Molecular analysis of *Pfmdr1* gene in *Plasmodium falciparum* in Thai-Cambodia border

Miss Papatchaya Pa-onta

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การวิเคราะห์ทางอณูโมเลกุลของยีนพีเอฟเอ็มดีอาร์วัน ของเชื้อพลาสโมเดียม ฟัลซิพารัม ในแถบชายแดนไทย-กัมพูชา

นางสาวปภัชญา พาอ่อนตา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Miss Papatchaya Pa-onta
Field of Study	Public Health Sciences
Thesis Advisor	Assistant Professor Naowarat Kanchanakhan, Ph.D.

Accepted by the College of Public Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the College of Public Health Sciences

(Professor Sathirakorn Pongpanich, Ph.D.)

THESIS COMMITTEE

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.....Thesis Advisor

(Assistant Professor Naowarat Kanchanakhan, Ph.D.)

.....Examiner

(Tepanata Pumpaibool, Ph.D.)

.....External Examiner

(Jitravee Cheychom, Ph.D.)

ปภัชญา พาอ่อนตา : การวิเคราะห์ทางอณูโมเลกุลของยีนพีเอฟเอ็มดีอาร์วัน ของเชื้อพลาสโมเดียม ฟัลซิพารัมในแถบชายแดนไทย-กัมพูชา (Molecular analysis of *Pfmdr1* gene in *Plasmodium falciparum* in Thai-Cambodia border) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. เนาวรัตน์ กาญจนาคาร, 127 หน้า.

การดื้อยาของเชื้อมาลาเรียชนิดฟัลซิพารัมยังคงเป็นปัญหาสำคัญต่อการควบคุมโรคมาลาเรีย การกำหนดนโยบายยาในการรักษายังใช้ผลการศึกษาการตอบสนองต่อยาของเชื้อมาลาเรียด้วยวิธีทดลองในคนและในหลอดทดลองควบคู่กันไป ซึ่งทั้งสองวิธีนี้ต้องอาศัยผู้เชี่ยวชาญในการทำในห้องปฏิบัติการและการอ่านผลการทดลองที่แม่นยำ อีกทั้งใช้ระยะเวลานานในการทำการทดลองแต่ละครั้ง ดังนั้นการใช้เครื่องหมายทางชีววิทยาโมเลกุลจึงเป็นทางเลือกอีกทางหนึ่งที่จะใช้ในการเฝ้าระวังและติดตามประสิทธิภาพของยารักษาโรคมาลาเรีย

มีการศึกษาพบว่าการเกิดการกลายพันธุ์และการเพิ่มจำนวนชุดของยีนพีเอฟเอ็มดีอาร์วันมีความสัมพันธ์กับการดื้อยาผสมระหว่างอาร์ทีซูเนตและเมฟโฟลควิน (ACTs) วัตถุประสงค์ในการศึกษาในครั้งนี้ จึงต้องการศึกษาความชุกของเชื้อฟัลซิพารัมที่ดื้อยาโดยการตรวจหาการกลายพันธุ์ของยีนพีเอฟเอ็มดีอาร์วันที่ตำแหน่ง N86Y Y184F และ S1034C ด้วยวิธีพีซีอาร์-อาร์เอฟแอลพี และตรวจหาการเพิ่มจำนวนชุดของยีนพีเอฟเอ็มดีอาร์วันโดยวิธีแทคแมนโพลบเรียลไทม์พีซีอาร์ ผลที่ได้จะถูกวิเคราะห์เปรียบเทียบกับผลของการรักษาด้วยยาผสมเอซีที

ตัวอย่างเชื้อฟัลซิพารัมมาลาเรียจำนวน 73 ตัวอย่าง ซึ่งได้จากโครงการศึกษาประสิทธิผลของยาเอซีทีใน 2 จังหวัดที่มีการระบาดของเชื้อมาลาเรียในประเทศไทย คือจังหวัดจันทบุรีและจังหวัดตราด จากผลการทดลองพบว่าเชื้อฟัลซิพารัมมาลาเรียที่จังหวัดตราดมีการเพิ่มจำนวนชุดของยีนที่มากกว่า 3 ชุด จำนวน 9.09 เปอร์เซ็นต์ แต่ในจังหวัดจันทบุรีไม่พบว่ามีการเพิ่มจำนวนชุดของยีนพีเอฟเอ็มดีอาร์วันที่มากกว่า 3 ชุด ค่าเฉลี่ยของจำนวนชุดของยีนพีเอฟเอ็มดีอาร์วันของเชื้อฟัลซิพารัมมาลาเรียที่เก็บได้จากจังหวัดตราดและจันทบุรีมีจำนวน 2.2 ชุด และ 1.5 ชุด ตามลำดับ ในทางกลับกันไม่พบการกลายพันธุ์ของยีนนี้ในเชื้อฟัลซิพารัมมาลาเรียที่เก็บมาจากจังหวัดตราด แต่พบว่าเชื้อที่เก็บมาจากจังหวัดจันทบุรีมีการกลายพันธุ์ที่ตำแหน่ง N86Y 20 เปอร์เซ็นต์ (วิเคราะห์จากตัวอย่าง 15 ตัวอย่าง) และตำแหน่ง S1034C 28.57 เปอร์เซ็นต์ (วิเคราะห์จาก 7 ตัวอย่าง) และไม่พบการกลายพันธุ์ที่ตำแหน่ง Y184F ในเชื้อที่เก็บจากทั้งสองจังหวัด และผลการทดลองนี้มีความสอดคล้องกับผลการรักษาด้วยยาผสมเอซีที ดังนั้น การกลายพันธุ์และการเพิ่มจำนวนชุดของยีนพีเอฟเอ็มดีอาร์วันอาจใช้เป็นเครื่องมือในการติดตามการดื้อยาของเชื้อฟัลซิพารัมมาลาเรียทั้งในทางปฏิบัติการและในการศึกษาการระบาดวิทยาขนาดใหญ่

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Drug resistant *Plasmodium falciparum* is a major problem for malaria control. Policy makers currently depend on *in vivo* and *in vitro* tests to adjust antimalarial regimens for malarial treatment guideline. These two methods are required expertise for interpretation and time consuming. Therefore the alternative reliable molecular markers of antimalarial resistance could play an important role in the surveillance of drug efficacy. *Pfmdr1* gene has been shown to be a reliable marker of resistance for *P. falciparum* related to artesunate and mefloquine combination therapies. The propose of this study are to investigated the prevalence of *P. falciparum* multidrug resistance by determined *Pfmdr1* point mutations at codon N86Y, Y184F, S1034C by PCR-RFLP and investigated *Pfmdr1* copy number by using real-time quantitative PCR with TaqMan and compared to efficacy of ACTs (Artemisinine Combination Therapies).

Seventy-three infected blood samples were collected from the therapeutic efficacy of ACTs project tested in 2 provinces in malaria endemic areas of Thailand which are Chantaburi and Trat. The results showed that in Trat province exhibited the higher percentage of *P. falciparum* with three or more copies of *Pfmdr1* than in Chantaburi (9.09% and 0%, respectively). The mean of *Pfmdr1* copy number in *P. falciparum* collected from Trat and Chantaburi provinces were 2.2 and 1.5 respectively. In contrast, there were no mutations in *Pfmdr1* gene in *P. falciparum* from Trat whereas in Chantaburi found point mutations in N86Y (20%, n=15), S1034C (28.57%, n=7) and no mutations were found in Y184F both in *P. falciparum* collected from Trat and Chantaburi.

These results were correlated to the ACTs efficacy tests. *Pfmdr1* mutations and copy number may be used as a high throughput tool to investigate the role of drug resistance of malaria parasites in laboratory studies or large scale epidemiological surveys.

Field of Study: Public Health Sciences Student's Signature .....

Academic Year: 2015 Advisor's Signature .....

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CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µg	Microgram
µg/ µl	Microgram/ microliter
µl	Microliter
ACTs	Artemisinin-based combination therapies
bp	Base pair
bps	Base pair sizes
C	Cytosine
cDNA	Complementary DNA
℃	Degree celsius
D	Aspartic acid
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates
dsDNA	Double-stranded deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
F	Phenylalanine
g/mol	gram/mole

M	Molar
mg	Milligram
MGB	Minor-groove binding
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Milliliter
mRNA	Messenger RNA
N	Asparagine
PBS	Phosephate buffer saline
PCR	Polymerase chain reaction
S	Serine
ssDNA	Single-stranded deoxyribonucleic acid
TBE	Tris Borate EDTA buffer
Tm	Melting temperature
VRAC.	Volume-regulated anion channels
WHO	World Health Organization
Y	Tyrosine

CHAPTER I
INTRODUCTION

Malaria is potentially infectious disease which is a major worldwide public health problem with appearance resistant of anti-malarial drugs, especially *Plasmodium falciparum* that develop to resist all classes of anti-malarial drugs including of the artemisinin derivatives. Furthermore, malaria is caused by protozoa parasite, *Plasmodium* species. There are five species of *Plasmodium* parasite can cause infectious malaria in human; *P. falciparum, P. malariae, P. ovale, P. vivax, and P. knowlesi*. The most severity is *P. falciparum* infection that causes life-threatening condition, cerebral malaria and this species also develop to resist all classes of anti-malarial drugs including the artemisinin. In human, the infection can occur by intermediate hosts essentially female Anopheles mosquitoes. The incident of this disease frequently occurs more than 100 countries in tropical and subtropical area, commonly in Africa, India, Latin America, and South-East Asia [WHO: International Travel and Health 2012]. In Thailand, malaria is potential infection disease in international border areas, Thai-Myanmar, Thai-Laos, Thai-Malaysia, and Thai-Cambodia such as Trat province and Chanthaburi province.

Nowadays the most effective treatment for the malaria infection is artemisinin-based combination therapy (ACTs). In Thailand, artesunate-mefloquine is suggested by World Health Organization (WHO) that an assumption is the combination-drugs partner

can protect artemisinin resistance. However, recent documents indicated that artemisinnin resistance has already appeared along the international border of Thailand. The *Plasmodium* parasite becomes more resistant to these drugs especially *P. falciparum* species. A serious problem that limits the efficiency of malarial control program is the distribution of multidrug resistance to *P. falciparum* [Dondorp et al., 2009; Chaijaroenkul et al., 2010, Vijaykaga et al, 2006].

The *P. falciparum* multidrug resistance gene (*Pfmdr1*) has been reported that it is related to alterative susceptibility to different anti-malarial drugs. This gene locates on chromosome 5 and encodes 12 transmembrance-domain proteins, which is called P- glycoprotein homologue 1 (Pgh1) (Mungthin et al., 2014). This protein locates at digestive vacuole, where is the site of action of chloroquine and other quinoline-base antimalarial drugs. However, the relation between *Pfmdr1* and artemisinin drugs resistance have remained difficult to understand. Resistance mechanisms providing this mainly implicate mutations and amplifications of the gene encoding target enzymes or transporters. A single genetic mutation in Pgh1 is not involved the resistance of chloroquine and mefloquine but Reeds and colleagues mentioned that the mutations in Pgh1 can lead to mefloquine, quinine and halofantrine resistance [Reeds et al., 2000]. The same mutations impact parasite resistance towards chloroqiune in a strain-specific manner and the level of sensitivity unrelated to artemisinin [Poyomtip et al., 2012; Muhamad et al., 2013; Sidhu et al., 2005; Sidhu et al., 2006]. This is important

assumptions for the development of anti-malarial agents (Reed, et al., 2000). Furthermore, the amplification of *Pfmdr1* gene has been reported to relate with malaria multi-drugs resistance, mefloquine resistant culture-adapted clone lines identified [Congpuong et al., 2005]. There have been various reports using *Pfmdr1* copy number as a molecular tool for monitoring anti-malarial drugs efficiency [Duah et al., 2013; Phompradit et al., 2011].

In this study, we investigate the prevalence of *P. falciparum* multi-drugs resistance by determining *Pfmdr1* copy number and *Pfmdr1* point mutations of *P. falciparum* endemic in Thai-Cambodia border, Trat and Chanthaburi provinces of Thailand.

Objectives

General Objective:

To evaluate the alteration of *Pfmdr1* gene in *P. falciparum* collected from Thai-Cambodia endemic area.

Specific Objective:

1. To analyse the *Pfmdr1* point mutations of *P. falciparum* endemic in Thai-Cambodia border.
2. To analyse the *Pfmdr1* copy number in *Pfmdr1* gene of *P. falciparum* endemic in Thai-Cambodia border.

CHAPTER II

REVIEW OF RELATED LITERATURES

1. Malaria

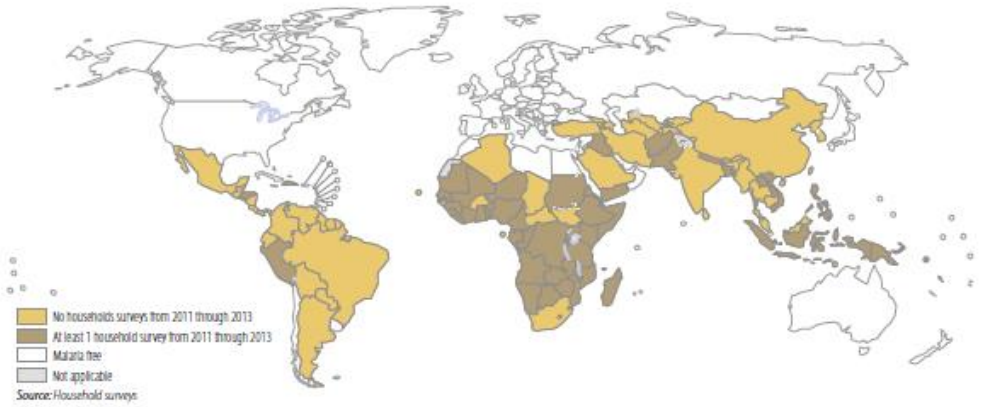


Figure 1 Malaria at risk for transmission [World Health Organization, 2013]

Malaria is caused by protozoan parasites in genus *Plasmodium*, which was transmitted through human by female anopheles mosquito. Five species of the *Plasmodium* are malaria infectious agent in human including *Plasmodium falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi*. Among these five species, *Plasmodium falciparum* is the most dangerous parasite because it can cause the cerebral malaria which leads to coma and death. *Plasmodium vivax* and *P. ovale* are relatively less virulent but its hypnozytic (dormant) stage in liver cell can cause relapse fever. However, *P. malariae,* and *P. ovale* are most prevalence in Africa. Their incidences in Southest Asia are much less. The last species, *P. knowlesi* is a zoonostic parasite which

accidentally transmits from macaque to human. The incidence of *P. knowlesi* infection has been found to restrict in South East Asia [WHO: World Malaria Report. 2013].

Figure 2 The situation of malaria in the Thai border area [http://travel.stackexchange.com/questions/3796/where-in-thailand-should-i-be-cautious-about-malaria]

2. **The Situation of malaria in Thailand**

In Thailand, the situation of malaria cases has decreased but there is still found along border areas of Thai-Myanmar, Thai-Cambodia, Thai-Laos and Thai-Malaysia (Figure 2). The border areas with Myanmar and Cambodia are worst affected. Non-immune migrant workers occupied in forests, logging, agriculture and construction are the most vulnerable and most affected. A high mobility of migrant and cross-border population encourage the spread of multi-drugs resistance of *P. falciparum* malaria from the Thai-Cambodia border to the Thai-Myanmar border. This has becoming a serious and growing problem in this area. (WHO: Malaria in the Greater Mekong Subregion, 2010).

In 2003, the *Plasmodium falciparum* strains become more resistance to mefloquine in some endemic area, especially, the provinces that located along Thai-Myanmar; Tak, Ranong and Kanchanaburi and Thai-Cambodia; Trat and Chanthaburi. The efficacy of mefloquine alone declined to less than 50% in 2002. As a result, the drug policy has been change to drug combination between mefloquine and aetesunate in these areas. Nowadays, the efficacy of a drug combination between mefloquine and aetesunate declined to less than 79% in Trat. The high failure rate of ACT use in Thai-Combodia border may relate to *Plasmodium falciparum* resistance to artemisinin-derivatives [Vijaykaga et al., 2006].

3. The life cycle of malaria parasites

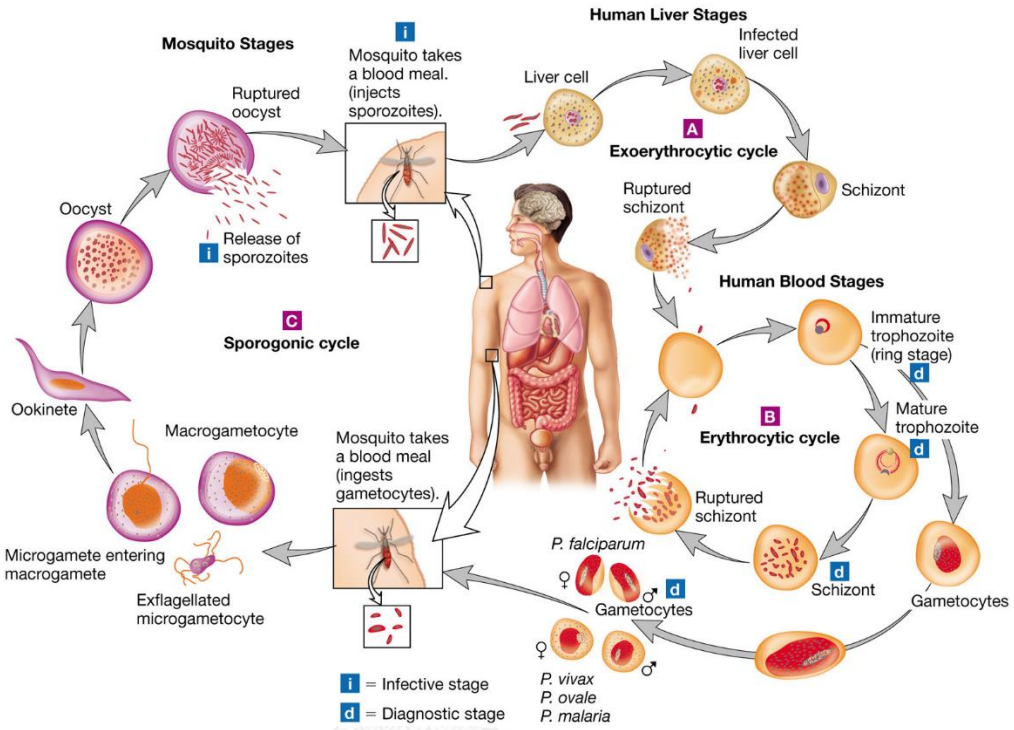


Figure 3 Life cycle of *Plasmodium* species
[http://galleryhip.com/schistosomiasis-life-cycle.html]

Malaria is cause by protozoa parasites in genus *Plasmodium*. This parasite is transmitting into the blood by mosquito bites. The *Plasmodium* parasites are unicellular organisms. The life cycle of parasite can be divided to 2 phases. An asexual occur in human host another is a sexual stage are found in human host and mosquito vector (Figure 3).

3.1 The parasite in the mosquito host (sporogonic cycle)

This phase started by a female mosquito bite the infected human host. The male and female gametocytes developed to be the exflagellated microgametocytes and macrogametocytes. After that, they fertilize in the gut of mosquito, formed to zygote and developed to ookinete. Then, transfer to gut wall (epithelial layer) and transform to the oocyst. The oocyst increases in the size and produces elongated sporozoites. Finally, released and move to mosquito salivary gland. The new infection starts when a female mosquito takes a blood and injects sporozoites into human bloodstream.

3.2 The parasite in the vertebrate host

3.2.1 Exo-erythrocytic cycle (human liver stage)

The mosquito bites the human kost. The sporozoite transfers into the bloodstream and remain in the blood. While many sporozoites are dead by phagocytes, there are some parasites are move and infect into liver cell. After that, the sporozoites develop into pre-erythrocytic schizonts and produce a lot of merozoites. Finally, the merozoites are released from the liver cells.

3.2.2 Erythrocytic cycle (human blood stage)

While the liver cells break down, the merozoites infect to the red blood cell by using their apex organelles. The parasite developed to ring form in red blood cell and develops to the trophozoites, early shizonts and mature shizonts. As the parasites develop, the parasite digested of haemoglobin, which is the main source of food for the parasites. The trophozoites and schizonts develops grain of dark brownish pigment appear in the cytoplasm of the parasites. It is a product of digestion in haemoglobin by the parasite. As the parasites grown, the schizont destroys its host blood cell. The merozoite releases into the blood stream and infected new red blood cell again. Sometimes, the parasite may be developing to male and female gametocytes [Hanssen et al., 2010; Chang et al., 2013].

4. Malaria treatment

The drug of use for treatment of malaria is target different phases to life cycle in malaria. The most of drug targets act on the intra-erythrocytic phases (Figure 4).

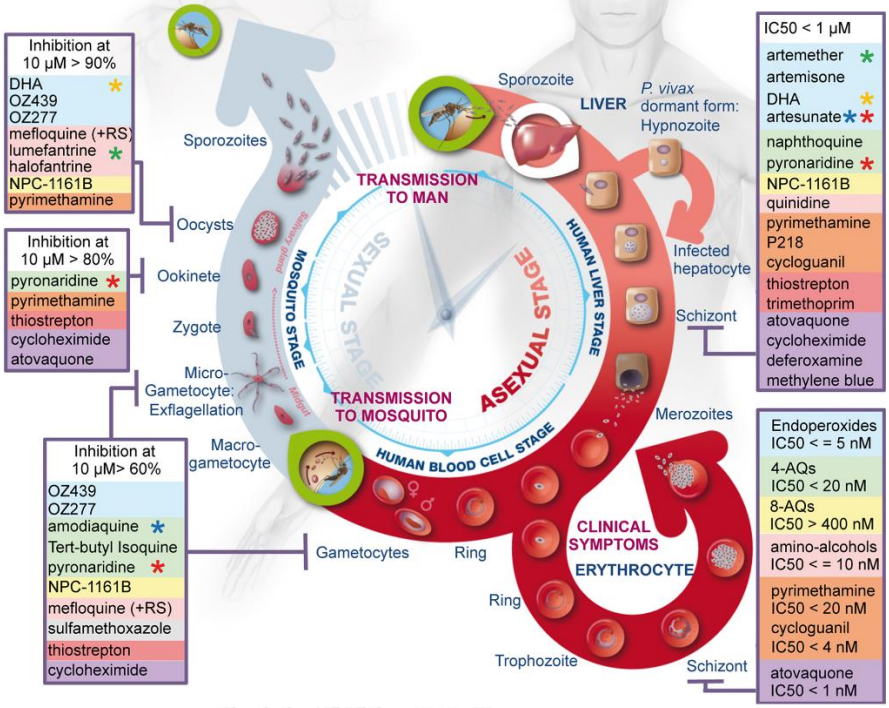


Figure 4 Antimalarial drug target phases in *Plasmodium* life cycle [http://www.plosmedicine.org/article/info%3Adoi%2F10.1371%2Fjournal.pmed.1001169]

The main type of antimalarial drug can be divide by mode of action into 2 groups (a summary showed in below).

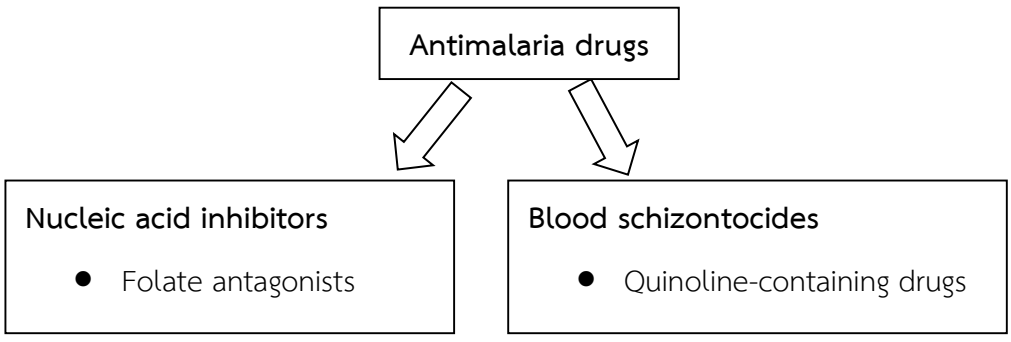


Figure 5 The main type o antimalarial drugs

4.1 Blood schizontocides

4.1.1 Artemisinin-type compound

The artemisinin is the active ingredient of the Chinese herb, in Chinese name known as qinghao or blue-green herb for relatively common plant differently known as *Artemisia annua* or sweet wormwood. It has been used in China for treatment of fever over two thousand years and also continues to common use in Chinese herbal medicine up to the current day [White, 2008; Weina, 2008]. It is a powerful and rapid acting blood schizontocide and is functioning against all *Plasmodium* species. It has an extraordinarily extensive activity against asexual parasites, killing all stages from young rings to schizonts and also kills the gametocytes [Olliaro, 2001]. In addition, artemisinin produced a clinical cure rapidly with clearance of fever and asexual parasites in blood of almost all patients, without toxicity certainly and faster parasite clearance than quinine. However, there were high reappearance rates. This problem causes to use the drug in combination with second antimalaria, the so called artemisinin combination therapies (ACT). ACT was innovated as a replacement for mefloquine monotherapy in Thailand during the early 1990 for temporary stopped the spread of mefloquine resistance and decreased the rate of occurrence of malaria disease. Since 2003, there have been isolated reports of high failure rates and reduced *in vitro* responses to artemisinin in some parts of Asia [Maude et al., 2010; Vijaykadga et al., 2006].

