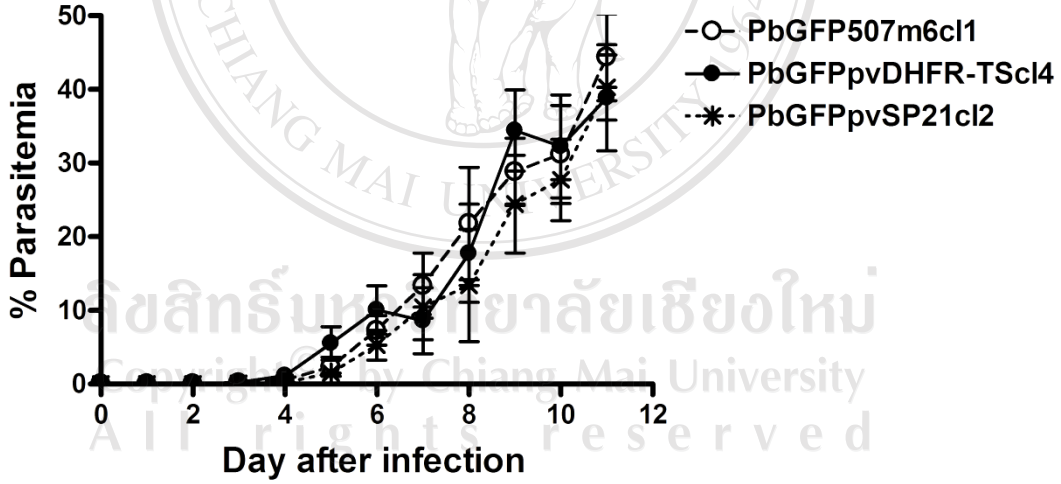


Figure 3-25 Comparison of parasitemia between the transgenic and its parental *P. berghei*. Three groups of mice (5 mice/group) were infected with either $1x10^6$ erythrocytes harboring control PbGFP507m6cl1, transgenic PbGFPpvDHFR-TScl4, or PbGFPpvSP21cl2 clone. The parasitemia was assessed daily on Giemsa stained thin blood smear.

3.7 Drug sensitivity analysis of transgenic *P. berghei* parasite stably expressing *P. vivax* dihydrofolate reductase-thymidylate synthase enzyme

Finally, to validate these transgenic parasites expressing *Pv*DHFR-TS as *in vivo* models for drug screening, the primary assessment of antimalarial drugs, pyrimethamine, chloroquine and artesunate, efficacy *in vivo* against the transgenic parasites was performed. Using the standard 4-day suppressive test, mice were inoculated with approximately $1x10^7$ parasitized erythrocytes intravenously. The infected mice were then treated orally by gavage once daily for 4 successive days from the same day of parasite inoculation. The course of parasitemia in untreated mice (control group) and in mice treated with different doses of three standard antimalarials (pyrimethamine, chloroquine, and artesunate) were monitored by flow cytometric enumeration as previously mentioned in Materials and Methods section.

After inoculation intravenously with $1x10^7$ parasitized erythrocytes, mice in the untreated control groups showed a progressively increase parasitemia, and all the mice died by day 11. Pyrimethamine is a standard antifolate drug and used as primary compound to test this system. The transgenic PbGFPpvDHFR-TScl4 demonstrated a drug susceptibility profile similar to that of the wild type parental PbGFP507m6cl1 line at ED_{50} of 0.53 and 0.69 mg/kg, respectively. This demonstrated that *Pv*DHFR-TS wild type was at least equally susceptible to antifolate compounds, compared with wild type *Pb*DHFR-TS. In contrast, transgenic PbGFPpvSP21cl2 was considerably less susceptible to this inhibitor. It had ~60-fold increased ED_{50} value to pyrimethamine (**Figure 3-26A**), compared with PbGFP507m6cl1, but was 40-fold more resistance to pyrimethamine, compared with PbGFPpvDHFR-TScl4. Therefore,

the double mutant DHFR-TS of *P. vivax* was very effective in conferring a high level of resistance to pyrimethamine in *P. berghei*. In our system, we found that pyrimethamine at the concentration of 50 mg/kg was found to be lethal to mouse as all animals died soon after treatment with this dose by oral administration. Hence, the maximum dose for this experiment was performed at 30 mg/kg.

In contrast to pyrimethamine, chloroquine is a 4-aminoquinoline drug that inhibits hemozoin formation in the food vacuole of the parasites. It was used as a standard non-antifolate compound. As shown in **Figure 3-26B**, all parasite lines were susceptible to chloroquine treatment, and parasitemia was progressively increasing until all infected mice died by day 11. However, PbGFPpvSP21cl2 was ~2.5-fold and ~1.4-fold more resistant to chloroquine than PbGFP507m6cl1 and PbGFPpvDHFR-TScl4, respectively.