From the previous study, the deriveatives of artemisinin including dihydroartemisinin, artesunate, artemether, arteether (Figure 6) have an extraordinarily mode of action involving the iron catalyzed generation of a carbon-centered free radical followed by the alkylation of malaria-specific proteins. Selectivity inhibits a P-type ATPase of the malaria parasite. These results validate P-type ATPases as a drug target of human pathogens [Eckstein-Ludwing at al., 2003].

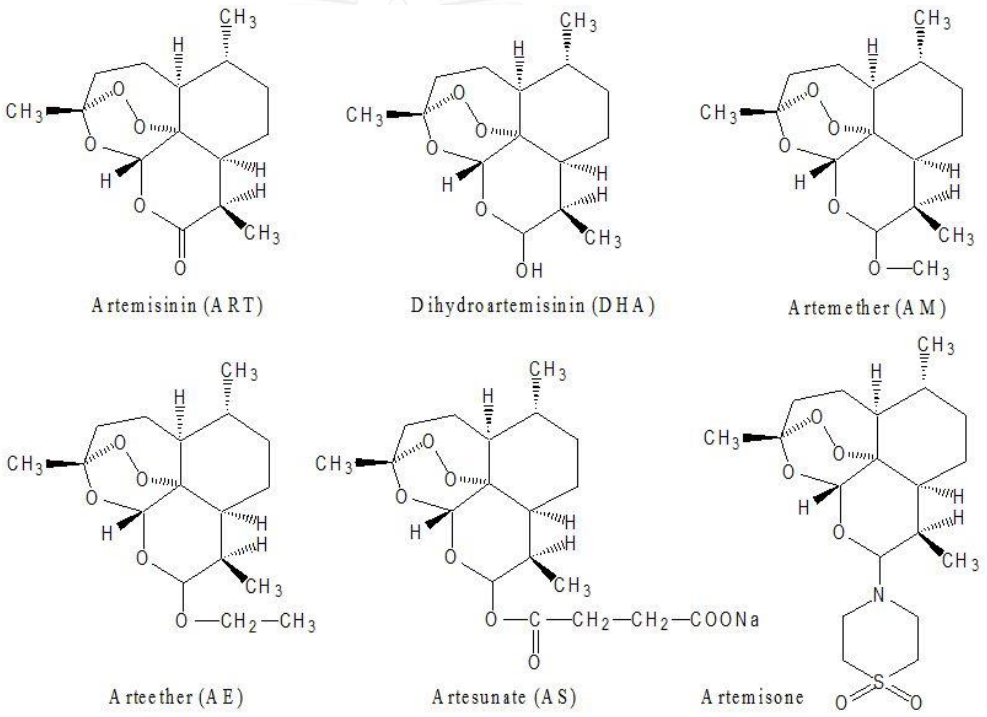


Figure 6 Chemical structure of artemisinin and its derivative-dihydroartemisinin, artemether, arteether, artesunate, artemisone [http://www.intechopen.com/books/research-directions-in-tumor-angiogenesis/the-use-of-artemisinin-compounds-as-angiogenesis-inhibitors-to-treat-cancer].

4.1.2 Quinoline-containing drugs

Quinoline derivative include chloroquine, amodiaquine, quinine, quinidine, mefloquine, primaquine, lumefantrine and halofantrine. These drugs have activity against the erythocytic stage of malarial infection. The drugs perform by collecting in the parasite food vacuole and forming a complex with heme that precludes crystallization in the plasmodium food vacuole. Heme polymerase activity is inhibited, resulting in accumulation of cytotoxic-free heme. The first used of quinoline-containing drugs is quinine which active ingredient is from the cinchona bark. Quinine has had the longest period of effectiveness, but the resistance has been reported [White, 1992]. The most common antimalarial drugs are included in the quinoline-containing drugs which are divided into two types:

(a) Type-I drugs (4-aminoquinolines chloroquine and Mannich-base amodiaquine, pyronaridine). These drugs are weak bases, diprotonated and hydrophilic at neutral pH. Because chloroquine is a diprotic weak base, it is attracted to the acidic pH of the parasite food vacuole. Once in the vacuole, it becomes deprotonated and membrane-impenetrable, and accumulates in the vacuole.

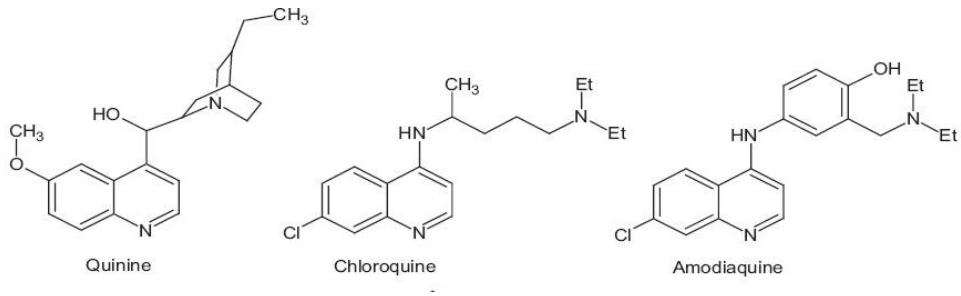


Figure 7 Chemical structures of Quinine, (2-ethenyl-4-azabicyclo[2.2.2]oct-5-y)-(6-methoxyquinolin-4-yl)-methnol, Chloroquine, N′- (7-chloroquinolin-4-yl)-N,N-diethyl-pentane-1,4-diamine and amodiaquine 4-(7-chloroquinolin-4-yl)amino-2-(diethylaminomethyl) phenol [http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3147106/?report=classic]

(b) Type-II drugs (the aryl-amino alcohols quinine and quinidine, mefloquine, halofantrine). These drugs are weaker bases and lipid soluble at neutral pH. The quinolines are thought to disrupt or prevent effective formation of hemozoin by binding to heme, resulting in heme-mediated toxicity to the parasite [Ridley et al, 1997].

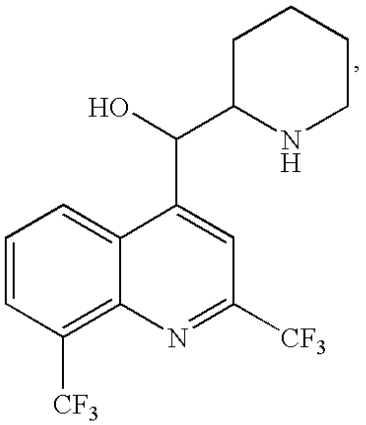
The prior study has been shown that the target for chloroquine action is ferriprotoporphyrin IX (FP), a self-toxic protein related to the pathway of polymerization, haem to hemozoin (malaria pigment). FP is necessary as plasmodia lack heme oxygenase enzymes. The direct mechanism of this polymerization is still under examination, and current theories are disagreeing. Chloroquine is can obstructing the process of polymerization. It has been shown that saturation of chloroquine uptake is mediated by binding to FP. The

chloroquine-FP complex may perform as a catalytic toxin to the polymerization reaction. Chloroquine activates against asexual forms of pathogenic malaria parasites. However, it is ineffective against gametocytes or exoerythrocytic liver forms [Zhang et al., 1999].

Mefloquine, the other main derivative, inhibits the uptake of chloroquine in infected cells by blocking consumption of haemoglobin. Deficiency of haemoglobin interrupts generation of FP to which chloroquine would bind. This mechanism describes the antagonistic effect parasite resistant to chloroquine parallels an increased sensitivity to mefloquine [Olliaro, 2001].

3.1.3 **Mefloquine** *[Klimpt et al., 2011; Valderramos et al., 2006; Price et al., 2004]*

Mefloquine is a **α**-2-piperidinyl-2, 8-bis(trifluoromethyl)-4-quinolinemethanol and is associated to quinoline compounds. It is soluble in alcohol but only very slightly soluble in water. The racemic mefloquine, first synthesis of was reported in 1971. The drug is productive against all forms of malaria.



Mefloquine

Figure 8 Chemical structures of Mefloquine [Alaíde B. *et al*, 2009]

4-aminoquinolines, arylaminoalcohols as mefloquine primary that against the intra-erythrocytic asexual stage of parasite development, inhibiting heme polymerization within the food vacuole, moreover inhibit both the hemozoin formation and the oxidative and glutathione-dependent degradation of heme. The effecting quinoline-heme complexs formed are poisonous for the parasite. The mefloquine influence volume-regulated anion channels (VRAC). This transmembrane pathway would be utilized by the parasite to carry substrates necessary to its growth and to allow the efflux of toxic compounds.

In addition, the first resistance to mefloquine has reported in Thailand, in 1982 and is distribution worldwide. The molecular mechanisms of resistance depend on the chemical class of a drug and its mechanisms of action. Usually, resistance originates from mutations in genes encoding the drug target and

influx-efflux pumps which drugs concentrate regulation at the target location. Resistance to mefloquine and the derivatives related to arylaminoalcohols *in P. falciparum* outcome from amplifications in *Plasmodium falciparum* Multidrug Resistance 1 (*Pfmdr1*) gene, which encodes an energy insistently requesting P-glycoprotein homologue pump (Pgh1). This protein mainly located on the food vacuole membrane. Moreover, there are several studies suggested that mefloquine selection of mefloquine-resistance *in vitro* leads to amplification and over manifestation of the *Pfmdr1* gene. An increasing of resistance to the quinoline compounds correlated with a transport of the drug out of the cell.

5. Mechanisms of Resistance

The resistance to chloroquine is seemingly conferred by multiple gene mutations. Several previous researches have been discussed about the theories of resistance. One of the interesting theories is associated with multidrug resistance product which is produced by multidrug resistance gene (ATP-binding cassette transporters). In *Plasmodium falciparum*, *Pfmdr1 and Pfmdr2* are MDR like gene homologues. The *Pfmdr1* protein product Pgh1 appears to be located in the digestive vacuole membrane. Pgh1 would work as a proton/chloride pump and adjustment in the membrane potential and/or food vacuole pH. Hence, the mutation in Pgh1 involves with decreased chloroquine

accumulation in food vacuole of chloroquine resistance strain. In the recent research examined that mutations in Pgh1 confer resistance to mefloquine, quinine, and halofantrine, and that they also attraction parasite resistance to chloroquine in a strain-specific manner, as well as the artemisinin compounds [Volkman et al., 1993; Cowman et al., 1991; Reed et al., 2000]. Moreover, this study also investigated whether changes in the copy number of these genes could account for resistance but found that resistant parasites retained the same number of copies as their sensitive progenitors [Afonso et al., 2006].

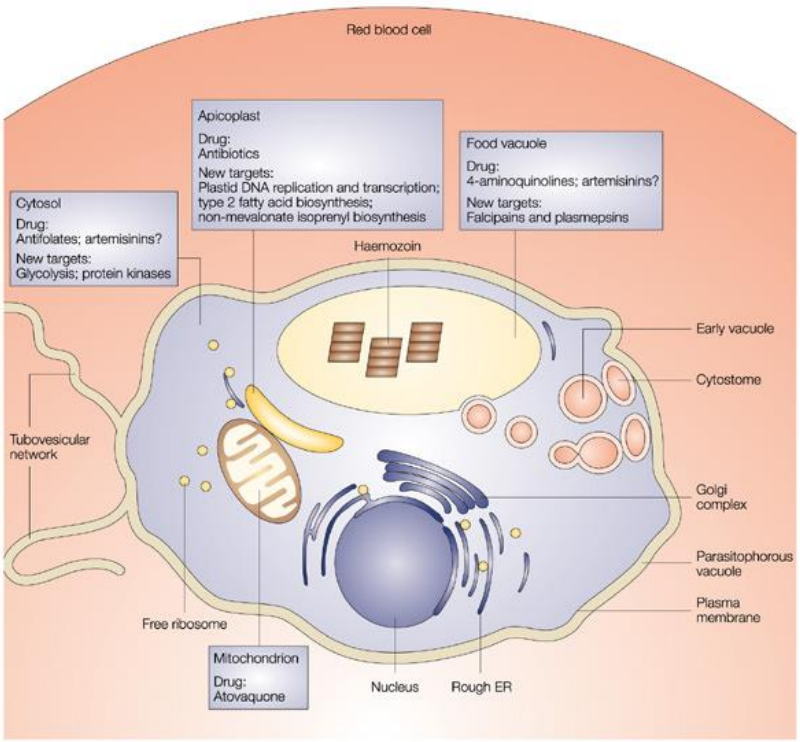


Figure 9 Representation of an intra-erythocytic *Plasmodium falciparum* trophozoit, highlighting key parasite intracellular compartment and the site of action of some of the major class of antimalarial drugs [http://www.nature.com/nrd/journal/v3/n6/fig_tab/nrd1416_F3.html]

6. Epidemiology of Antimalarial drugs resistance

Drug treatment failure in *P. falciparum* is associated with the development of drug resistance, which has limited the susceptibility of available drugs, include chloroqiune, tetracycline, sufadoxine, proquanil, pyrimethamine, primaquine, mefloquine, halofantrine, pyronaridine, atovaquone, artimesinin and also primitive drug as quinine [Kain et al., 2001].

Quinine resistance was first reported in 1910 and now resistance to quinine monotherapy appears inconstantly in Southeast Asia and Western Oceania. Widespread use of quinine in Thailand in the early 1980s as an interval therapy in the face of decline sulfadoxine-pyrimethamine effectiveness resulted in significant decreasing of its sensitivity [Wernsdorfer, 1994]. In the late 1950s, resistance to chloroquine was reported on the Thai-Cambodia border and in Cambodia. All endemic areas in South America were affected by 1980 and almost all in Asia and Oceania by 1989 [Wernsdorfer, 1991]. Resistance to sulfadoxine-pyrimethamine was first reported on the Thai-Cambodia border in the mid-1960s [Bjorman and Phillips-Howard, 1990] and it became an operative problem in the same area within a few years of its establishment to the malaria-control program in 1975 [Hurwitz et al., 1981]. Mefloquine resistance was first noticed near the Thai-Cambodia border in the late 1980s [Wongsrichanalai et al., 2001, 2002]. The powerful use of quinine just before the introduction of mefloquine may have affected the start of mefloquine resistance in Thailand.

The summary of introduction and first report of antimalarial drug resistance is listed in Table 1

Table 1 Dates of introduction and first Documented Resistance [Wongsrichanalai et al., 2002].

Antimalarial Agent	**Introduced**	**First report Resistance**	**Difference (Year)**
Quinine	1632	1910	278
Cloroquine	1945	1957	12
Proguanil	1948	1949	1
Sulfadoxine-pyrimenthamine	1967	1967	0
Mefloquine	1977	1982	5
Atovaqoune	1996	1996	0

Presently, the use of combination chemotherapy is used for imitating with the emerging of drug resistance. Regrettably, multi-drugs resistance of *P. falciparum* was appeared, determined previously as resistance to more than

two operational antimalarial compounds of different chemical classes [Wernsdorfer, 1994].

The Figure 10 shows the estimated multidrug-resistance areas together with those where resistance to both chloroquine and sulfadoxine-pyrimethamine has been documented, which thus have the potential for emergence of multi-drugs resistance.

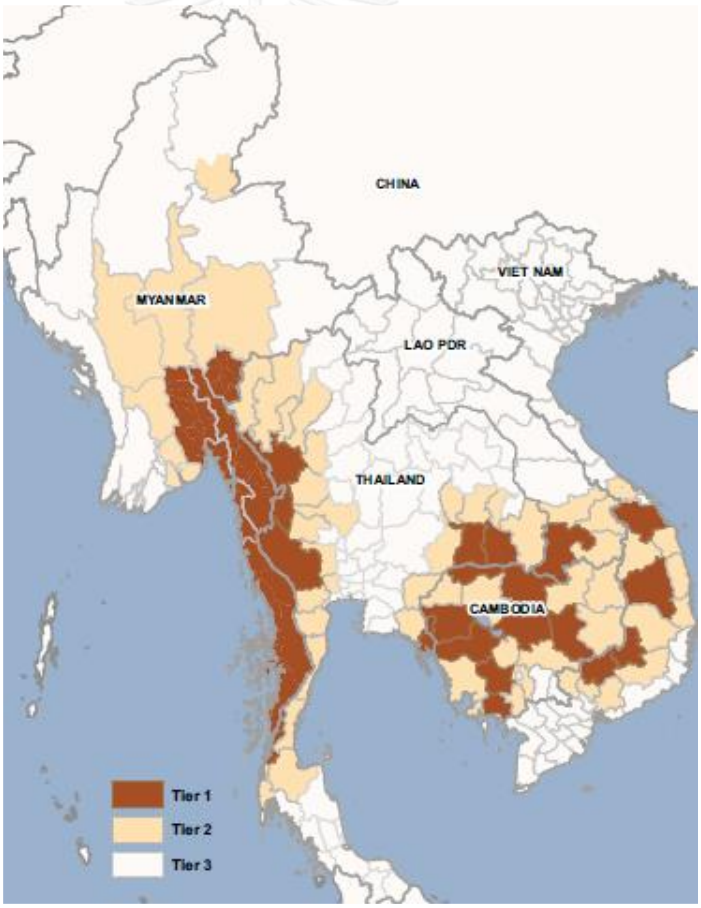


Figure 10 Area where there is credible evidence of artemisinin resistance [GLOBAL Malaria Programme, WHO, November, 2013]

7. *Plasmodium falciparum* multidrug resistance1 *(Pfmdr1)* gene

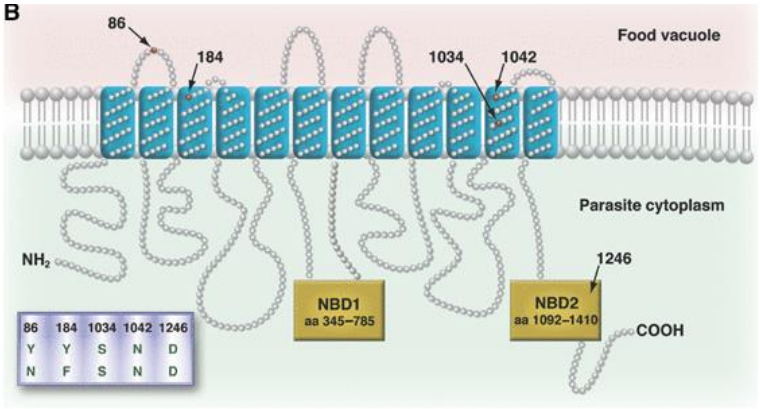


Figure 11 The Pgh1 protein of *P. falciparum*. Polymorphic amino acids are indicated [Duraisingh et al., 2005].

Plasmodium falciparum multidrug resistance1 (*Pfmdr1*) is the gene that located on chromosome 5 encodes 12 transmembrane-domain proteins, also call Pgh1 (P-glycoprotein homologue 1). This protein located in digestive vacuole that perform the transport proteins on the plasma membrane of the parasite's food vacuole, where is the site of action of chloroquine and other quinoline-base antimalarial drugs [Djimdé et al., 2001; Ferreira et al., 2011; Mungthin et al., 2014].

The *P. falciparum* multidrug resistance gene (*Pfmdr1*) has been reported to association about the alteration of susceptibility to several of current antimalarial drugs. However, evidences are conclusive that a direct causative association between *Pfmdr1* and resistance to these anti-malarial drugs has remained elusive, and a single genetic cross has proposed that Pgh1 is not involved in resistance to chloroquine and mefloquine. However, Reeds and colleagues furnish direct proof that mutations in Pgh1 can confer resist to mefloquine, quinine and halofantrine. The same mutations influence parasite resistance towards chloroqiune in a strain-specific manner and the level of sensitivity to the structurally unrelated compound, artemisinin. This has important assumptions for the development and effectiveness of future antimalarial agents (Reed 2000). The amplification of *Pfmdr1* gene has been reported to associate with malaria multidrug resistance [Chaijaroenkul et al., 2010; Duah et al., 2013; Muhamad et al., 2013; Phompradit et al., 2013].

In Thailand, there are several researches showed that the *Pfmdr1* gene mutations and gene amplifications are related to drugs resistance in *P. falciparum*. One of these researches examined pattern of *Pfmdr1* which collected from different endemic area in Thailand showed the mutation pattern at codons 86, 184, 1034, 1042 and 1246. In addition, *Pfmdr1* copy

number enhanced more resistant to malarial drugs such as mefloquine, quinine, artemisinin, and artesunate [Alker et al., 2007; Duraisingh et al., 2005].

8. **DNA extraction by Chelex resin method**

Chelex 100 resin is a styrene divinylbenzene copolymer containing paired iminodiadetate ions, which act as chelating groups in binding polyvalent metal ions. The carboxylic groups of Chelex 10 resin classify it as a weak cation exchange resin, but it differs from other exchanges in this class by featuring uniquely high selectivity for metal ions and much higher bond strengths. The resin can be used to ultra-purify buffers and ionic reagents: it will scavenge metal contaminants without altering the concentration of nonmetal ions.

Chelex chelating ion exchange resin has unusually high preference for copper, iron, and other heavy metals over monovalent cations such as sodium and potassium. Its selectivity for divalent over monovalent ions is approximately 5,000 to 1, and it has a very strong attraction for transition metals, even in highly concentrated salt solution.

Chelating resin is available as Analytical Grade Chelex 100 resin, Biotechnology Grade Chelex 100 resin, and Technical Grade Chelex 20 resin. The Analytical Grade Chelex 100 resin has been exhaustively sized, purified, and converted to make it suitable for accurate, reproducible analytical

techniques. Biotechnology Grade Chelex 100 resin is analytical grade resin which is certified to contain less than 100 micro-organisms per gram of resin. Technical Grade Chelex 20 resin is coarse mesh resin useful for large scale clean-up, for example metals from waste waters, where analytical purity is not a major concern. Chelex 100 resin and Chelex 20 resin are styrene divinylbenzene copolymers containing paired iminodiacetate ions which act as chelating groups in binding polyvalent metal ions. Chelex chelating resin is classed with the weakly acidic cation exchange resins by virtue of its carboxylic acid groups, but it differs from ordinary exchangers because of its high selectivity for metal ions and its much higher bond strength. Chelex chelating resin is efficiently regenerated in dilute acid and operates in basic, neutral, and weakly acidic solutions of pH 4 or higher. At very low pH, the resin acts as an anion exchanger.

9. Polymerase Chain Reaction Technique and Application

Polymerase Chain Reaction (PCR) is the procedure for amplifying a specific DNA segment. PCR amplification technique demanded the DNA template, the heat-stable DNA polymerase- *Taq* DNA polymerase and the specific sequence of oligonucleotides which are forward and reverse primers.

The amplification process is comprised of three main steps: denaturation step, annealing step and extension step.

In the denaturation step, the double strands of DNA sample are separated by heated up. In the annealing step, the DNA sample is cooled moderately, allowing the primers to bind and in the extension step, the sample is incubated at 72° C so that the *Taq* DNA polymerase can extend the primers, a long complemental strands of DNA are created. After polymerization, the procedure is reproduced for 25 to 30 cycles. The thermostable DNA polymerase *Taq*I (from *Thermus aquaticus*, bacteria growing in hot spring) is not denatured by the heating steps.

PCR technique is an essential tool for many applications. For example, it can be used to amplify a sample of DNA when there is not sufficiency to examine (e.g. a sample of DNA from a crime scene, archeological samples), as a process of distinguishing gene interest, or to examination for disease. In addition, PCR technique can be adapted for P. falciparum identification and diagnosis by detection the amplified specific DNA segment [Wooden et al, 1992; Mullis, 1990; Nelson et al., 2000; Snounou et al., 1993; Schoone et al., 2000].

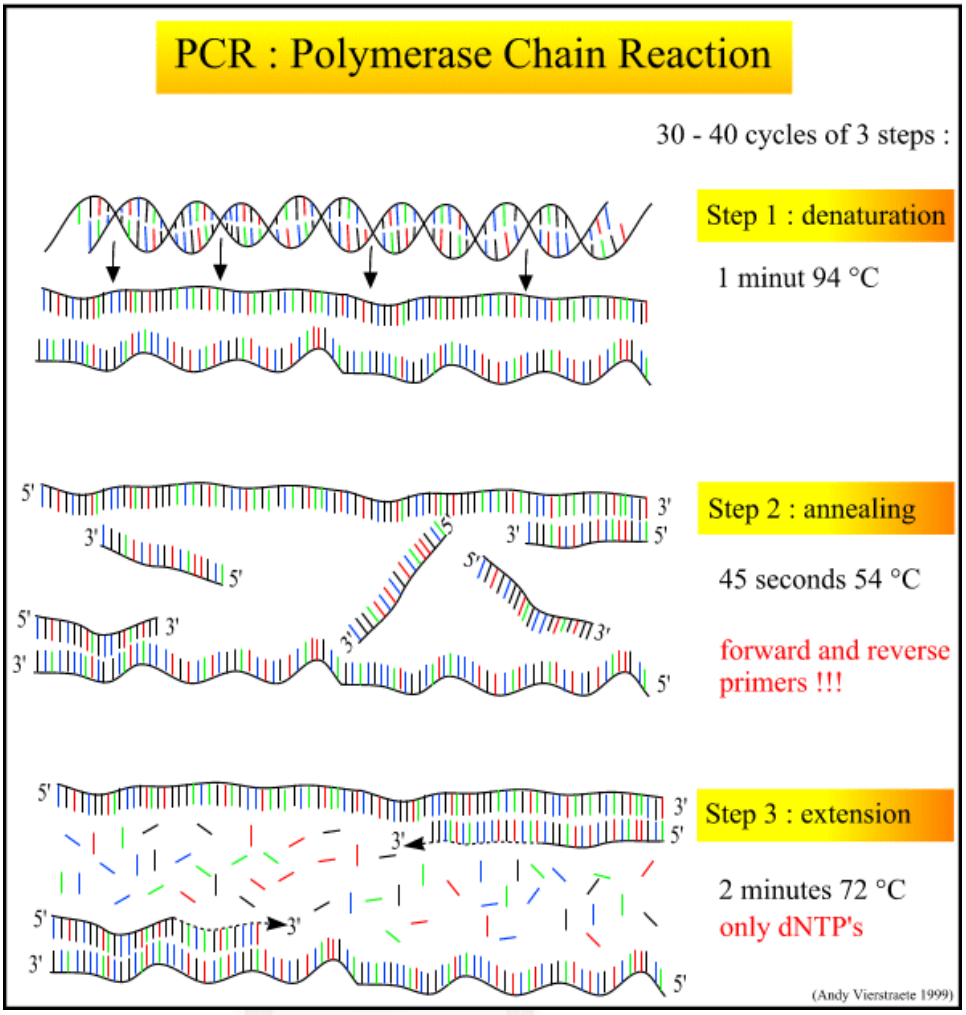


Figure 12 The characteristic of PCR
[http://users.ugent.be/~avierstr/principles/pcr.html]

10. Real-Time Polymerase Chain Reaction Technique

Real-Time Polymerase Chain Reaction Technique is one of the highly sensitive quantifying techniques. Real-Time PCR is utilized to determine the increasing of PCR product at real time. This technique is used for several application including quantitative gene expression testing, copy number, genotyping, drug target validation, biomarker discovery, pathogen detection

and measuring RNA interference. The reaction of real-time PCR monitors the accumulation of PCR product using fluorescent reporter molecule or probe or dye. The exponential phase was furnishing the most collect and correct data for quantitative. This phase is used to estimate the threshold and C_T value. The threshold value is the level of detection at which a reaction stretches to a florescent intensity above background. The C_T value is adapted in absolute or relative quantitation. This value is the PCR cycle at which the sample stretches to the threshold. The benefits of real-time PCR are generation of accurate quantitative data, enlargement dynamic range of detection, expulsion of post-PCR procedure, detection down one copy, increased precision to determine smaller fold charge and increased throughput [Promega, 2014].

10.1 Real-Time PCR Phase

The real-time PCR reaction had been three phase, the exponential phase is the doubling of product occurs in every cycle at 100% reaction efficiency. The best reaction efficacy appears because all of the reagents are fresh and available. The kinetics of the reaction forces the reaction to support doubling of amplicon. At linear phase, some of the reagents are consumed as an effect of amplification. The reaction starts to decelerate and PCR product is no continuing over doubled at each cycle. Plateau is the last phase of reaction.

At this phase the reaction discontinue, no more products are inventing and the PCR products initiate to degrade (Figure 13).

The result of the real-time PCR was concentrate on the exponential phase. This phase is furnishing the most accurate data for quantification. The threshold and C_T value were calculating. The threshold is level of detection at which a reaction reaches a fluorescent intensity above background. The C_T value is the PCR cycle at which the sample reaches the threshold (Figure 13).

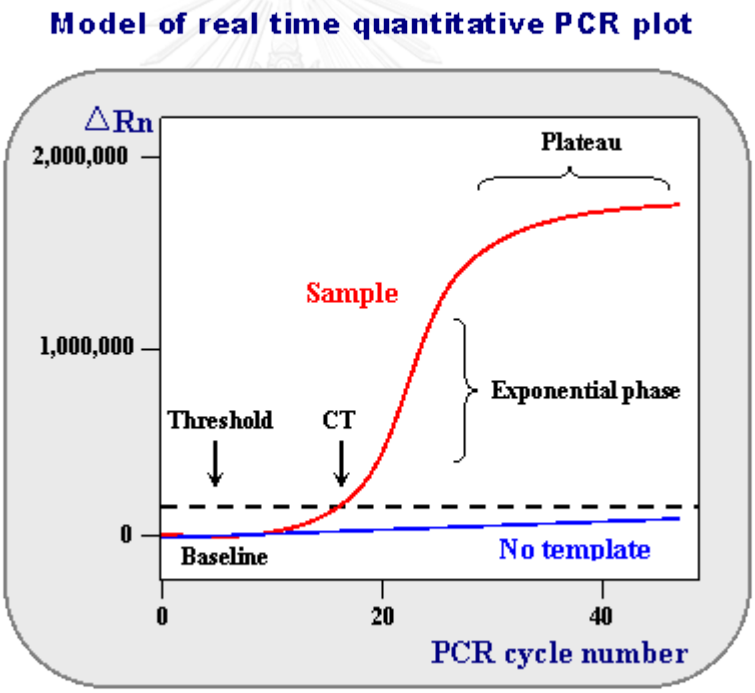


Figure 13 Model of real-time Quantitative PCR plot [29]
http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechQPCR.shtml

10.2 Real-time PCR fluorescence detection system

10.2.1 TaqMan probe types

TaqMan probes may be divided into two types, MGB and non-MGB. The first TaqMan probes could be classified as non-MGB. They used a dye called TAMRA dye as the quencher. Early in the development of real-time PCR, extensive testing revealed that TaqMan probes required an annealing temperature significantly higher than that of PCR primer to allow cleavage to take place. TaqMan probes were therefore longer than primer. A one-base mismatch in such long probes had a relatively mild effect on probe binding, allowing cleavage to take place. However, for many applications involving high genetic complexity, such as eukaryotic gene expression and SNPs, a higher degree of specificity was desirable.

TaqMan MGB probes were a later refinement of the TaqMan probe technology. TaqMan MGB probes possess a minor-groove binding (MGB) molecule on the 3'end. Where the probe binds to the target, a short minor groove is formed in the DNA, allowing the MGB molecule to bind and increase the melting temperature; thus strengthening probe binding. Consequently, TaqMan MGB probes can be much shorter than PCR primers. Because of the MGB moiety, these probes can be shorter than TaqMan probes and still achieve a high melting temperature. This enables TaqMan MGB probes to bind to the target more specifically than primers at higher temperatures. With the shorter

probe size, a one-base mismatch has a much greater impact on TaqMan MGB probe binding. And because of this higher level of specificity, TaqMan MGB probes are recommended for the most genetic complexity applications.

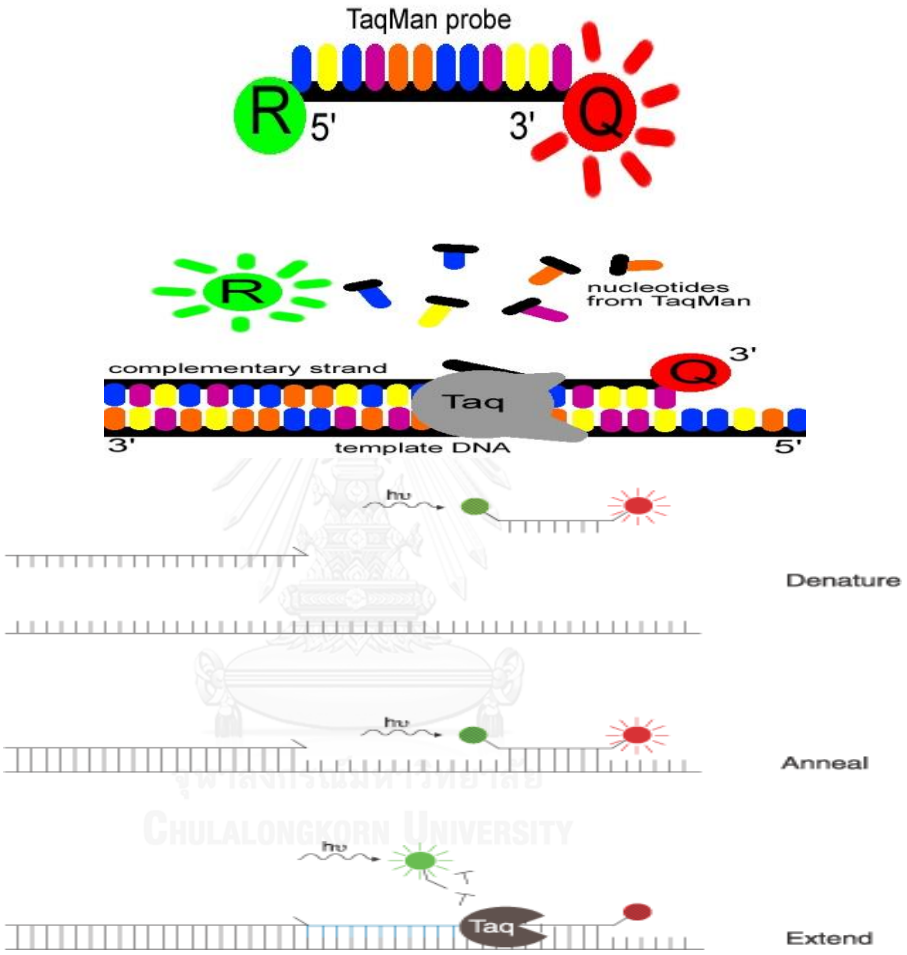


Figure 14 TaqMan probe. The TaqMan probe has a gene-specific sequence and is designed to bind the target between the two PCR primers. Attached to the 5' end of the TaqMan probe is the “reporter”, which is a fluorescent dye that will report the amplification of the target. On the 3' end of the probe is a quencher, which quenches fluorescence from the reporter in intact probes. The quencher also blocks the 3' end of the probe so that it cannot be extended by thermostable DNA polymerase.

10.2.2 SYBR Green I dye

The SYBR Green I dye is the fluorescent DNA binding dye, binding to the minor groove of any double-stranded DNA. Excitation of DNA-bound SYBR Green dye produces a much stronger fluorescent signal compared to unbound dye. A SYBR Green dye-based assay typically consists of two PCR primers. Under ideal conditions, a SYBR Green assay follows a similar amplification pattern as a TaqMan probe-based assay. In the early PCR cycle, a horizontal baseline id observed. If the target was present in the sample, sufficient accumulated PCR product will be produced at the same point so that amplification signal becomes visible [Ferreira et al, 2006].

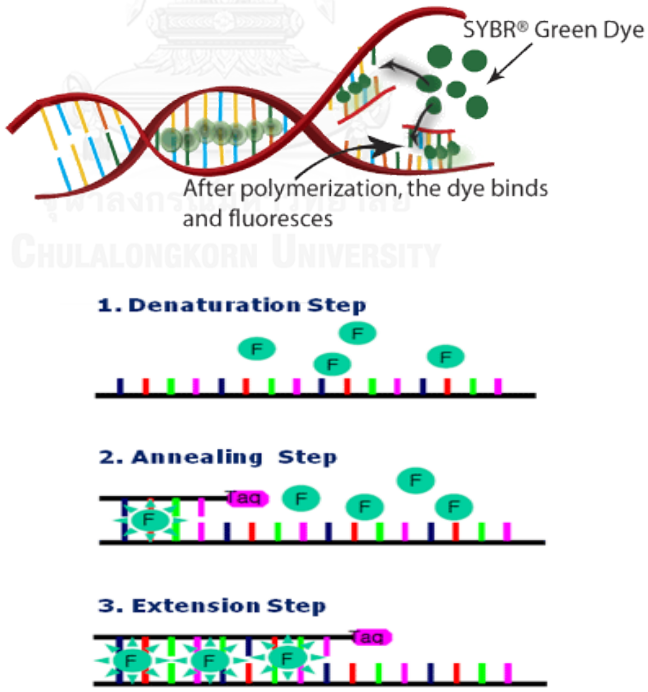


Figure 15 SYBR Green Detection Method
[http://projects.nfstc.org/pdi/Subject03/pdi_s03_m05_07_a.htm]

10.3 Real-time PCR analysis methods

10.3.1 Comparative C_T ($\Delta\Delta C_T$) method (Relative quantification)

In the $\Delta\Delta C_T$ method of quantitation polymerase chain reaction data analysis, the C_T values from two different RNA samples are directly normalized by the *β-actin* gene (housekeeping gene) and then compared. The $\Delta\Delta C_T$ method assumes that the amplification efficiencies of the target gene and the reference genes (housekeeping gene) are close to 100 percent. The difference between the ΔC_T of the target gene and the reference gene is calculated in each sample. Then, the difference of the ΔC_T value is calculated between the experimental and control samples. The fold-change in expression of the interest gene of between the two samples is $2^{-(\Delta\Delta C_T)}$.

10.3.2 Standard curve method (Absolute quantification)

A dilution series of known temperature concentrations can be used to establish a stand curve for determining the initial starting amount of the target template in experimental samples or for assessing the reaction efficiency (Figure 16). The log of each known concentration in the dilution series (x-axis) is plotted against the C_T value for that concentration (y-axis). From this standard curve, information about the performance of the reaction as well as various reaction parameters including slope, y-intercept, and correlation coefficient can

be derived. The concentrations chosen for the standard curve should encompass the expected concentration range of the target in the experimental samples.

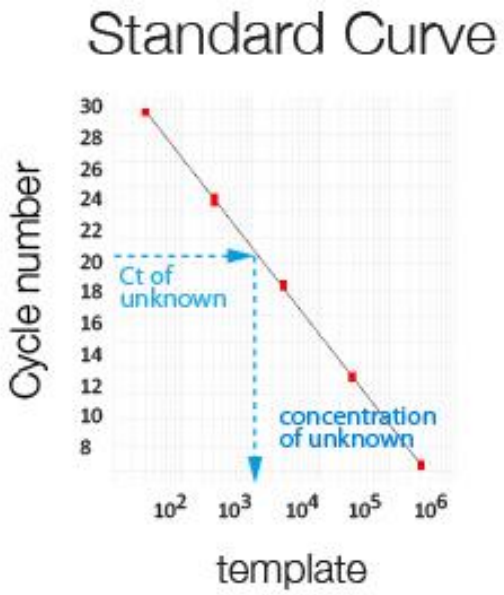


Figure 16 Example of standard curve of real-time PCR data. A standard curve shows threshold cycle (C_T) on the y-axis and starting quantity of RNA or DNA target on the x-axis. Slope, y-intercept, and correlation coefficient values are used to provide information about the performance of the reaction.

10.3.3 Melting curve (Dissociation curve)

A melting curve charts the change n fluorescence observed when double-stranded DNA (dsDNA) with incorporated dye molecules dissociates, or "melts" into single stranded DNA (ssDNA) as the temperature of the reaction is raised. For example, when double-stranded DNA bound with SYBR Green I dye is heated, a sudden decrease in fluorescence is detect when the melting point

(T_m) is reached, due to dissociation of DNA strands and subsequent release of the dye. The fluorescence is plotted against temperature, and then the $-\Delta F/\Delta T$ (change in fluorescence/ change in temperature) is plotted against temperature to obtain a clear view of the melting dynamics.

Post-amplification melting-curve analysis is a simple, straight forward way to check real-time PCR reactions for primer-dimer artifacts and to ensure reaction specificity because the melting temperature of nucleic acids is affected by length, CG content, and the presence of base mismatches, among other factors, different PCR products can often be distinguished by their melting characteristics. The characterization of reaction products (e.g., primer-dimers vs. amplicons) via melting curve analysis reduces the need for time-consuming gel electrophoresis.

11. Single nucleotide polymorphism (SNP) genotyping methods

Single nucleotide polymorphisms (SNPs) are individual base positions in the genome that show natural variation in a population. SNP genotyping is expected to help identify gene affecting complex diseases and responses to drugs or environmental chemicals. SNP genotyping methods consist of two complements, a method for determining the type of base present at a given SNP locus (allele discrimination), and a method for reporting the presence of

the allele (single detection). There are three general allele discrimination methods: hybridization/annealing (with or without a subsequent enzymatic discrimination step), primer extension, and enzyme cleavage [Twyman et al., 2005].

11.1 Allele-specific hybridization

Allele-specific hybridization is the method using allele-specific oligonucleotide (ASO) probes to discriminate between allele at the SNP locus. Two probes are required, one specific for each allele and stringency conditions are employed such that a single-base mismatch is sufficient to prevent hybridization of the non-matching probe. The ASO probe can be used as labeled or unlabeled pairs and distinct labels can be used for each probe, e.g., different fluorophores or mass tags. More sophisticated assays, such as *TaqMan*, use allele-specific hybridization as the primary discriminating reaction, but additional enzymatic steps are required to detect the signal. Allele-specific polymerase chain reaction (PCR) is a modification of a hybridized people. However, this technique allows the extension of a mismatched primer/template if stringency conditions are not optimized [Twyman et al., 2005].

11.2 Allele-specific-base primer extension

In allele-specific single-base primer extension (also called mini-sequencing), primers that anneal one nucleotide upstream of the polymorphic site are designed, and allele discrimination depends on the ability of this perfectly annealed primer to be extended. This is distinct to allele-specific PCR where the discriminatory position lies within one of the primers and extension depends on the ability of the primer to anneal to its template. A much greater diversity of labeling strategies can be used. For example, the free nucleotides in solution can be labeled with four different of multiple SNPs in parallel [Twyman et al., 2005].

11.3 Allele-specific enzymatic cleavage

Allele-specific enzymatic cleavage or restriction fragment length polymorphism (RFLP) analysis is one of the most widely used. This works on the principle of allele-specific enzymatic cleavage. An RFLP is generated when an SNP occurs at a restriction endonuclease recognition sequence, and one allele preserves the sequence while the other destroys it [Twyman et al., 2005].

12. Restriction Fragment Length Polymorphism (RFLP)

RFLP or Restriction Fragment Length Polymorphism is a distinction in homologous DNA sequences that can be detected by the presence of fragment lengths after digestion of the DNA samples with specific restriction endonucleases.

The advantages of the PCR-RFLP technique consist of inexpensiveness and unnecessary the advanced equipment. Moreover, the design of PCR-RFLP analyses mainly is easy and can be performed using public available programs. However, the disadvantageous points include the requirement for specific endonucleases and identify difficultly the exact variation in the event that several SNPs influence the same restriction enzyme recognition step, it is relatively time-consuming. Eventually, the technique is inappropriate for the simultaneous analysis of a large number of different SNPs caused the requirement for a specific primer pair and restriction enzyme for each SNP.

After finish of digestion of amplicons with the selected restriction enzymes, the derivable fragments are determined by electrophoresis. Habitually, this is complete using slab gel electrophoresis with polyacrylamide or agarose as molecular sieving matrix. Recently, capillary electrophoresis and microchannel electrophoresis have become favorites increasingly. They proffer higher resolving capability and throughput than conventional slab gels.

Visualisations of the restriction enzyme-treated amplicons can be done using fluorescent-labelled amplification primers. Nevertheless, most regularly PCR-RFLP analyses are managed with unlabeled primers. In that event, visualization of restriction fragments is completed by complexation of DNA fragments with ethidium bromide or another fluorescent dye during the electrophoresis. For PCR-RFLP analysis with covalently labeling of primers, the restriction-enzyme treated fragments are normally heated and analysed by denaturing electrophoresis in a single-strand state to examine fragment sizes arising the procedure for genotyping of microsatellite. The advantage of this is that the size determination originally depends upon fragment lengths. In contrast, size examination of DNA fragments using electrophoresis under non-denaturing conditions may be attracted by the formation of the DNA fragments [Rasmussen]

CHAPTER III

RESEARCH METHODOLOGY

1. Malaria samples

Finger prick blood samples had already obtained from malaria patients attended at malaria clinics, Trat province and Chanthaburi province during 2008-2010. After confirmation of *P. falciparum* infection by microscopic observation of thick Giemsa-stained blood films, the blood sample were spotted onto Whatman filter paper and then returned to perform DNA extraction in the laboratory. These blood samples were the samples from the therapeutic efficacy of Malarone^{TM} project of Mrs. Saowanit Vijaykadga (Ministry of Public Health, Thailand).

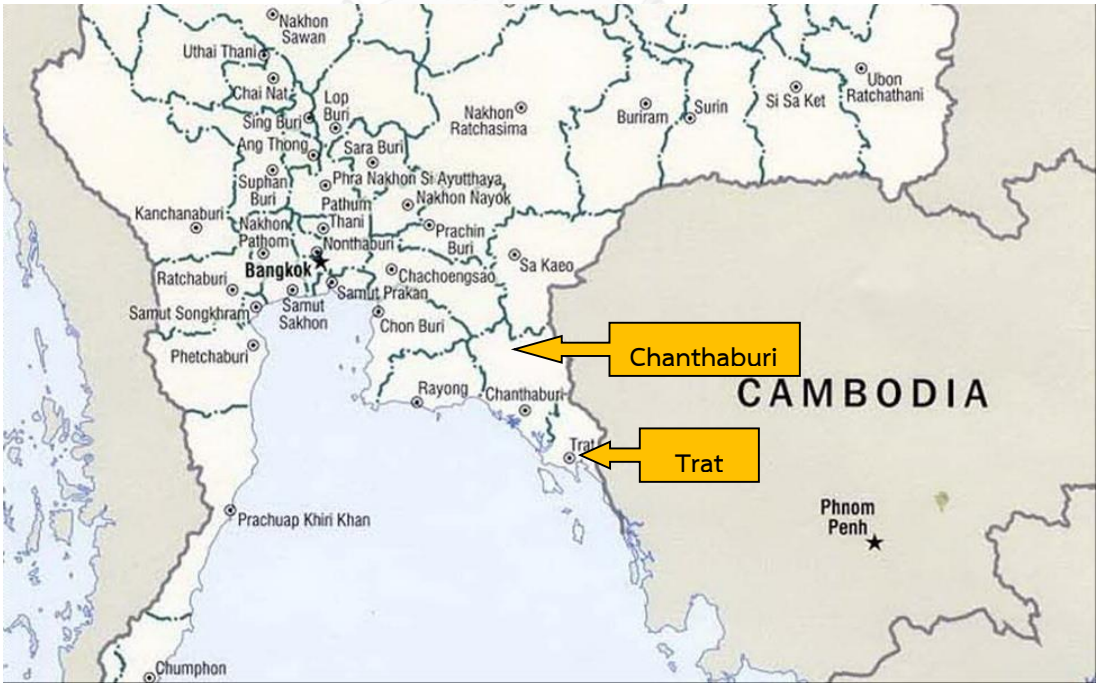


Figure 17 The present study locations including Trat province, Chanthaburi province

2. DNA extraction

Blood samples collected in the Whatman filter papers were incubated in PBS solution overnight at 4°C. After that the PBS solutions were replaced with 0.5% saponin in PBS and incubated at 4°C for 10 minutes. Then treated filter papers were transferred to hot 5% chelex-100 resin solutions (Bio-Rad Laboratories, CA) and incubate at 95°C for 10 minutes, vortex briefly between incubations. Supernatants were kept until used for parasite genotyping after centrifugation [Plowe et al, 1995].