Artesunate is part of the artemisinin group of drugs that are used to treat malaria. It is a semi-synthetic derivative of artemisinin that is water-soluble and may therefore be given by injection. In addition, artesunate is extremely potent against highly pyrimethamine-resistant parasites. All parasite lines were susceptible to artesunate treatment with ED_{50} of 5.43, 7.43, and 7.59 mg/kg in PbGFP507m6cl1, PbGFPpvDHFR-TScl4, and PbGFPpvSP21cl2, respectively (**Figure 3-26C**).

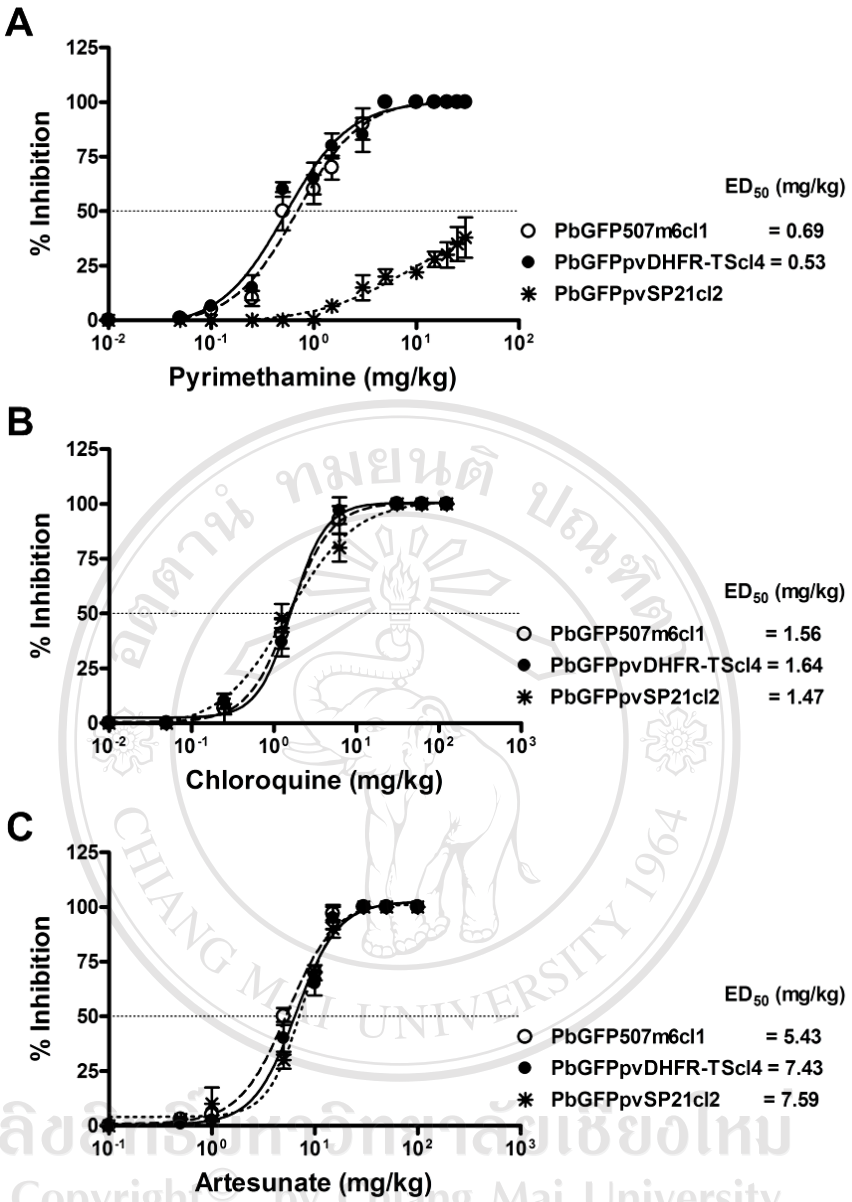


Figure 3-26 Sensitivity of *P. vivax* DHFR-TS-expressing *P. berghei* to (A) pyrimethamine, (B) chloroquine and (C) artesunate. Drugs were prepared as described in Materials and Methods section. Three groups of mice (5 mice/group) were infected intravenously with either $1x10^7$ PbGFP507m6cl1 parental parasite, transgenic PbGFPpvDHFR-TScl4, or PbGFPpvSP21cl2 parasitized erythrocytes. Drugs were given orally by gavage with different doses for 4 consecutive days. Untreated controls were given DMSO or water only.