3. Detection of Pfmdr1 polymorphisms by PCR-RFLP

Genomic DNA was extract using Chelex-resin (Biorad Co.Ltd., USA) according to the method of Plowe and colleague in 1995. Previously published and PCR-RFLP methods were employed to detect *Pfmdr1* at the codons 86, 184, and 1034 [Duraisingh and Cowman, 2005; Duraisingh et al., 2000]. The primers and reaction conditions used were according to the previously described methods [Duraisingh and Cowman, 2005; Duraisingh et al., 2000; Fidock et al., 2000]. PCR were performed in a total volume of 25 μl with the following reaction mixture: 0.1 μM of each primer, 2.5 mM $MgCl_2$, 100 μM of each deoxynucleotide triphosphate, 1×PCR buffer (100 mM KCL, 20 mM Tris-HCL pH 8.0), 2 μl of genomic DNA. One cycle of 94°C for 2 min; 40 cycles of 94°C for 1 min; 45°C for 1 min and 72°C for 1 min; one cycle of 72°C for 5 min in a reaction.

Genomic DNA extracted from 3D7, K1 and PFK12 *P. falciparum* clones were used as positive controls, which contain different types of mutations, whereas water was used as a negative control.

Table 2 *Pfmdr1* genotype of 3D7, K1, and PFK12

Codon	86	184	1034	1042	1246
3D7	Asn (AAT)	Try (TAT)	Ser (AGT)	Asn (AAT)	Asp (GAT)
K1	Try (TAT)	Try (TAT)	Ser (AGT)	Asn (AAT)	Asp (GAT)
PFK12	Asn (AAT)	Try (TAT)	Cys (TGT)	Asp (GAT)	Asp (GAT)

The primer sequences for all PCR reactions are described in Table3. Restriction sites were already present for the polymorphisms at codon 86 (*Apo* I and *Afl* III digested when the asparagine and tyrosine codons were presented respectively); for the 184 (*Dra* I digested when the phenylalanine codon was presented); and for 1034 (*Dde* I digested when the serine was presented) (Figure18).

Table 3 Sequences of primers used for typing polymorphisms at position 86, 184, and 1034 in *Pfmdr1*

Primers	Sequences	Nucleotides
A1(f)	5´ TGTTGAAAGATGGGTAAAGAGCAGAAAGAG 3´	9...21
A2(r)	5´ TACTTTCTTATTACATATGACACCACAAACA 3´	584...552
A3(r)	5´ GTCAAACGTGCATTTTTTATTAATGAACCATTA 3´	648...618
A4(f)	5´ AAAGATGGTAACCTCAGTATCAAAGAAGAG 3´	24-54
O1(f)	5´ AGAAGATTATTTCTGTAATTTGATACAAAAAGC 3´	3003...3035
O2(r)	5´ ATGATTCGATAAATTCATCTATAGCAGCAA 3´	3889...3860
1034f	5´ AGAATTATTGTAAATGCAGCTTTATGGGGACTC 3´	3067...3099
1042r	5´ AATGGATAATATTTCTCAAATGATAACTTAGCA 3´	3299...3267
1246f	5´ ATGATCACATTATATTAAAAAATGATATGACAAAT 3´	3545...3589

Accession number for *Pfmdr1*: S53996

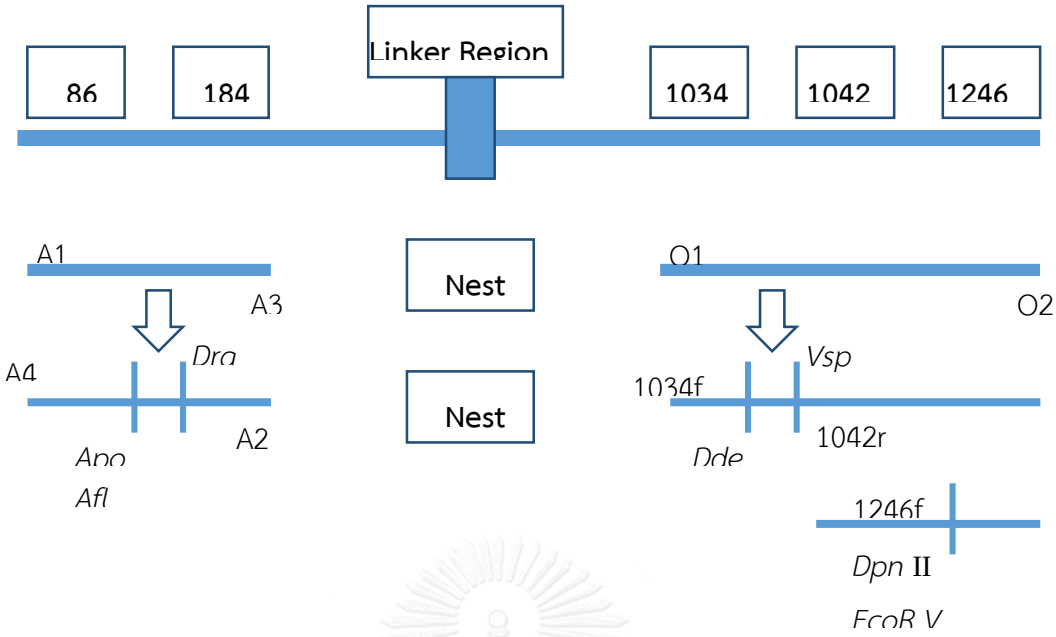


Figure 18 Schematic representation used of the nested system for the detection of polymorphisms in *Pfmdr1* gene of *P.falciparum* Primers and restriction sites used for the detection of each variant are indicated. [Duraisingh et al., 2000].

Unless otherwise stated, PCR products were analyzed by electrophoresis using 2% agarose gels, running in 1x TBE (Tris Borate EDTA buffer) containing ethidium bromide (0.5 µg/l in 1x TBE). Samples were loaded into wells after the addition of 1/5 volume of orange G dye (Sigma, U.K.) loading dye. DNA ladders were used as molecular weight markers and to aid in the size determination of PCR products. Electrophoresis was carries out at 100 V until the dye had electrophoresed about three fourth of length of the gel. Separated PCR products were visualized by UV trans illumination (medium wavelength 302 nm).

4. Detection of gene copy number of *Pfmdr1* by TaqMan real-time PCR

Pfmdr1 copy number was assessed by TaqMan real-time PCR (Applied Biosystems, Warrington, UK). The primers and the probe are specific to a conserved region of *Pfmdr1,* β*-Actin*, and the designed sequences of these primers and probes were shown in Table 4. *Pfmdr1* copy number was analyzed according to the modified method developed by Price [Price et al., 2004]. Briefly, the amplification reactions were done as multiplex PCR in MicroAmp 48-well plates (Applied Biosystem) in a 25 μl reaction mixture containing TaqMan buffer pH 8.3 (8% glycerol, 0.625U DNA polymerase, 5.5 mmol/l $MgCl_2$, 300 μmol/l dNTP, 600 nmol/l passive reference dye 5-carboxy-Xrhodamine), 300 nmol/l of each forward and reverse primer, 100 nmol/l of each probe, and 5 μl of templates DNA. Fluorescence data was expressed as normalized reporter signal, calculated by dividing the amount of reporter signal by the passive reference signal. The detection threshold was set above the mean baseline value for fluorescence of the first 15 cycles, the threshold cycle (C_T) was done. So, that the reactions were performed for 40 cycles (Pre-Incubation at 95 °C for 2 min, 1 cycle, amplification program: denature at 94 °C 2 min, annealing at 45 °C 40 sec, extension 72 °C 1 min, melting 72 °C 5 min and a final cooling step to 40 °C). When the increase in reporter signal was first detected above baseline, the results were analyzed by a comparative C_T method based on the assumption that the target (*Pfmdr1*) and reference (β*-actin*) was amplified with the same efficiency with in an

appropriate range of DNA concentrations. The comparative $\Delta\Delta C_T = C_TE - C_TB$, where C_TE denotes the experimental C_T and C_TB the baseline C_T. Every TaqMan run contained the reference DNA samples from 3D7 clones which contained only one copy numbers of *Pfmdr1*. All reactions were performed in triplicate and results were rejected in case of nonexponential kinetics.

Table 4 Primer sequences

	Primers	**Sequences**
TaqMan	*Pfmdr1*-1F	AAACTTTAAAGCTTGAATATTTAAGAAGTGTTTT
	Pfmdr1-1R	GAACTCACTTGTTCTAAATAAAAATCTAAATCAGATCTTAAT
	Pfmdr1-probe	FAM-CAAGATGGACAATTTC-MGB
	Actin-1F	CCAGAAGCTTTATTCCAACCATCCT
	Actin-1R	CATTTTTTAATAGAGTTGAAAGTAGTTGTGTGGA
	Actin-probe	FAM-CCTGCTGCTTCTTTTC-MGB

CHAPTER IV
RESULTS

4.1 **Malaria samples**

73 *P falciparum* infected blood samples were obtained from malaria patients attended at malaria clinics at Trat and Chantaburi provinces during 2006 to 2010. 49 samples were originally collected from Trat while 24 samples were received from Chantaburi province as shown in Table 5. All of these samples were collected by using filter paper from which their genomic DNA were extracted and analysed for *Pfmdr1* gene mutation and copy number. These samples were the samples from the patients participated in the the ATCs (Artesunate+Mefloquine+Primaquine) treatment efficacy Project of Mrs Saowanit Vijaykadga (Ministry of Public Health, Thailand). The *in vivo* treatment followed the WHO guideline with all blood samples were collected in Day 0 before ACTs treatment. After ACTs treatment, the patients had been followed up for 42 days. The outcomes of ACTs treatment were used for interpretation in the part of analysis of *Pfmdr1* copy number as shown in Table 10 and Table 11.

Table 5 Isolates of *P. falciparum* collecting during year 2006-2010 from Trat and Chanthaburi provinces

No.	Isolates	Years	ACTs Efficacy Treatment	From
1	Br1/4	2006	S	Amphoe Bo Rai, Trat
2	Br1/5	2006	S	Amphoe Bo Rai, Trat
3	Br1/22	2006	S	Amphoe Bo Rai, Trat
4	Br1/23	2006	S	Amphoe Bo Rai, Trat
5	Br1/24	2006	S	Amphoe Bo Rai, Trat
6	Br1/25	2006	S	Amphoe Bo Rai, Trat
7	Br1/26	2006	S	Amphoe Bo Rai, Trat
8	Br1/27	2006	S	Amphoe Bo Rai, Trat
9	Br2/50	2006	S	Amphoe Bo Rai, Trat
10	Br3/8	2006	S	Amphoe Bo Rai, Trat
11	Br3/50	2006	S	Amphoe Bo Rai, Trat
12	Br4/50	2006	S	Amphoe Bo Rai, Trat
13	Br18/50	2006	S	Amphoe Bo Rai, Trat

No.	Isolates	Years	ACTs Efficacy Treatment	From
14	Br2	2006	S	Amphoe Bo Rai, Trat
15	Br4	2006	S	Amphoe Bo Rai, Trat
16	Br5	2006	S	Amphoe Bo Rai, Trat
17	Br6	2006	S	Amphoe Bo Rai, Trat
18	Br7	2006	S	Amphoe Bo Rai, Trat
19	Br8	2006	S	Amphoe Bo Rai, Trat
20	Br9	2006	S	Amphoe Bo Rai, Trat
21	Br10	2006	S	Amphoe Bo Rai, Trat
22	Br18	2006	S	Amphoe Bo Rai, Trat
23	Br19	2006	S	Amphoe Bo Rai, Trat
24	Br22	2006	S	Amphoe Bo Rai, Trat
25	Br23	2006	S	Amphoe Bo Rai, Trat
26	Br24	2006	S	Amphoe Bo Rai, Trat
27	Br25	2006	S	Amphoe Bo Rai, Trat
28	Br26	2006	S	Amphoe Bo Rai, Trat

No.	Isolates	Years	ACTs Efficacy Treatment	From
29	Br28	2006	S	Amphoe Bo Rai, Trat
30	Br29	2006	S	Amphoe Bo Rai, Trat
31	Br30	2006	S	Amphoe Bo Rai, Trat
32	Br31	2006	S	Amphoe Bo Rai, Trat
33	Br32	2006	S	Amphoe Bo Rai, Trat
34	Br33	2006	S	Amphoe Bo Rai, Trat
35	KS4	2006	S	Amphoe Khao Saming, Trat
36	KS5	2006	S	Amphoe Khao Saming, Trat
37	KS6	2006	S	Amphoe Khao Saming, Trat
38	TR14	2006	S	Trat
39	TD433	2010	S	Trat
40	TD502	2010	S	Trat
41	TD522	2010	S	Trat
42	TD523	2010	S	Trat
43	TD524	2010	S	Trat

No.	Isolates	Years	ACTs Efficacy Treatment	From
44	TD525	2010	S	Trat
45	TD526	2010	S	Trat
46	TD529	2010	S	Trat
47	TD530	2010	S	Trat
48	TD531	2010	S	Trat
49	TD533	2010	S	Trat
50	CB3	2006	S	Chanthaburi
51	CB4	2006	S	Chanthaburi
52	CB58	2006	S	Chanthaburi
53	CB59	2006	S	Chanthaburi
54	CB64	2006	S	Chanthaburi
55	CB65	2006	S	Chanthaburi
56	CB70	2006	S	Chanthaburi
57	CB73	2006	S	Chanthaburi
58	CB74	2006	S	Chanthaburi

No.	Isolates	Years	ACTs Efficacy Treatment	From
59	CB75	2006	S	Chanthaburi
60	CB76	2006	S	Chanthaburi
61	CB82	2006	S	Chanthaburi
62	CB83	2006	S	Chanthaburi
63	CB87	2006	S	Chanthaburi
64	CB88	2007	S	Chanthaburi
65	CB89	2007	S	Chanthaburi
66	CB90	2007	S	Chanthaburi
67	CB91	2007	S	Chanthaburi
68	CB92	2007	S	Chanthaburi
69	CH13	2010	S	Chanthaburi
70	CH15	2010	S	Chanthaburi
71	CH25	2010	S	Chanthaburi
72	CH28	2010	S	Chanthaburi
73	CH31	2010	S	Chanthaburi

* 3D7, K1, and PFK12 were used as the positive controls

* S = Sensitive to ACT treatment

4.2 **Analysis of *Pfmdr1* gene mutation of *P. falciparum* by PCR-RFLP**

73 genomic DNA of *P. falciparum* blood samples (49 samples from Trat and 24 from Chantaburi) were extracted using Chelex-resin according to the method of Plowe and colleague in 1993. The DNA were then analyse for point mutation of *Pfmdr1* gene in the codons 86, 184 and 1034 by PCR-RFLP method. After conducting the PCR with the specific primers of each codon, the amplified products were cut by restriction enzymes and revealed the specific bands size as shown in Table 6. The results of *Pfmdr1* gene point mutation analysed in samples collected from Trat were shown in Table 7 and the results of *Pfmdr1* gene point mutation analysed in Chantaburi samples were shown in Table 8. The comparisons of the results between 2 endemic areas were shown in Table 9. In addition, Figure 19 to Figure 42 were the figures of the PCR products after cutting with restriction enzyme and running on 2 % agarose gel electrophoresis.

The results revealed that there is no mutation in *Pfmdr1* gene in *P. falciparum* from Trat whereas the samples collected from Chantaburi found point mutation in N86Y (20%, n=15), S1034C (28.57%, n=7) and no mutation was found in Y184F.

Table 6 Primers and Enzymes condition and expected nucleic acid sizes for detection of amino acid mutation of *Pfmdr1*

Target residue	Primer	Restriction enzyme	Incubation condition	Expected size (bp)	
				Wild type	Mutation
N86Y	A2 A4	*Afl III* *Apo I*	37°	560 505	232+328 249+256
F184Y	A2 A4	*Dra I*	37°	242+204	242+173
S1034C	1034f 1042r	*Dde I*	37°	172+60	205+27

* The incubation reactions were performed overnight.

Table 7 *Pfmdr1* gene point mutations of *P. falciparum* from Trat province. The mutation positions were reported as amino acid residue. 3D7, K1, and PFK12 clones were used as controls.

No.	Isolates	Years	Mutations (amino acid residues) *Pfmdr1* genes		
			N86Y	Y184F	S1034C
1	TD433	2010	N	Y	S
2	TD502	2010	N	Y	S
3	TD522	2010	N	Y	S
4	TD523	2010	N	Y	S
5	TD524	2010	N	Y	S
6	TD525	2010	N	Y	S
7	TD526	2010	N	Y	S
8	TD529	2010	N	Y	S
9	TD530	2010	N	Y	S
10	TD531	2010	N	Y	S
11	TD533	2010	N	Y	S
12	Br1/4	2006	N	Y	S
13	Br1/5	2006	N	Y	S

No.	Isolates	Years	Mutations (amino acid residues) *Pfmdr1* genes		
			N86Y	Y184F	S1034C
14	Br1/22	2006	N	Y	S
15	Br1/23	2006	N	Y	S
16	Br1/24	2006	N	Y	S
17	Br1/25	2006	N	Y	S
18	Br1/26	2006	N	Y	S
19	Br1/27	2006	ND	ND	ND
20	Br2/50	2006	N	Y	S
21	Br3/8	2006	N	Y	S
22	Br3/50	2006	N	Y	S
23	Br4/50	2006	ND	ND	ND
24	Br18/50	2006	N	Y	S
25	Br2	2006	ND	ND	ND
26	Br4	2006	N	Y	ND
27	Br5	2006	ND	ND	ND
28	Br6	2006	N	Y	S

No.	Isolates	Years	Mutations (amino acid residues) *Pfmdr1* genes		
			N86Y	Y184F	S1034C
29	Br7	2006	N	Y	S
30	Br8	2006	N	Y	ND
31	Br9	2006	N	Y	S
32	Br10	2006	ND	ND	ND
33	Br18	2006	ND	ND	ND
34	Br19	2006	ND	ND	ND
35	Br22	2006	N	Y	ND
36	Br23	2006	N	Y	S
37	Br24	2006	N	Y	S
38	Br25	2006	N	Y	S
39	Br26	2006	ND	ND	ND
40	Br28	2006	N	Y	S
41	Br29	2006	ND	ND	ND
42	Br30	2006	N	Y	S
43	Br31	2006	N	Y	ND

No.	Isolates	Years	Mutations (amino acid residues) *Pfmdr1* genes		
			N86Y	Y184F	S1034C
45	Br33	2006	N	Y	S
46	KS4	2006	N	Y	S
47	KS5	2006	ND	ND	ND
48	KS6	2006	N	Y	ND
49	TR14	2006	N	Y	ND
	3D7		N	Y	S
	K1		Y	Y	S
	PFK12		N	Y	C

* ND = No Data

* N= Asn, Y= Tyr, F= Phe, S= Ser

Table 8 Point mutations of *P. falciparum* from Chanthaburi province in the *Pfmdr1* gene.

No.	Isolates	Years	Mutations (amino acid residues) *Pfmdr1* genes		
			N86Y	Y184F	S1034C
1	CH13	2010	Y	Y	C
2	CH15	2010	Y	Y	C
3	CH25	2010	Y	Y	ND
4	CH28	2010	N	Y	ND
5	CH31	2010	N	Y	ND
6	CB3	2006	N	Y	ND
7	CB4	2006	ND	ND	ND
8	CB58	2006	ND	ND	ND
9	CB59	2006	N	Y	ND
10	CB64	2006	ND	ND	ND
11	CB65	2006	ND	ND	ND
12	CB70	2006	N	Y	ND
13	CB73	2006	N	Y	ND

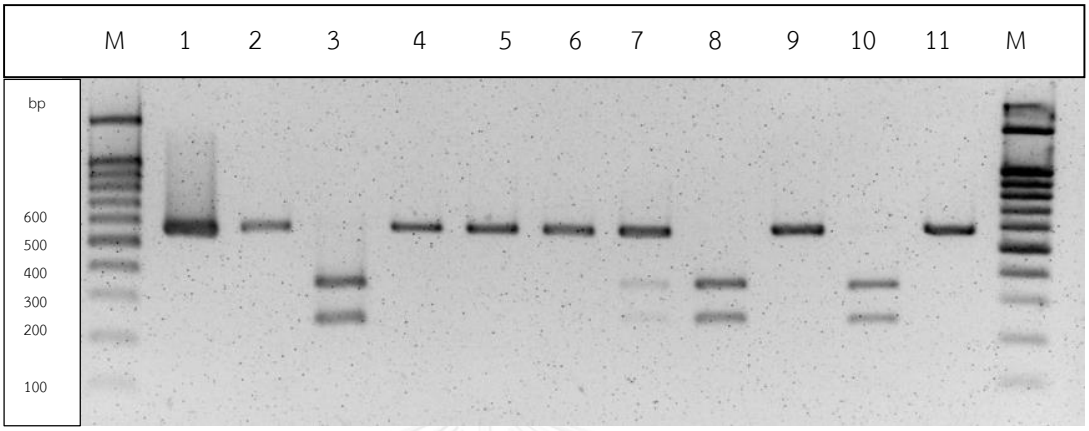
No.	Isolates	Years	Mutations (amino acid residues) *Pfmdr1* genes		
			N86Y	Y184F	S1034C
14	CB74	2006	ND	ND	ND
15	CB75	2006	N	Y	ND
16	CB76	2006	ND	ND	ND
17	CB82	2006	ND	ND	ND
18	CB83	2006	ND	ND	ND
19	CB87	2006	ND	ND	ND
20	CB88	2007	N	Y	S
21	CB89	2007	N	Y	S
22	CB90	2007	N	Y	S
23	CB91	2007	N	Y	S
24	CB92	2006	N	Y	S
	3D7		N	Y	S
	K1		Y	Y	S
	PFK12		N	Y	C

* ND = No Data * N= Asn, Y= Tyr, F= Phe, S= Ser

Table 9 Prevalence of *Pfmdr1* point mutation in *Plasmodium falciparum* from Trat province and Chanthaburi province

Polymorphisms of *Pfmdr1*		Trat	Chantaburi
Pfmdr1 N86Y	86N	38 (100%, n=38)	12 (80%, n=15)
	86Y	0 (0%, n=38)	3 (20%, n=15)
Pfmdr1 Y184F	184Y	38 (100%, n=38)	24 (100%, n=24)
	184F	0 (0%, n=38)	0 (0%, n=24)
Pfmdr1 S1034C	1034S	32 (100%, n=32)	5 (71.42%, n=7)
	1034C	0 (0%, n=32)	2 (28.57%, n=7)

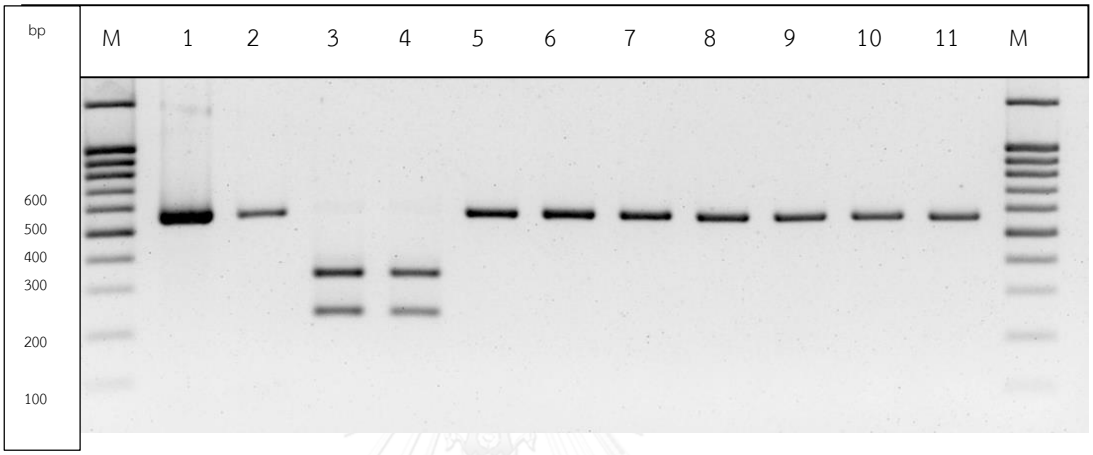
Figure 19 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Afl III* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = Uncut 2 = 3D7 3 = K1 4 = TD530 5= TD531 6= TD5

7= CH13 8= CH15 9= CH18 10=CH25 11=CH28

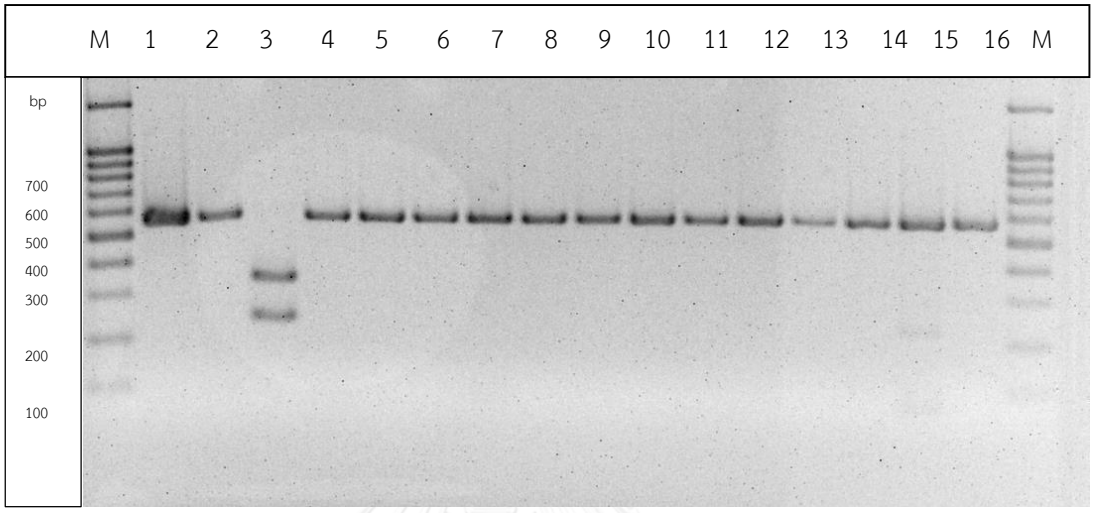
Figure 20 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Afl III* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = Uncut 2 = 3D7 3 = K1 4 = RC17 5= TD502 6= TD522

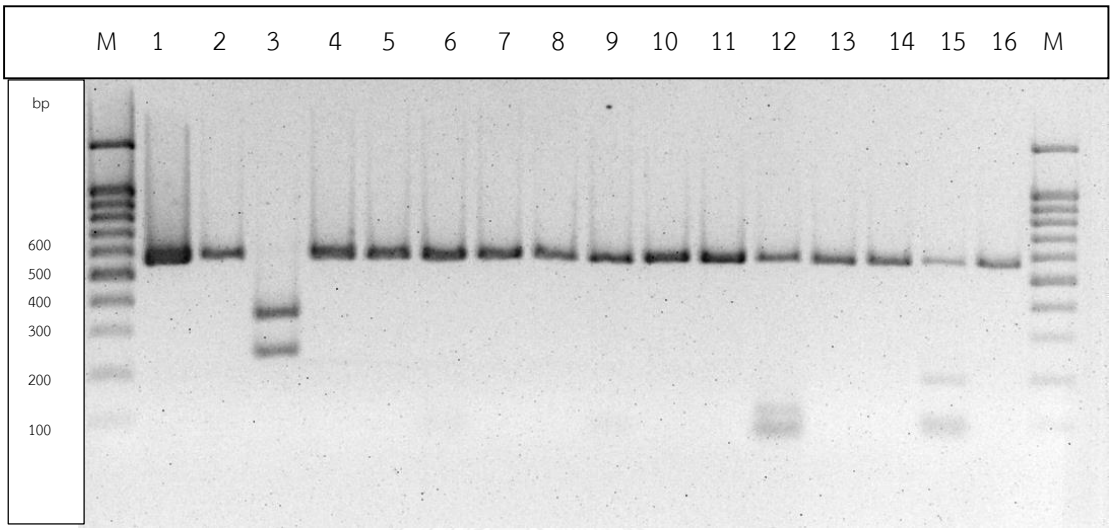
7= TD523 8= TD524 9= TD525 10=TD526 11=TD529

Figure 21 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Afl III* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control.



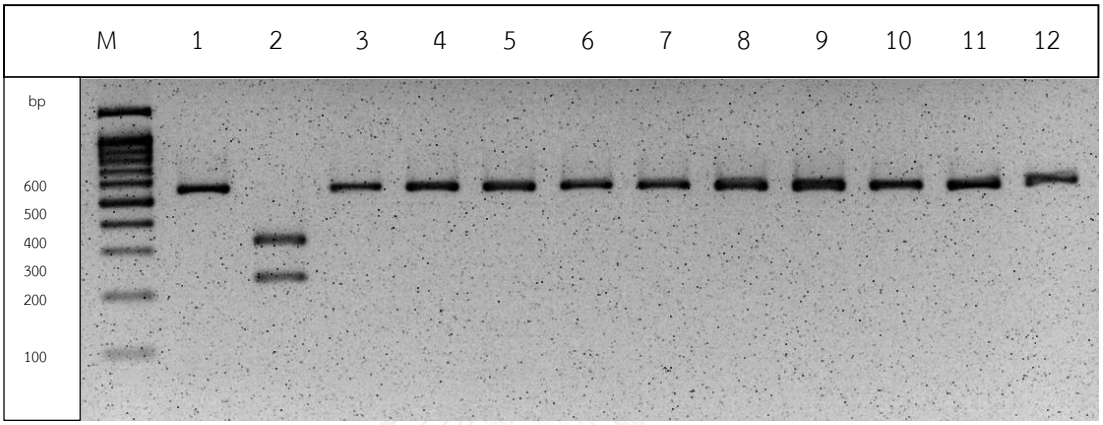
1 = Uncut	2 = 3D7	3=K1	4 = BR4	5 = BR6	6= BR8
7= BR9	8= BR23	9= BR25	10= BR28	11= BR32	12= BR33
13=CB3	14=CB59	15=CB70	16=Br3/50		

Figure 22 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Afl III* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = Uncut	2 = 3D7	3=K1	4 = KS4	5 = TR14	6= TD433
7= CH31	8= Br1/4	9= BR7	10= BR22	11= BR24	12= KS6
13=BR30	14= BR31	15=CB73	16= CB75		

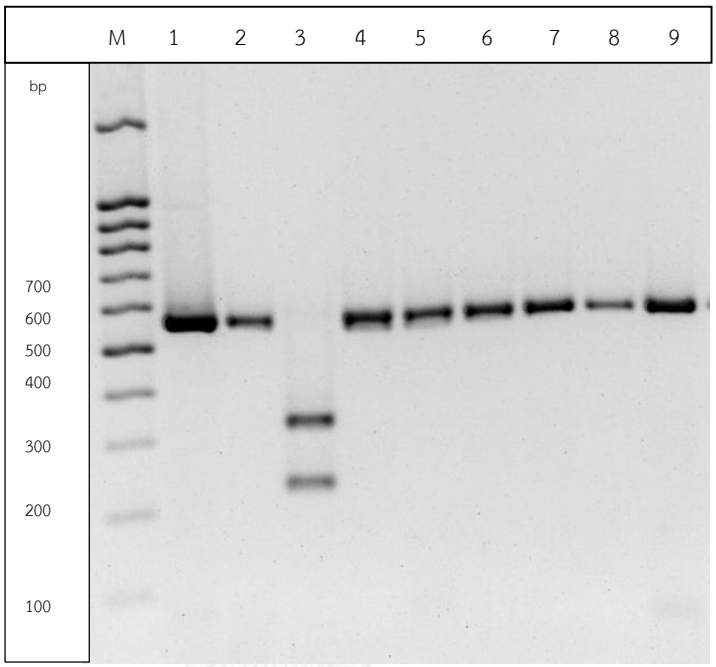
Figure 23 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Afl III* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = Uncut 2 = K1 3 = 3D7 4 = Br1/5 5= Br1/22 6= Br1/23

7= Br1/24 8= Br1/25 9= Br1/26 10= Br2/50 11= Br3/8 12=Br18/50

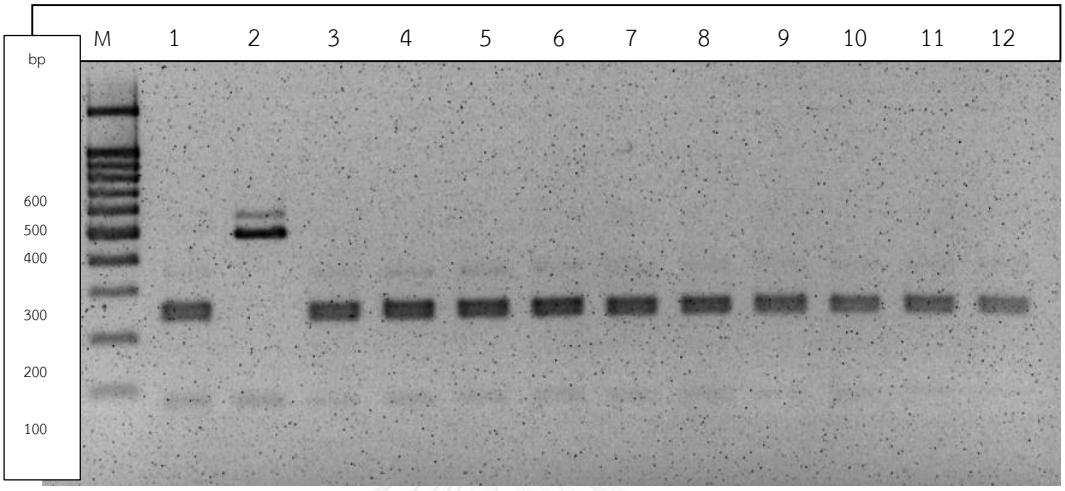
Figure 24 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Afl III* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = Uncut 2 = 3D7 3=K1 4 = CB90 5 = CB91 6= KS3

7= CB88 8= CB89 9= BR31

Figure 25 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Apo I* restriction enzyme. M: 100 bp marker; K1 is mutant-type positive control and 3D7 is wild-type positive control.

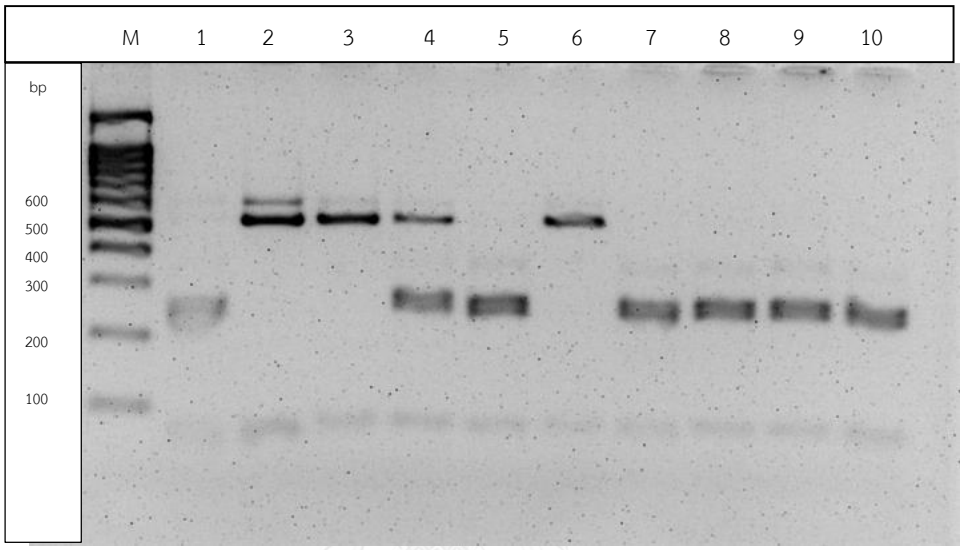


1 = 3D7 2 = K1 3 = TD502 4= TD522 5= TD523

6= TD524 7= TD525 8= TD526 9=TD529 10=TD530 11=TD531

12=TD533

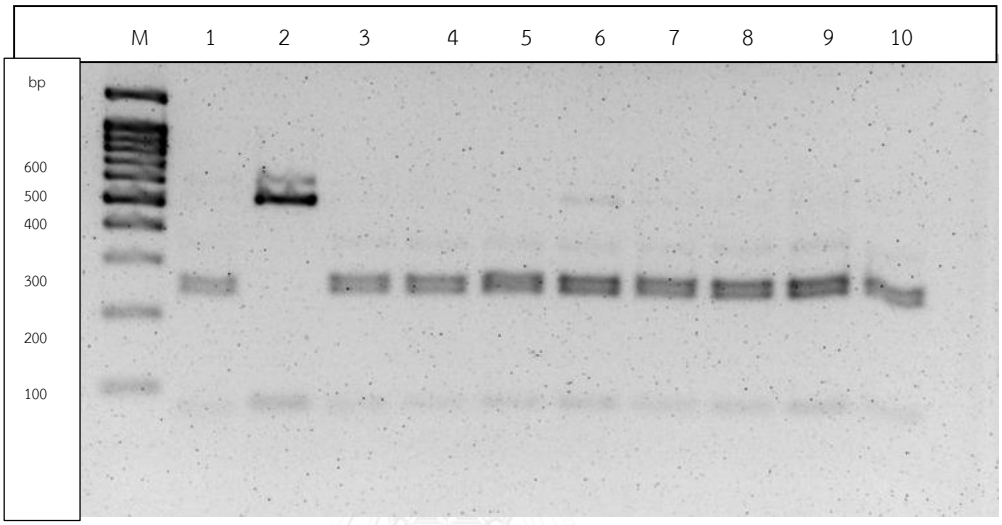
Figure 26 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Apo I* restriction enzyme. M: 100 bp marker; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = 3D7 2 = K1 3 = CH13 4= CH15 5= CH18 6= CH25

7= CH28 8= CH31 9=Br1/5 10=Br1/22

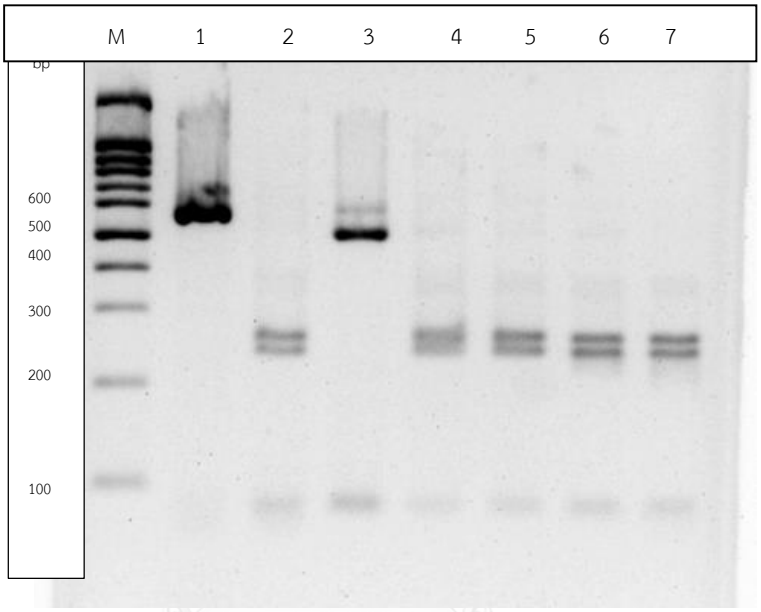
Figure 27 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Apo I* restriction enzyme. M: 100 bp marker; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = 3D7 2 = K1 3 = Br1/23 4= Br1/24 5= Br1/25 6= Br1/26

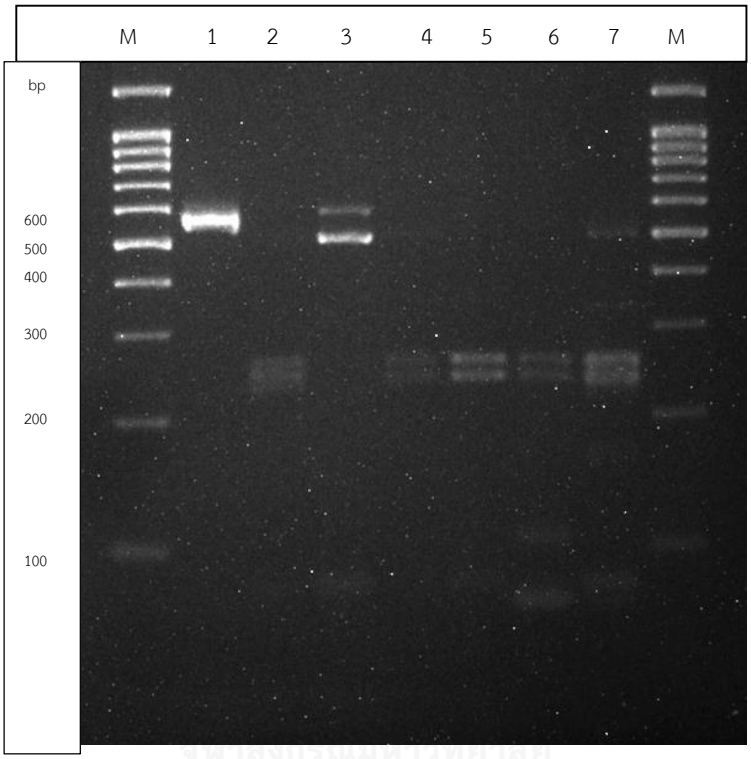
7= Br2/50 8= Br3/8 9=Br3/50 10=Br18/50

Figure 28 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Apo I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = Uncut 2 = 3D7 3 = K1 4 = TD433 5= Br1/4 6= BR6 7= CB75

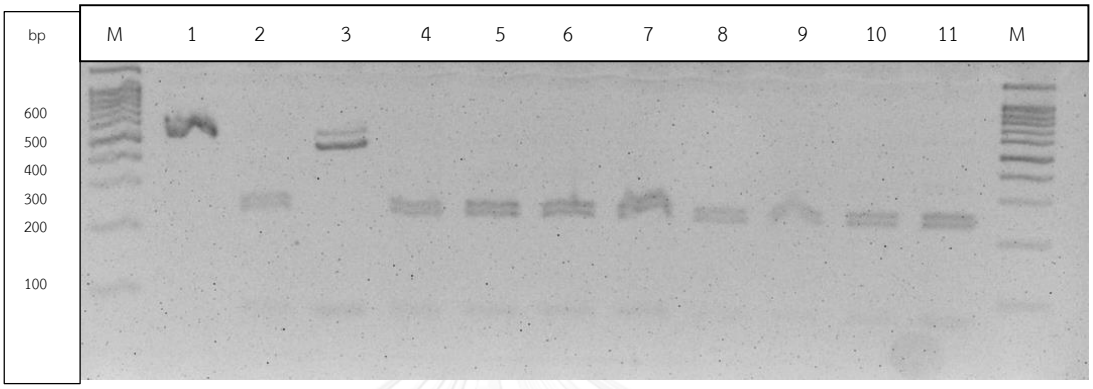
Figure 29 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Apo I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = Uncut 2 = 3D7 3 = K1 4 = CB3 5= CB59 6= KS6

7= CB70

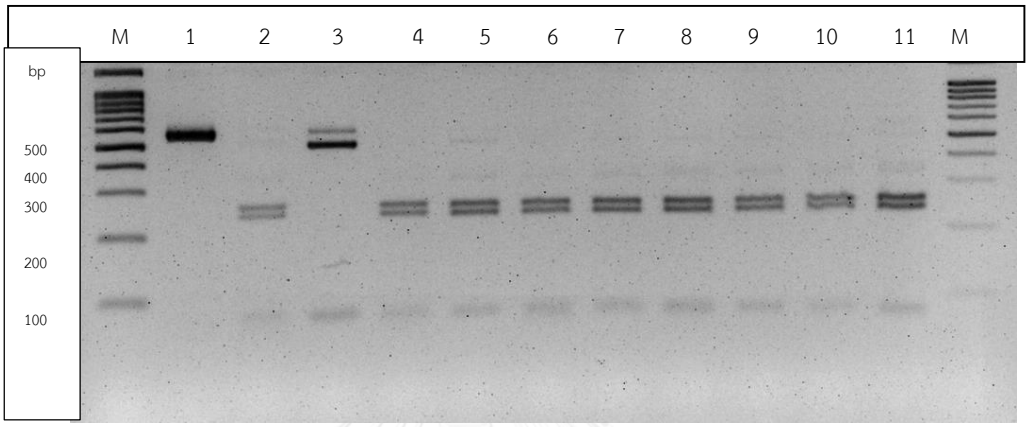
Figure 30 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Apo I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = Uncut 2 = 3D7 3 = K1 4 = BR4 5= BR6 6= BR7

7= BR8 8= BR9 9= BR22 10= BR23 11= BR24

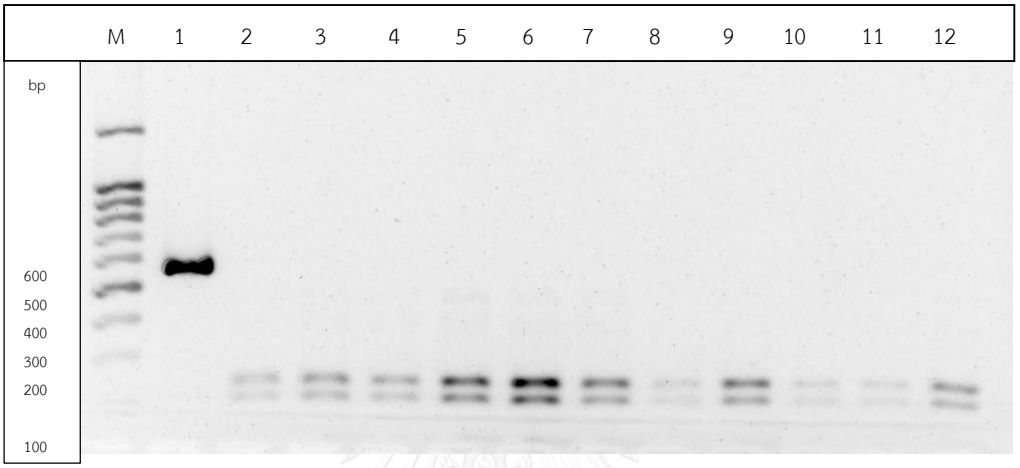
Figure 31 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Apo I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control.



1 = Uncut 2 = 3D7 3 = K1 4 = BR25 5= KS4 6= TR14

7= BR28 8= BR30 9= BR31 10= BR32 11= BR33

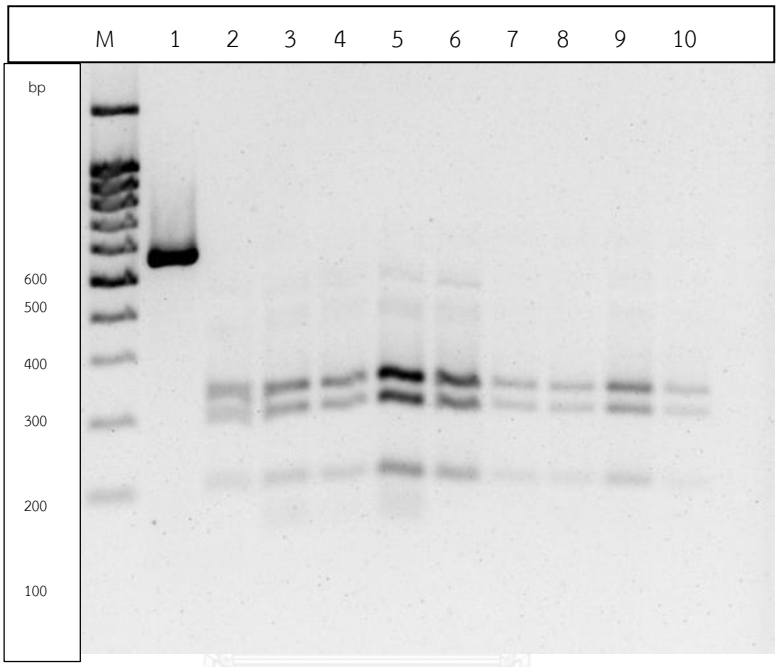
Figure 32 Detection of *Pfmdr1* polymorphism at codon 184, which was digested by *Dra I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 and 3D7 are wild-type positive control.



1 = Uncut 2 = 3D7 3=K1 4 = TD433 5 = TD502 6 = TD522

7 = TD523 8= TD524 9= TD525 10= TD526 11= TD529 12= TD530

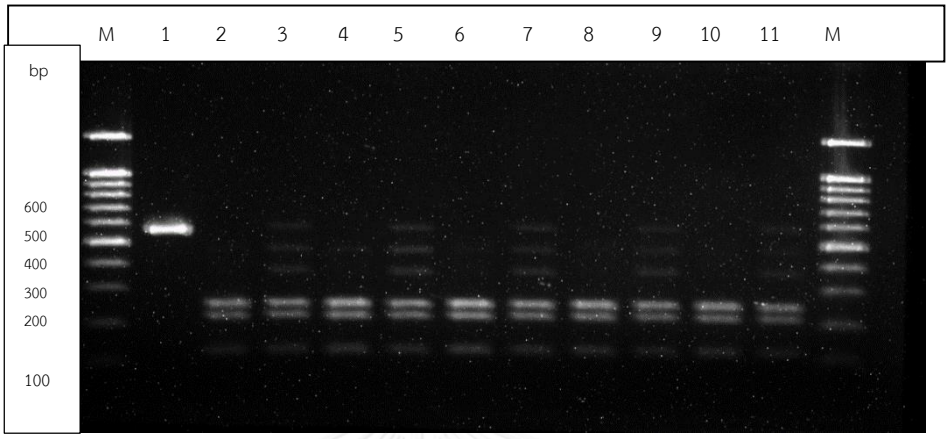
Figure 33 Detection of *Pfmdr1* polymorphism at codon 184, which was digested by *Dra I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; 3D7 are wild-type positive control.



1 = Uncut 2 = 3D7 3= TD531 4= TD533 5= CH13 6= CH15

7= CH18 8=CH25 9=CH28 10=CH31

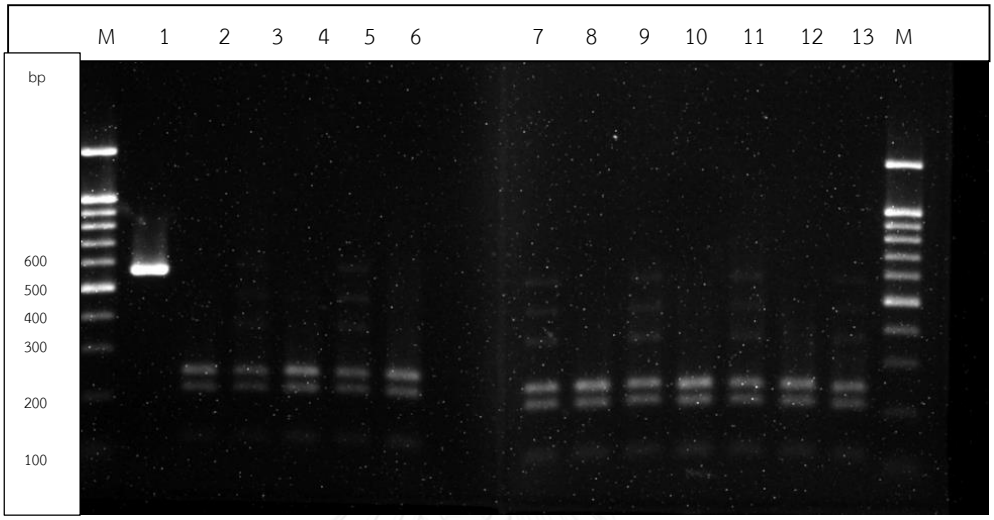
Figure 34 Detection of *Pfmdr1* polymorphism at codon 184, which was digested by *Dra I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; 3D7 are wild-type positive control.



1 = Uncut 2 = 3D7 3 = Br1/4 4 = Br1/5 5= Br1/22 6= Br1/23

7= Br1/24 8= Br1/25 9= Br1/26 10= Br1/27

Figure 35 Detection of *Pfmdr1* polymorphism at codon 184, which was digested by *Dra I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; 3D7 are wild-type positive control.

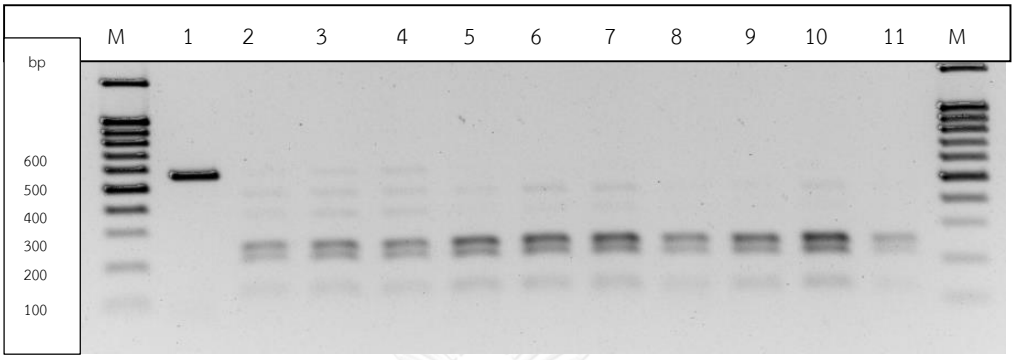


1 = Uncut 2 = 3D7 3 = Br2/50 4 = Br3/8 5= Br3/50 6= Br18/50

7= BR4 8= BR6 9= BR7 10= BR9 11= BR18 12= BR23

13= BR24

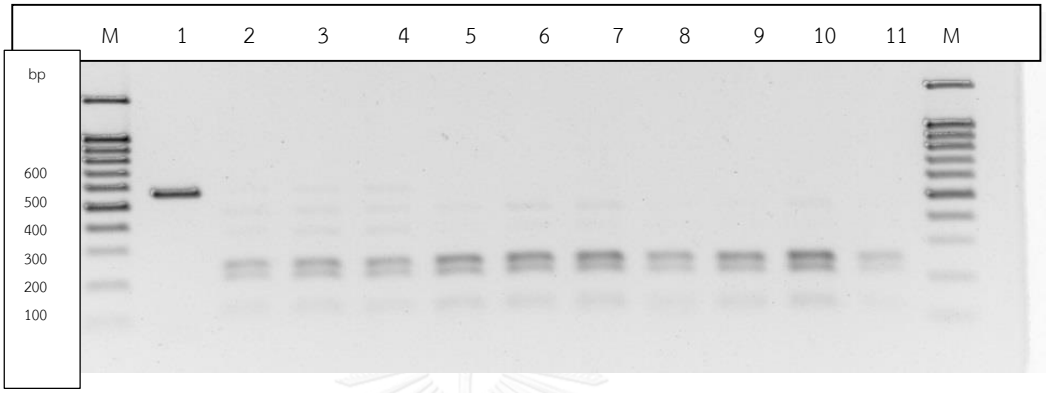
Figure 36 Detection of *Pfmdr1* polymorphism at codon 184, which was digested by *Dra I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; 3D7 are wild-type positive control.



1 = Uncut 2 = 3D7 3 = BR25 4 = BR28 5= BR29 6= BR30

7= BR31 8= BR32 9= BR733 10= CB3 11= CB4

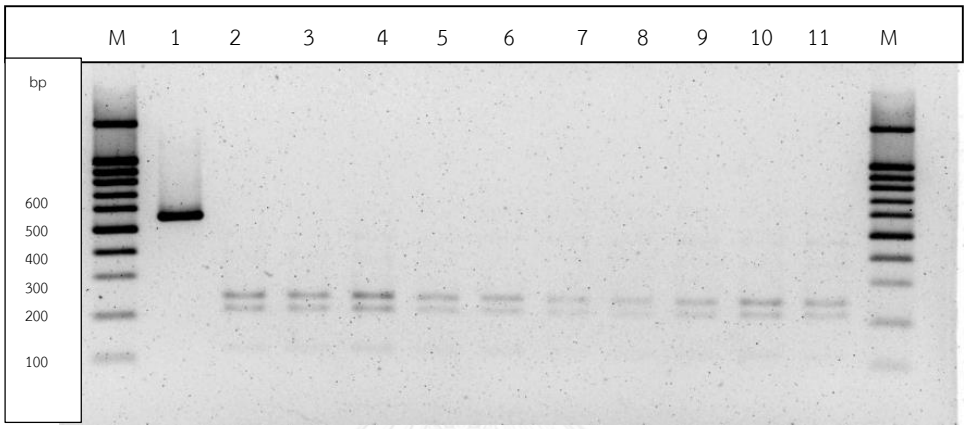
Figure 37 Detection of *Pfmdr1* polymorphism at codon 184, which was digested by *Dra I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 and 3D7 are wild-type positive control.



1 = Uncut 2 = 3D7 3 = K1 4 = CB58 5= CB59 6= 64

7= CB65 8= CB70 9= CB73 10= CB74 11= CB75

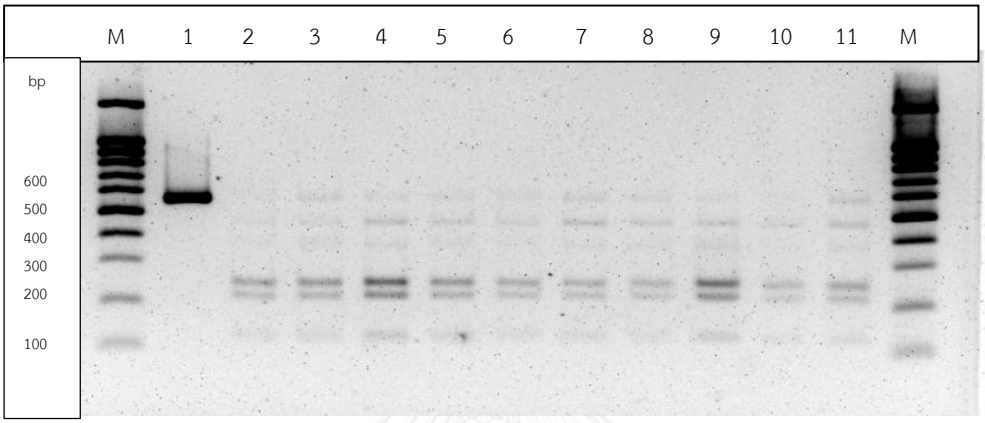
Figure 38 Detection of *Pfmdr1* polymorphism at codon 184, which was digested by *Dra I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; 3D7 are wild-type positive control.



1 = Uncut 2 = 3D7 3 = CB76 4 = CB82 5= CB83 6= CB87

7= CB88 8= CB89 9= CB90 10= CB92 11= CB92

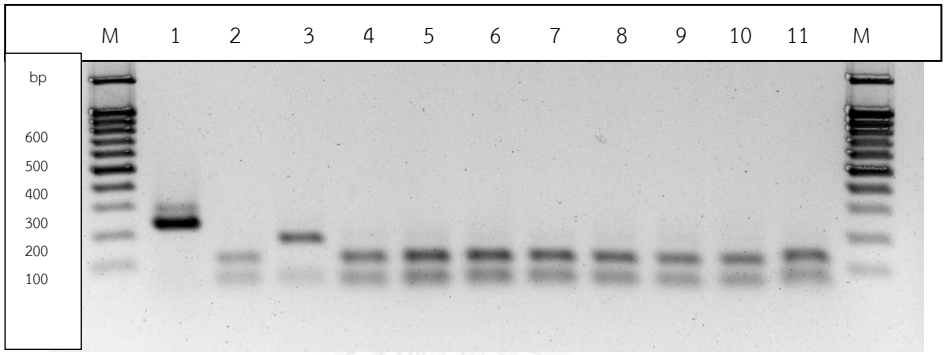
Figure 39 Detection of *Pfmdr1* polymorphism at codon 184, which was digested by *Dra I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 and 3D7 are wild-type positive control.



1 = Uncut 2 = 3D7 3 = K1 4 = KS3 5= KS4 6= KS5

7= KS6 8= TR14 9= RC17 10= PFK12 11= G112

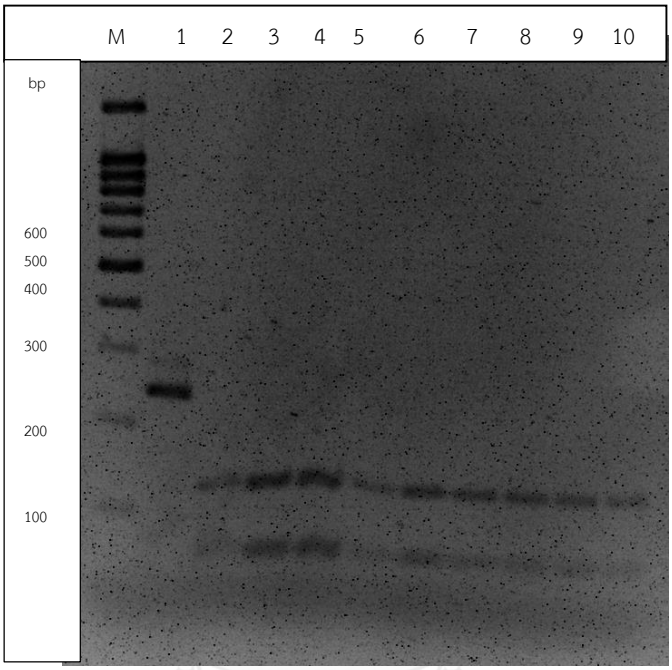
Figure 40 Detection of *Pfmdr1* polymorphism at codon 1034, which was digested by *Dde I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; PFK12 is mutant-type positive control and 3D7 are wild-type positive control.



1 = Uncut 2 = 3D7 3 = PFK12 4 = TD530 5= TD531 6= TD533

7= CH13 8= CH15 9= CH18 10=CH25 11=CH28

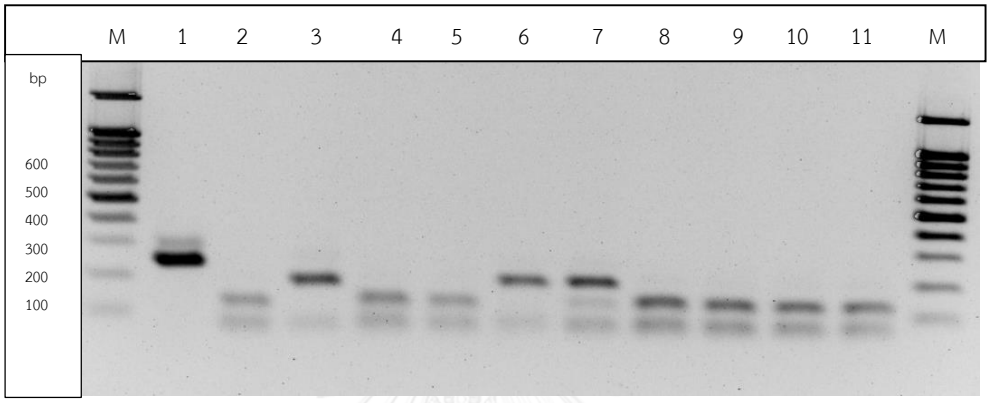
Figure 41 Detection of *Pfmdr1* polymorphism at codon 1034, which was digested by *Dde I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; PFK12 is mutant-type positive control and 3D7 are wild-type positive control.



1 = Uncut 2 = 3D7 3 = TD433 4 = TD502 5= TD522 6= TD523

7= TD524 8= TD525 9= TD526 10= TD529

Figure 42 Detection of *Pfmdr1* polymorphism at codon 1034, which was digested by *Dde I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; PFK12 is mutant-type positive control and 3D7 are wild-type positive control.



1 = Uncut	2 = 3D7	3 = PFK12	4 = TD530	5= TD531	6= CH13
7= CH15	8= TD533	9=Br1/4	10=Br1/5	11=Br1/22	

4.3 **Analysis of *Pfmdr1* gene copy number in *P. falciparum***

Detection of *Pfmdr1* gene copy number was determined by using TaqMan® real time qPCR. The primers and the probe specific to a conserved region of *Pfmdr1* and the primers and a probe specific to *β-actin* were designed. The *P. falciparum* 3D7 clone was determined in parallel as control samples. 3D7 carry only one gene copy of *Pfmdr1*. At the end of each reaction, cycle threshold (C_T) and melting curves were generated for further analysis. *Pfmdr1* gene was determined by relative quantification between *Pfmdr1* gene and *β-actin* gene based on the $\Delta\Delta C_T$ method. The *β-actin* is housekeeping genes that carry only a single copy of *Pfmdr1* in all parasite isolates and thus allow the comparison of the gene copy number. The target gene (*Pfmdr1*) and reference gene (*β-actin*) were amplified with the same efficiency with an appropriate range of DNA concentrations. The copy number of *Pfmdr1* was calculated using the comparative C_T method, also called $2^{-\Delta\Delta CT}$ method, where $\Delta C_{T,sample} = C_{T,pfmdr1} - C_{T,reference}$, $\Delta C_{T,sample}$ is the C_T value for any sample normalized to the endogenous house-keeping gene, and $\Delta\Delta C_T = \Delta C_{T,sample} - \Delta C_{T,3D7.}$

In all the samples, the $2^{-\Delta\Delta CT}$ refers to N-fold change of *pfmdr1* copy number relative to 3D7 *pfmdr1* copy number. Therefore, the 3D7 *pfmdr1* copy number was calculated by $\Delta C_{T,3D7.} = C_{T,pfmdr1} - C_{T,reference}$ $\Delta\Delta C_T = \Delta C_{T,3D7} - \Delta C_{T,3D7} = 0$. 3D7 *pfmdr1* copy number = $2^{-\Delta\Delta CT} = 2^{-0} = 2^0 = 1$. For each sample, $\Delta C_{T,E}$ denoting the experimental ΔC_T was determined by $\Delta C_{T,G} - \Delta C_{T,R}$,where $\Delta C_{T,G}$ is the target and $\Delta C_{T,R}$ is the

reference C_T. This value was then applied to the comparative $\Delta\Delta C_T$ method: $\Delta\Delta C_T = \Delta C_{T,E} - \Delta C_{T,3D7}$ where the $\Delta C_{T,3D7}$ is the ΔC_T of 3D7. The copy number of *pfmdr1* in each sample was revealed by $2^{-\Delta\Delta CT}$ as the N-fold copy number of *pfmdr1* when compare with 3D7 [Ferreira, 2006].

The results showed that in Trat province exhibited the higher percentage of *P. falciparum* copy number with three or more copies of *Pfmdr1* than in Chantaburi (9.09% and 0%, respectively). The mean *Pfmdr1* copy number in *P. falciparum* collected from Trat and Chantaburi were 2.2 and 1.5 respectively, as shown in Table 10, Table 11. The amplification curves and relative quantification values were shown in Figure 43 to Figure 44. Furthermore, in Table 12 was shown the comparison the results of both *Pfmdr1* point mutation and copy number in these two areas.

Table 10 *Pfmdr1* gene copy number of *P. falciparum* from Trat province.

No.	Isolate	*Pfmdr1* gene Copy number				ACTs Efficacy Treatment	Year of collection
		1	2	3	Mean ± SD		
1	TD522	1.84	4.40	2.48	2.90 ± 1.33	S	2010
2	TD523	2.57	ND	ND	2.57 ± 0.00	S	2010
3	TD524	2.24	ND	ND	2.24 ± 0.00	S	2010
4	TD525	1.01	2.39	2.43	1.94 ± 0.80	S	2010
5	TD526	1.76	2.85	ND	2.28± 0.77	S	2010
6	TD433	3.51	ND	2.71	3.11 ± 0.56	S	2010
7	TD502	2.60	ND	2.90	2.75 ± 0.20	S	2010
8	TD529	1.42	ND	1.8	1.65 ± 0.32	S	2010
9	TD530	1.30	ND	1.8	1.55 ± 0.35	S	2010
10	TD531	2.03	ND	1.53	1.78 ± 0.34	S	2010
11	TD533	1.45	ND	1.58	1.51 ± 0.08	S	2010
Mean total					2.20 ± 0.43		

* S= sensitive to ACT treatment

Table 11 *Pfmdr1* gene copy number of *P.falciparum* from Chanthaburi province.

No.	Isolate	*Pfmdr1* gene Copy number				ACTs Efficacy Treatment	Year of collection
		1	2	3	Mean ± SD		
1	CH13	1.77	1.29	1.78	1.61 ± 0.28	S	2010
2	CH15	1.91	1.18	2.16	1.75 ± 0.51	S	2010
3	CH18	1.98	1.91	2.32	2.07 ± 0.21	S	2010
4	CH25	0.95	1.83	1.14	1.31 ± 0.46	S	2010
5	CH28	1.03	1.08	1.15	1.09 ± 0.05	S	2010
6	CH31	0.73	0.91	1.56	1.07 ± 0.43	S	2010
Mean total					1.48 ± 0.32		

Table 12 Prevalence of *Pfmdr1* point mutations and copy number in *Plasmodium falciparum* from Trat province and Chanthaburi province

Polymorphisms of *Pfmdr1*		Trat	Chantaburi
Pfmdr1 N86Y	86N	38 (100%, n=38)	12 (80%, n=15)
	86Y	0 (0%, n=38)	3 (20%, n=15)
Pfmdr1 Y184F	184Y	38 (100%, n=38)	24 (100%, n=24)
	184F	0 (0%, n=38)	0 (0%, n=24)
Pfmdr1 S1034C	1034S	32 (100%, n=32)	5 (71.42%, n=7)
	1034C	0 (0%, n=32)	2 (28.57%, n=7)
Copy number of *Pfmdr1*		2.20 ± 0.43	1.48 ± 0.32

Figure 43 Relative quantification of *Pfmdr1* gene from Trat province.

3D7 is positive control (copy number = 1).

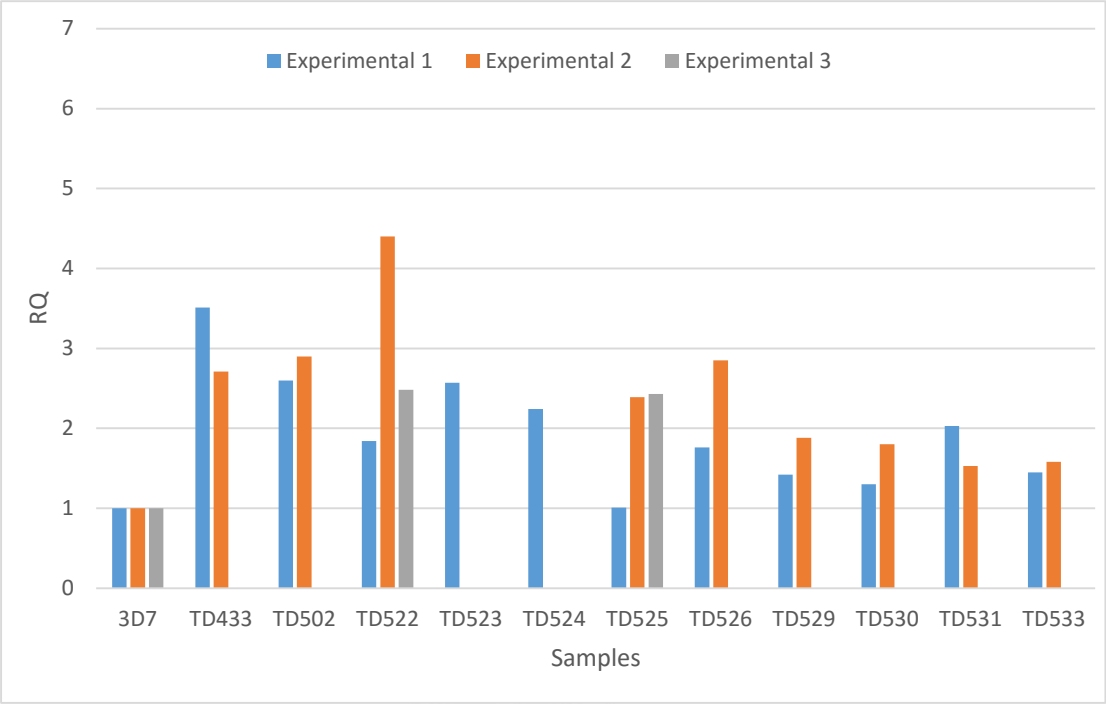
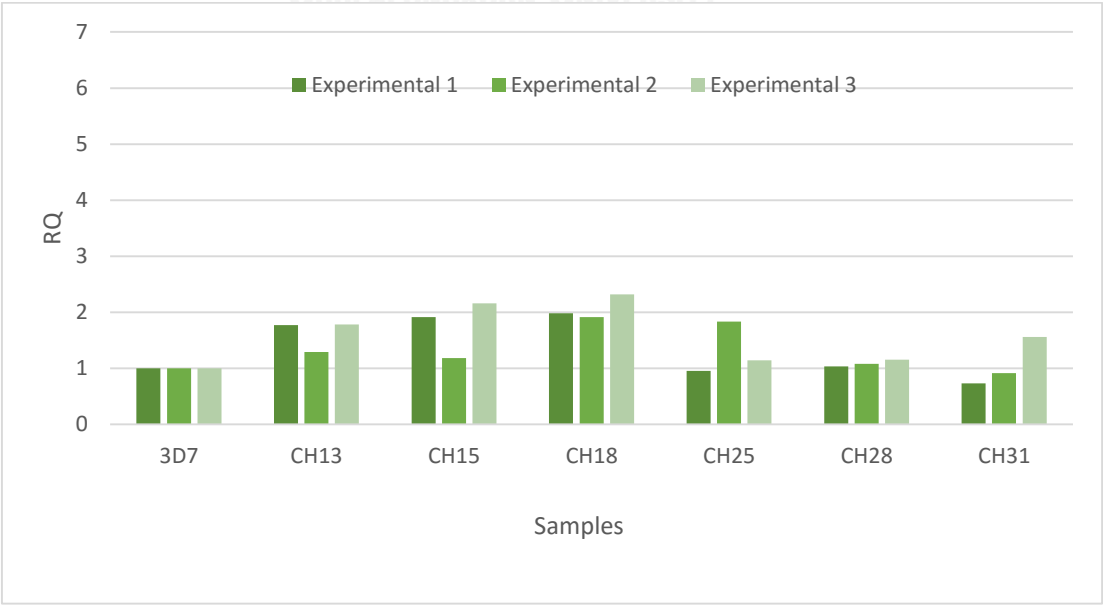


Figure 44 Relative quantification of *Pfmdr1* gene from Chanthaburi province.

3D7 is positive control (copy number = 1).



CHAPTER V
DISCUSSION AND CONCLUSION

Discussion

Multidrug-resistant falciparum malaria is now a serious problem in Southeast Asia, where resistance to chloroquine, mefloquine and quinine are frequently found (Wongsrichanalai et al., 2002). This resistance has been restrained to some degree through the use of artemisinin-based combination therapies, which is recommended by the world Health Organization as the first-line treatment of uncomplicated falciparum malaria in endemic areas (WHO, 2010). In Thailand anti-malarial drugs resistance has been spread over 50 years. Currently, the situation of drugs resistance is more seriously. The evidences both in vitro and in vivo of P. falciparum which is the most resistant strain has been confirmed. There are some studies reported that the falciparum malaria in Thailand already resistant to chloroquine, sulfadoxine-pyrimethamine, mefloquine and quinine (Wongsrichanalai et al., 2001). The declining of mefloquine susceptibility leading to the addition of artesunate in the first-line drugs regimen in Trat, Chantaburi, Sa Kaeo which are located in Thai-Cambodian border, and Tak in Thai-Myanmar border. The resistance of P. falciparum is the main factor contributing to the obstruction of malaria control program in Thailand.

The objectives of this study are to determine the distribution of the point mutations of *Pfmdr1* gene and to determine the *Pfmdr1* gene copy number of P.

falciparum endemic in Thai-Cambodia border. The study areas are 2 provinces, Chantaburi and Trat, which are highly resistant endemic areas. Several studies have shown that single nucleotide polymorphisms and amplification of the Pfmdr1 gene is associated with in vitro response and clinical efficacy of mefloquine, an arylaminoalcohol. Evidence suggests that the Pfmdr1 gene plays a role in the in vitro response to other quinolines such as quinine and lumefantrine and artemisinin derivatives. The results revealed that there was no mutation in *Pfmdr1* gene in *P. falciparum* from Trat where as in Chantaburi found point mutation in N86Y (20%, n=15), S1034C (28.57%, n=7) and no mutation was found in Y184F both *in P. falciparum* collected from Trat and Chantaburi. The prevalence of point mutation at codon 86 and 1034 in *Pfmdr1* gene of *P. falciparum* endemic in Chantaburi province was higher than in Trat province even though they both are situated in Thai-Cambodia border. There are no point mutations at codon 184 in *Pfmdr1* gene of *P. falciparum* endemic both in Chantaburi Province and Trat province. In contrast, *Pfmdr1* copy number in *P. falciparum* endemic in Trat province was higher than in Chantaburi province. The parasites exhibited *Pfmdr1* gene copy number lower than 3 copies were found sensitive to ACTs treatment patients in both of Trat and Chanthaburi provinces.

Previously, *P. falciparum* isolates collected in 1988-2003 from this area were genetically characterized. Similar to the present study, Mungthin et al. identified 86Y 9%, 184F 86%, 1034C18% from Trat and Chanthaburi provinces with a trend of

increasing prevalence of wild-type genotypes. The prominent pattern of *Pfmdr1* at condon 86/184/1034 was NFS with prevalence increasing from 40% to 90% and the prevalence of more than one copy number increasing from 17% to 62% during the 10-year period. Their results support to this study that found 184F 100% with F prevalence also increasing. *P. falciparum* exhibited more than one copy number of *Pfmdr1* 100% in Trat and 50% in Chanthaburi, from 2005-2010 [Mungthin et al., 2010]. Moreover, in Thai-Myanmar border area found 86Y 5%, more than one copy number 52% from the samples year 2006-2009 [Phompradit et al., 2011].

Moreover, in southern areas of Thailand, found 86Y 36%, 184F 63%, 1034C 0% in upper southern (Ranong and Chumphon) whereas in lower southern (Yala, Narathiwas, and Songkhla) found 86Y 96%, 184F 3%, 1034C 0.4%. These results show the *Pfmdr1* 184F allele was more common in the parasite from upper southern areas. However, the copy number from upper southern areas was significantly higher than lower southern areas with the mean 2.3 and 1.2 respectively, in year 2009-2010 [Mungthin et al., 2014].

In contrast, there are reported in Malaysia that found 86Y 5% from P. falciparum collected during 2007-2009 and revealed high predominance of wild type. However, this study found 1246C 5% that is rarely found in Thailand [Atroosh et al., 2012]. This results show that the different pattern of Pfmdr1 point mutation in different geography.

Pfmdr1 mutation and copy number may be used as an attractive alternative tool to investigate the role of drug resistance of malaria parasites in laboratory studies or large scale epidemiological survey.

Conclusion

The *P. falciparum* isolates were observed during the five-year observation period (2006-2010). *Pfmdr1* appear to be the keys genes that modulate multi-drugs resistance in *P. falciparum*. Trat and Chanthaburi provinces are the endemic areas to implications for anti-malarial multi-drugs resistance in Thailand.

The parasites from Trat province exhibited different resistant patterns compared to Chantaburi province even though these two provinces are located close to the border of Thai-Cambodia. The present study showed that *P. falciparum* isolated from different areas along the international border of Thailand exhibited different resistant phenotypic and genotypic patterns. However, this information from this study will be useful for anti-malarial drug policy in Thailand, early detection of emergence of anti-malarial drugs resistance. New candidate drugs should be adopted at least based on their activity against these phenotypic and genotypic parasites in different areas of Thailand.

REFERENCES

1) Atroosh, W. M, Al-Mekhlafi, H. M., Mahdy, M. A., and Surin, J. 2012. The detection of *pfcrt* and *pfmdr1* point mutations as molecular markers of chloroquine drug resistance, Pahang, Malaysia. Malaria Journal. 11:251.

2) Alker, A.P., Lim, P., Sem, R., Shah, N.K., Yi, P., Boutg, D.M., et al. 2007. *Pfmdr1* and *in vivo* resistance to artesunate-mefloquine in falciparum malaria on the Cambodia-Thai border. Am J Trop Med Hyg. 76(4):614-617.

3) Afonso, A., Hunt, P., Cheesman, S., Alves, A. C., Cunha, C. V., do Rosa' rio, V., and Cravo, P. 2006. Malaria Parasites Can Develop Stable Resistance o Artemisinin but Lack Mutations in Candidate Genes atp6 (Encoding the Sarcoplasmic and Endoplasmic Reticulum Ca2_ATPase), tctp, mdar1, and cg10. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY: 480-489.

4) Bjorman, A. and Phillips-Howard, P. A. 1990. The epidemiology of drug-resistant malaria. Trans R Soc Trop Med Hyg 84: 177-80.

5) Chaijaroenkul, W., Wisedpanichkij, R., and Na-Bangchang, K. 2010. Monitoring of in vitro susceptibilities and molecular markers of resistance of *Plasmodium falciparum* isolates from Thai-Myanmar Border to chloroquine, quinine, mefloquine and artesunate. Acta Tropica 113: 190-194.

6) Chang, H. H., Moss, E. L., Park, D. J., Ndiaye, D., Mboup, S., Volkman, S. K., Sabeti, P. C., Wirth, D. F., Neafsey, D. E., and Hartl, D. L. 2013. Malaria life cycle

intensifies both natural selection and random genetic drift. PNAS 110(50): 20129-20134.

7) Congpuong, K., Na-Bangchang, K., Mungthin, M., Bualombai, P., and Wernsdorfer, W.H. 2005. Molecular epidermiology of drug resistance markers of *Pasmodium falciparum* malaria in Thailand. Tropical Medicine and International Health 10(8): 717-722.

8) Cowman, A. F., Karcz, S., Galatis, D., and Culvenor, J. C. 1991. A P-glycoprotein homoloque of *Plasmodium falciparum* is located on the digestive vacuole. J Cell Biol 113: 1033-1042.

9) Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., Tarning, J., Lwin, K. M., Ariey, F., Hanppithakpong, W., Lee, S. J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Singhasivanon, P., Day, N. P. J., Lindegardh, N., Socheat, D., and White, N. J. 2009. Artemisinin Resistance in *Plasmudium falciparum* Malaria. The N Engl J Med. 361: 455-67.

10) Duah, N. O., Matrevi, S. A., Souza, D. K., Binnah, D. D., Tamakloe, M. M., Opoku, V. S., Onwona, C. O., Narh, C. A., Quashie, N. B., Abuaku, B., Duplessis, K. C. K., and Koeam, K. A. 2013. Increase *pfmdr1* gene copy number and the decline in *pcrt* and *pfmdr1* resistance alleles in Ghanaian Plasmodium falciparum isolates after the change of anti-malarial drug treatment policy. Malaria Journal. 12:337

11) Duraisingh, M.T. and Cowman, A.F. 2005. Contribution of the *Pfmdr1* gene to antimalarial drug resistance. Acta Trop 94(3): 181-190.

12) Duraisingh, M.T., Roper, C., Walliker, D., and Warhurt, D.C. 2000. Increased sensitivity to the antimalarials mefloqiune and artemisinin is conferred by mutations in the *Pfmdr1* gene of *Plasmodium falciparum*. Mol Microbiol 36:955-961.

13) Djimdé, A., Doumbo, O. K., Cortese, J. F., Kayentao, K., Doumbo, S., Diourté, Y., Dicko, A., Su, X.Z., Nomura, T., Fidock, D. A., Wellems, T. E., Plowe, C. V., and Coulibaly, D. 2001. A molecular marker for chloroqiun-reisitant falciparum malaria. N Engl J Med 344:955-961.

14) Eckstein-Ludwing, U., Webb, R. J., van Goethem, I. D. A., East, J. M., Lee, A. G., Kimura, M., O'Neill, P. M., Bray, P.G., Ward, S. A., and Krishna, S. 2003. Artemisinins target the SERCA of *Plasmodium falciparum*. Nature 424: 957-961.

15) Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M.T., Ursos, L. M., Sidhu, A. B., Naudé, B., Deitsch, K. W., Su, X. Z., Wootton, J. C., Roepe, P. D.,and Wellems, T. E. 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Molecular Cell 6: 861–871.

16) Ferreira, P. E., Holmgren, G., Veiga, M. I., Uhlén, P., Kaneko, A., and Gill, J. P. 2011. *Pfmdr1*: Mechanisms of Transport Modulation by Functional Polymorphisms. PLOS ONE 6(9): e23875.

17) Ferreira, I. D., Rosario, V. E., and Cravo, P. V. 2006. Real-time quantitative PCR with SYBR Green I detection for estimating copy numbers of nine drugs resistance candidate genes in Plasmodium falciparum. Malaria Journal 5: 1.

18) Hanssen, E., McMillan, P. J., and Tilley, L. 2010. Cellular architecture of Plasmodium-infected erythrocytes. International Journal for Parasitology 40: 1127-1135.

19) Hurwitz, E. S., Johnson, D., and Campbell, C. C. 1981. Resistance of *Plasmodium falciparum* malaria to sulphadoxine-Pryimenthamine ('Fansidar') in a refugee camp in Thailand. Lancet 1: 1068-1070.

20) Kain, K. C., Shanks, G. D., and Keystone, J.S. 2001. Malaria Chemoprophylaxis in the Age of Drug Resistance. I. Currently Recommended Drug Recommended Drug Regimens. Clinical Infectious Diseases 33: 226-234.

21) Klimpt, A. D., Jonet, A., Pillon, M., Mullié, C., and Sonnet, P. 2011. Mefloquine derivatives: synthesis, mechanism of action, antimicrobial activities. Formatex: 23-35

22) Maude, R. J., Woodrow, C. J., and White, L.J. 2010. Artemisinin Antimalarials: Preserving the “Magic Bullet”. Drug Development Research 71: 12-19.

23) Muhamad, P., Chaijaroenkul, W., Phompradit, P., Rueangweerayut, R., Tippawangkosol, P., and Na-Bangchang, K. 2013. Polymorphic patterns of *pfcrt* and *pfmdr1* in *Plasmodium falciparum* isolated along the Thai-Myanmar border. Asian Pac J Trop Biomed 3(12): 931-935.

24) Mungthin, M., Suwandittakul, N., Chaijaroenkul, W., Rungsrihirunrat, K., Harnyuttanakorn, P., Suegorn, A., and Na Bangchang, K. 2010. The patterns of mutation and amplification of *Plasmodium falciparum pfcrt* and *pfmdr1* genes in Thailand during the year 1988 to 2003.Parasiotol Res 107: 539-545.

25) Mungthin, M. Intanakom, S., Suwandittakul, N., Suida, P., Amsakul, S., Sitthichot, N, Thammapalo, S., and Leelayoova, S. 2014. Distribution of *pfmdr1* polymorphisms in *Plasmodium falciparum* isolated from Southern Thailand. Malaria Journal 13: 117

26) Mullis, K. B. 1990. The unusual origin of the polymerase chain reaction. Scientific American 36-43.

27) Nelson, D. L. and Cox, M.M. 2000. Lehninger principles of biochemistry. Worth Publishers, New York.

28) Olliaro, P. 2001. Mode of action and mechanisms of resistance for antimalarial drugs. Pharmacology & Therapeutics. 89: 207-219

29) Phompradit, P., Wisedpanichkij, R., Muhamad, P., Chaijaroenkul, W., and Na-Bangchang, K. 2011. Molecular analysis of *pfatp6* and *pfmdr1* polymorphisms and their association with in vitro sensitivity in Plasmodium falciparum isolates from the Thai-Myanmar border. Acta Tropica 120: 130-135.

30) Plowe, C. V., Djimde, A., Bouare, M., Doumbo, O., Wellens, T. E. 1995. Pyrimethamine and proguanil resistanceconferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am J Trop Med

31) Poyomtip, T., Suwandittakul, N., Sittichot, N., Khositnithikul, R., Tan-ariya, P., and Mungthin, M. 2012. Polomorphisms of the *pfmdr1* but not the *pfnhe-1* gene is associated with in vitro quinine sensitivity in Thai isolates of *Plasmmodium falciparum.* Malaria Journal 11:7.

32) Price, R. N., Uhlemann, A. C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S., White, N. J., Nosten, F. and Krishna, S. 2004. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Mechanisms of Disease 364: 438-447.

33) Promega. Real-Time qPCR and RT-qPCR Kits. 2014; Available from : http://worldwide.promega.com/products/pm/realtime=qpcr-and-rtpcr-kits.

34) Ridley, R., Dorn, A., Vippagunta, S., and Vennerstrom, J. 1997. Haematin (haem) polymerization and its inhibition by quinoline antimalarials. Ann. Trop. Med. Parasitol. 91: 559-566.

35) Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K., and Cowman, A. F. 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. Nature. 403: 906-909.

36) Rasmussen, H.B. Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP) and Gel Electrophoresis- Valuable Tool for Genotyping and Genetic Fingerprinting. Institute of Biological Psychiatry, Mental Health Centre Sct. Hans, Copenhagen University Hospitals, Roskilde, Denmark.

37) Schoone, G. J., Oskam, L., Kroon, N. C., Schallig, H. D., and Omar, S.A. 2000. Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification. J Clin Microbiol 38(11): 4072-5.

38) Sidhu, A. B., Valderramos, S. G. and Fidock, D. A. 2005. *Pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol Microbiol 57: 913-926.

39) Sidhu, A. B., Uhlemann, A. C., Valderramos, S. G., Valderramos, J. C., Krishna, S., and Fidock, D.A. 2006. Decreasing pfmdr1 copy number in *Plasmodium*

falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis 194: 528-535.

40) Snounou, G., Viriyakosol, S., Jarra, W., Thaithong, S., Brown, K. N. 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Mol Biochem Parasitol 58: 283-289.

41) Twyman R. M., 2005. Single nucleotide polymorphism (SNP) genotyping Techniques- An overview. Encyclopedia of Diagnotic Genomics and Proteomics.

42) Vijaykaga S., Rojanawatsirivej C., Cholpol S. et al., 2006. In vivo sensitivity monitoring of mefloquine monotherapy and artesunate-mefloquine combinations for the treatment of uncomplicated falciparum malaria in Thailand in 2003. Tropical Medicine and international Health. 11:211-219.

43) Vijaykadga, S., Rojanawatsirivej, C., Cholpol, S., Phoungmanee, D., nakavej, A., Wongsrichanalai, C., 2006. In vivo sensitivity monitoring of mefloquine monotherapy and artesunate-mefloquine combinations for the treatment of uncomplicated falciparum malaria in Thailand in 2003. Trop Med Int Health 11: 211-219.

44) Valderramos, S. G. and Fidock, D. A. 2006. Transporters involved in resistance to antimalarial drugs. TRENDS in Pharmacological Sciences 27(11): 594-601.

45) Volkman, S. K., Wilson, C. M., and Writh, D. F. 1993. Stage-specific transcripts of *Plasmodium falciparum Pfmar1* gene. Mol Biochem parasitol. 57: 203-212.

46) Weina, P.J. 2008. Artemisinins from folklore to modern medicine-transforming an herbal extract to life-saving drug. Parasitologia 50: 25-29.

47) Wernsdorfer, W. H. and Payne, D. 1991. The dynamics of drug resistance in *Plasmodium falciparum*. Pharmacol Ther 50: 95-121

48) Wernsdorfer, W. H. 1994. Epidemiology of drug resistance in malaria. Acta Trop 56: 143-56.

49) White, N. L. 1992. Antimalarial drug resistance: the pace quickens. J. Antimicrob. Chemother. 30: S71-S78.

50) White, N. J. 2008. Qinghaosu (Artemisinin): the price of success. Science 320(5874): 330-334.

51) WHO. World Health Organization. 2012. International Travel and Health.

52) WHO. World Health Organization. 2013. World Malaria Report, Geneva:

53) WHO. World Health Organization. 2010. Malaria in the Greater Mekong Sub region: Regional and country profiles. Geneva.

54) Wongsrichanalai, C., Sirichaisinthop, J., Karwacki, J. J., Congpuong, K., Miller, R.S., Pang, L. and Thimasarn, K. 2001. Drug resistant malaria on the Thai-Myanmar and Thai-Cambodian borders. Southeast Asian J Trop Med Public Heath 32: 41-49.

55) Wongsrichanalai, C., Pickard, A. L., Werndorfer, W. H., and Meshnick, S. R. 2002. Epidemiology of drug-resistant malaria. THE LANCET Infectious Diseases.

56) Wooden, J., Gould, E.E., Paull, A. T., Sibley, C. H. 1992. *Plasmodium falciparum*: a simple polymerase chain reaction method for differentiating strains. Exp Parasitol 75:207-212.

57) Zhang, J. K. M., Ginsburg, H. 1999. The fate of ferriprotorphyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. Mol Biochem Parasitol 99(1): 129-141.

APPENDIX

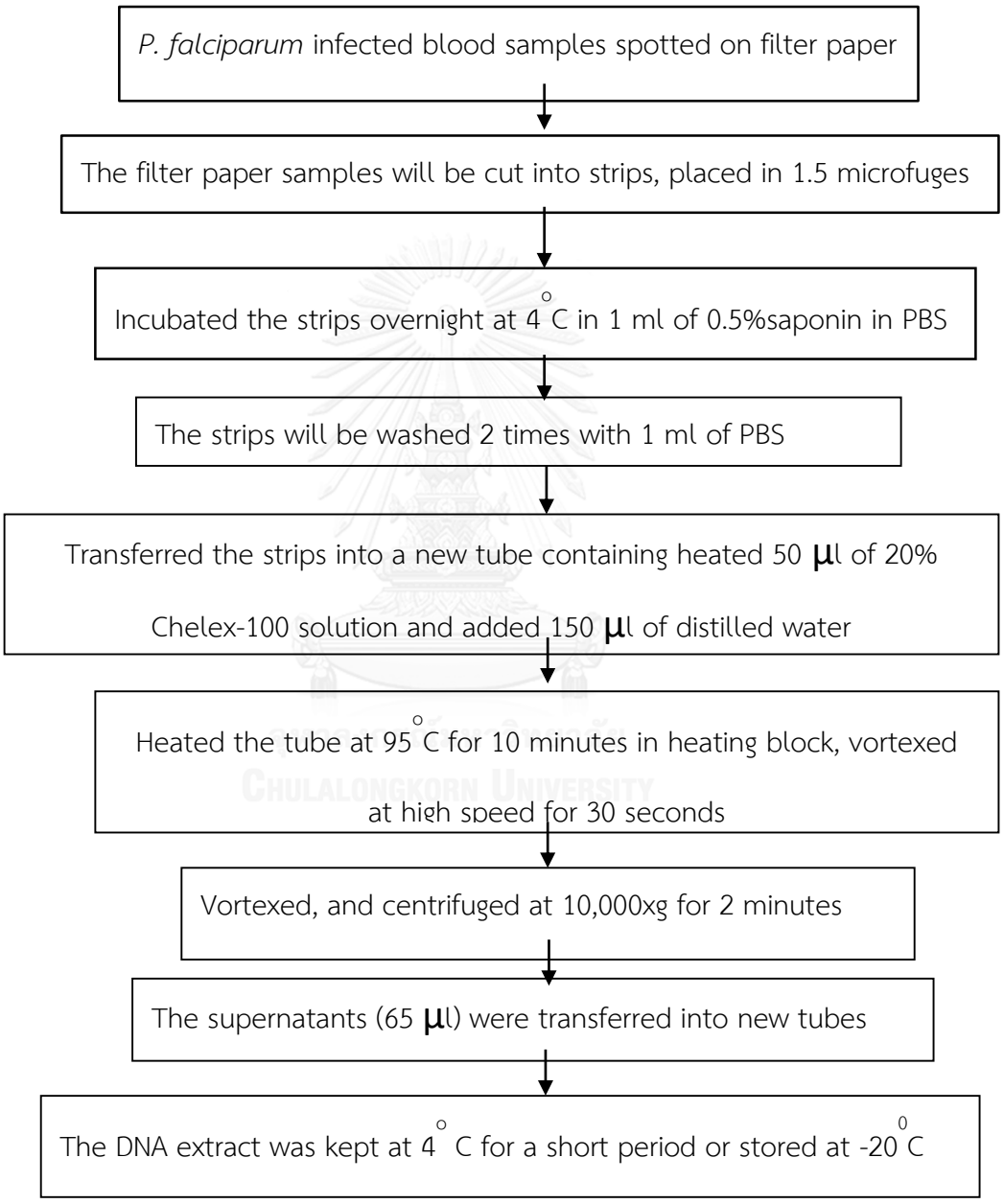
Materials and Methods

The reagents were used for genomic DNA preparation and gel electrophoresis. All reagents were sterilized by autoclave

Reagents	Composition
0.05% saponin	0.05 g of saponin Final volume 100 ml
6x phosphate buffer saline (PBS)	48.6 g of NaCl 3.06 g of KH_2PO_4 9.585 g of Na_2PO_4 Adjusted pH to 7.2 with NaOH Final volume 1,000 ml
0.5 M disodium ethylene diamine tetra acetrate (EDTA)	181.6 g of EDTA Adjusted pH to 8.0 with NaOH pellet Final volume 1,000 ml

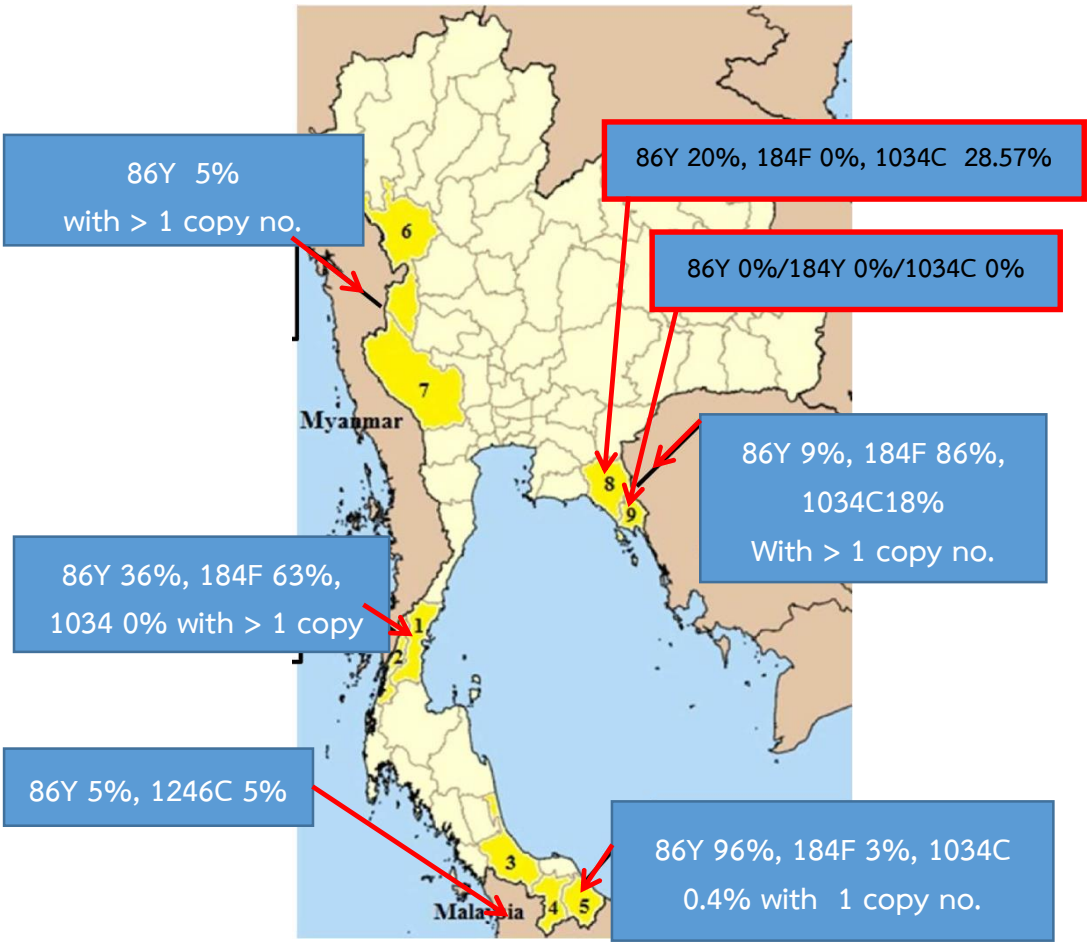
Reagents	Composition
10x TBE	121.14 g of Tris-HCl 5 g of boric acid 40 ml of 0.5 M EDTA pH 8.0 Final volume 1,000 ml
6x loading dye	0.4 mg of bromophenol blue 0.4 mg of xylene cyanol 3 ml of glycerine Final volume 10 ml
100 bp standard DNA ladder marker	0.1 µg/µl 1kb ladder 1x laoding buffer
Ethidium bromide (EtBr) staining solution	0.5 µl/ml EtBr

Chelex-100 extraction



Point mutations

Pfmdr1 point mutations and copy number in international border of Thailand and including some neighboring countries.



Pfmdr1=multiple-drug resistance [*Plasmodium falciparum*, GA3 clone of GH2 isolate, Genomic, 4260 nt]

GenBank: S53996.1

LOCUS S53996 4260 bp DNA linear INV 08-MAY-1993

ACCESSION S53996

ORIGIN

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1261 accatactaa aattaattga aagactttat gatccaaccg aaggagatat tattgtaaat

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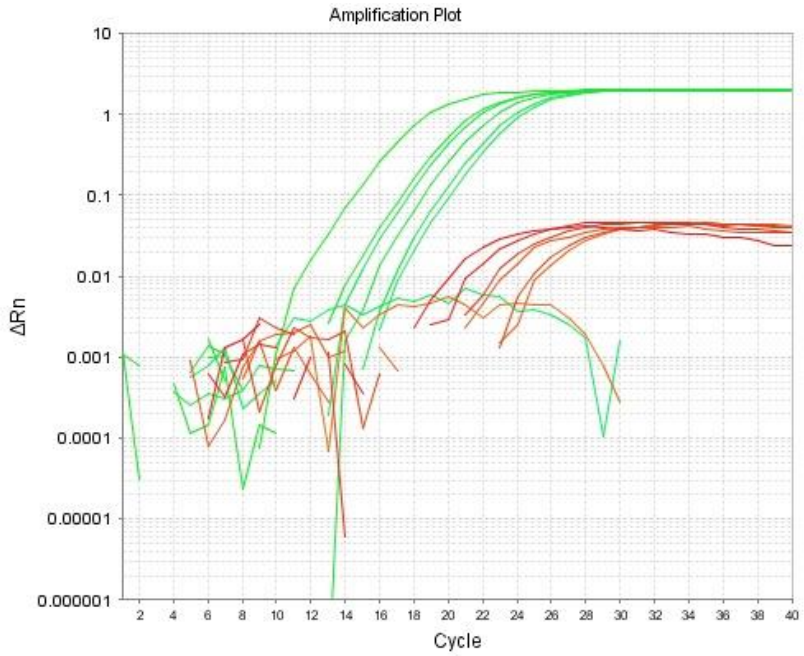
//

APPENDIX III

Pfmdr1 copy umber



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TD522	18	19.01	9	16.84
TD523	19	20.21	13	19.57
TD524	21	21.68	14	20.84
TD525	23	23.89	15	21.91
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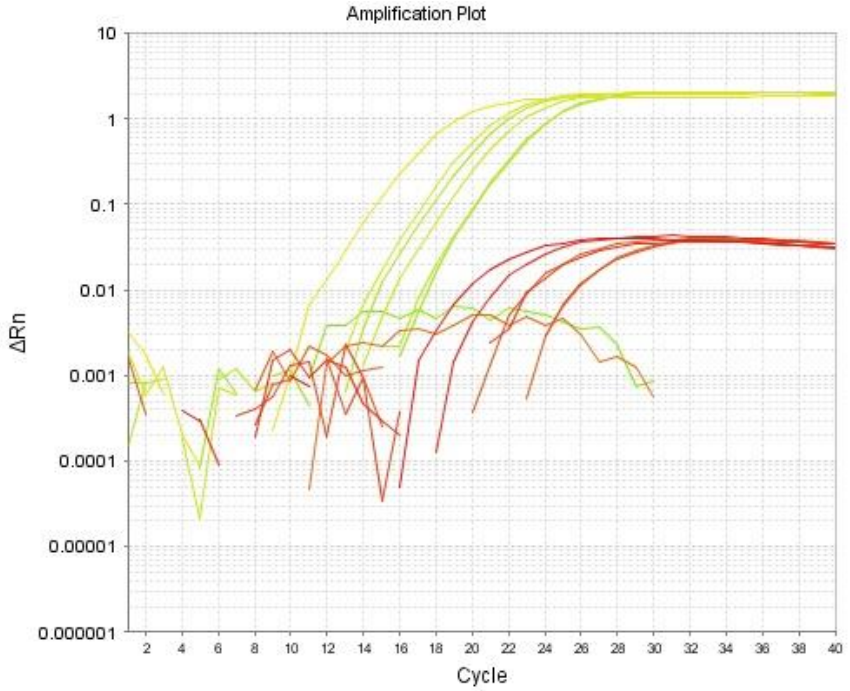

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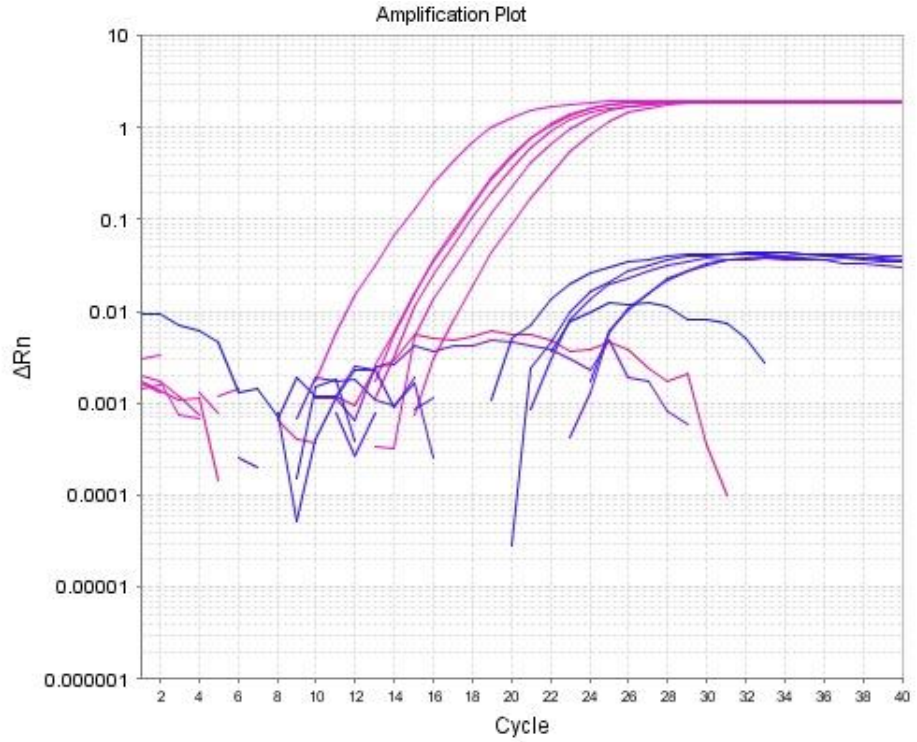

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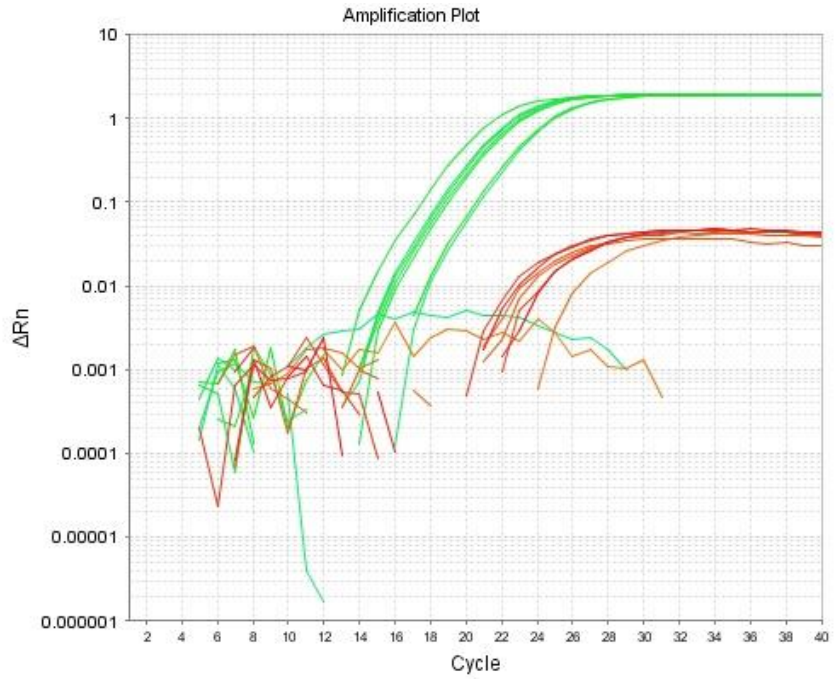

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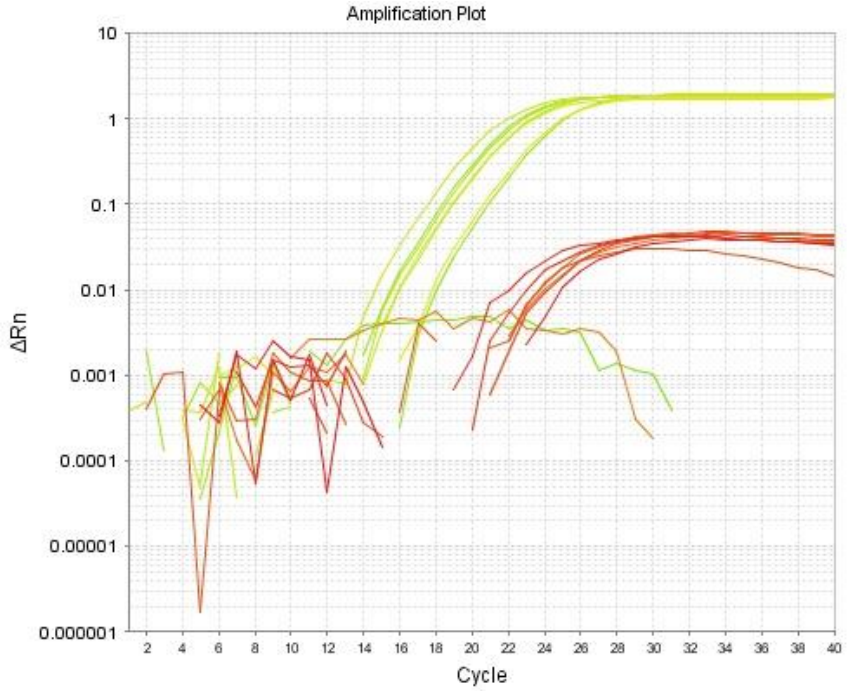

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TD502	19	20.73	14	20.89
TD529	20	21.90	13	19.50
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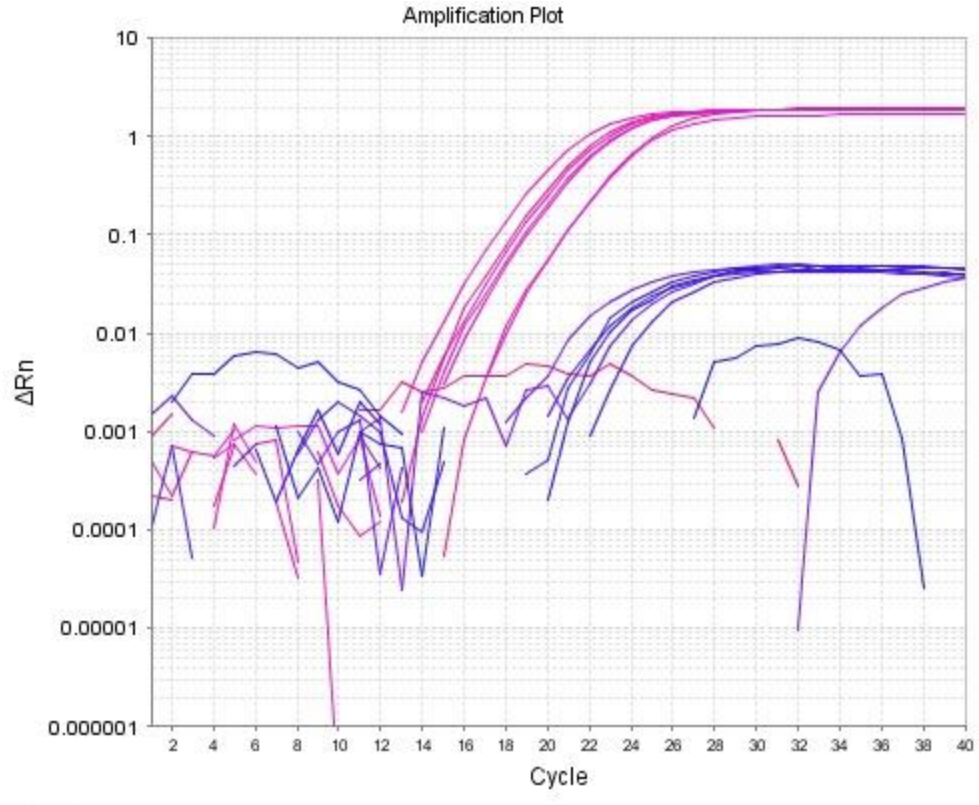

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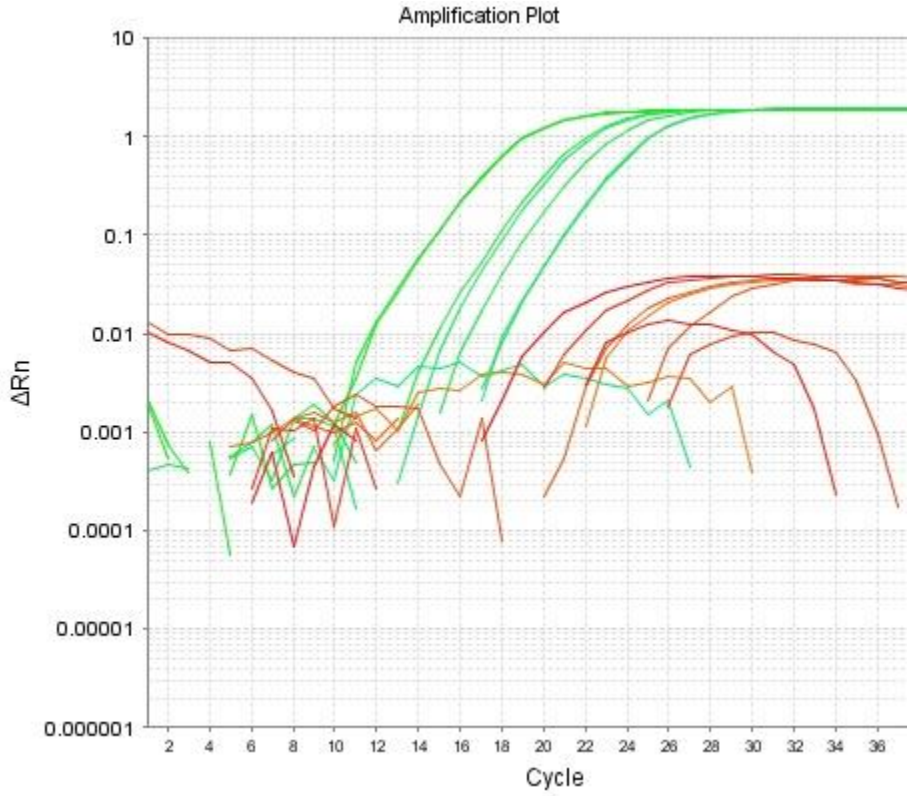
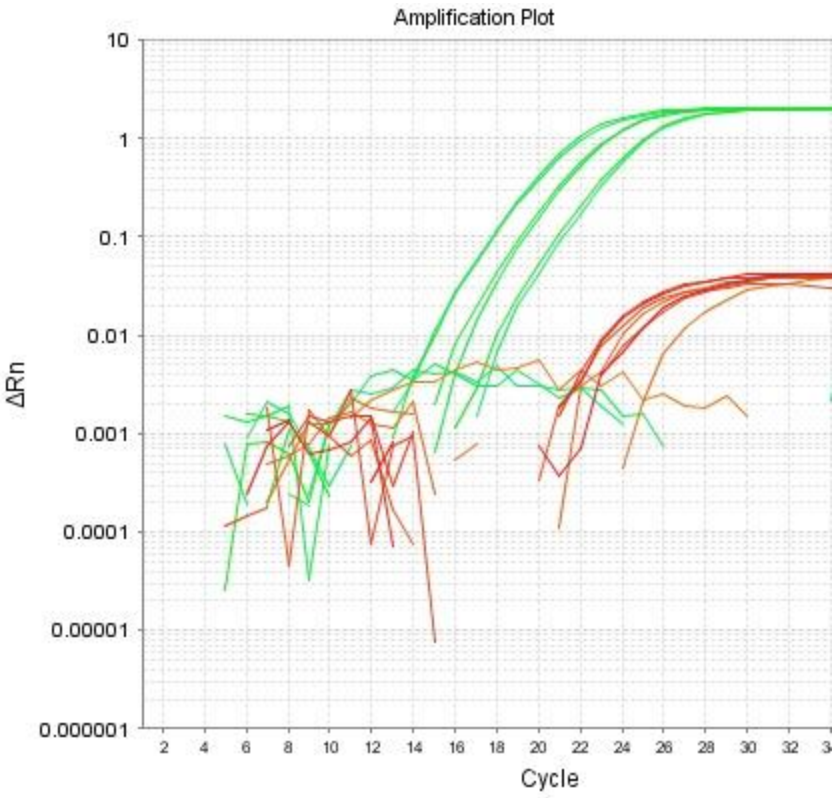
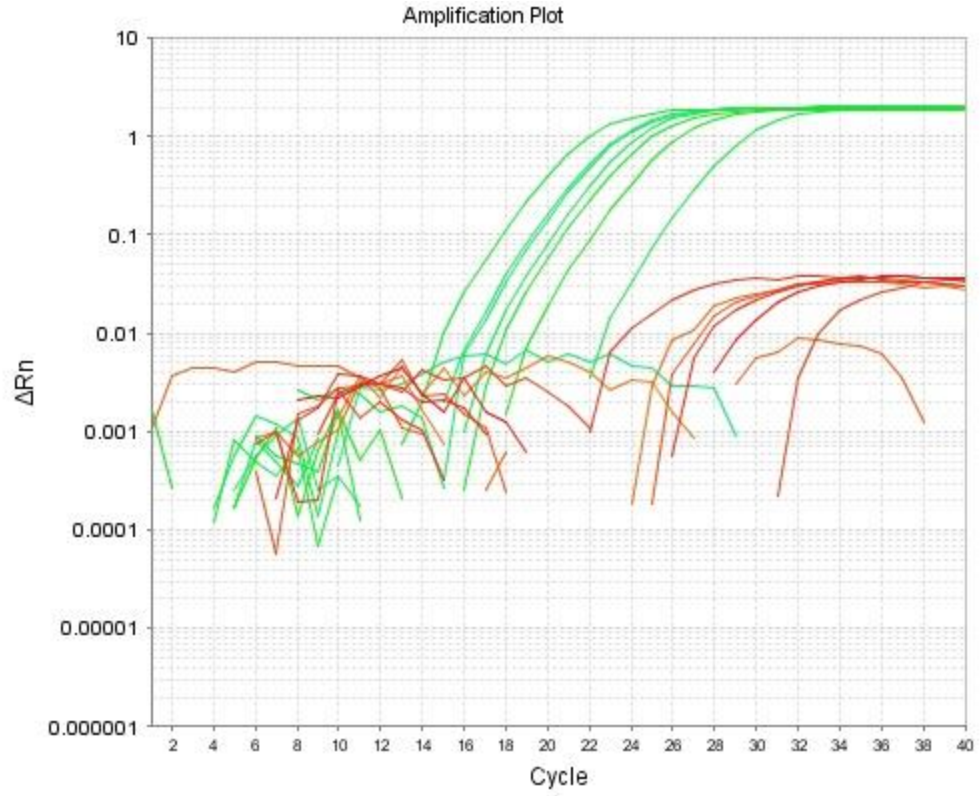
TD529	22	21.51	13	19.45
TD530	20	23.49	15	20.93
TD531	21	21.48	14	20.51
TD533	18	22.37	13	20.27



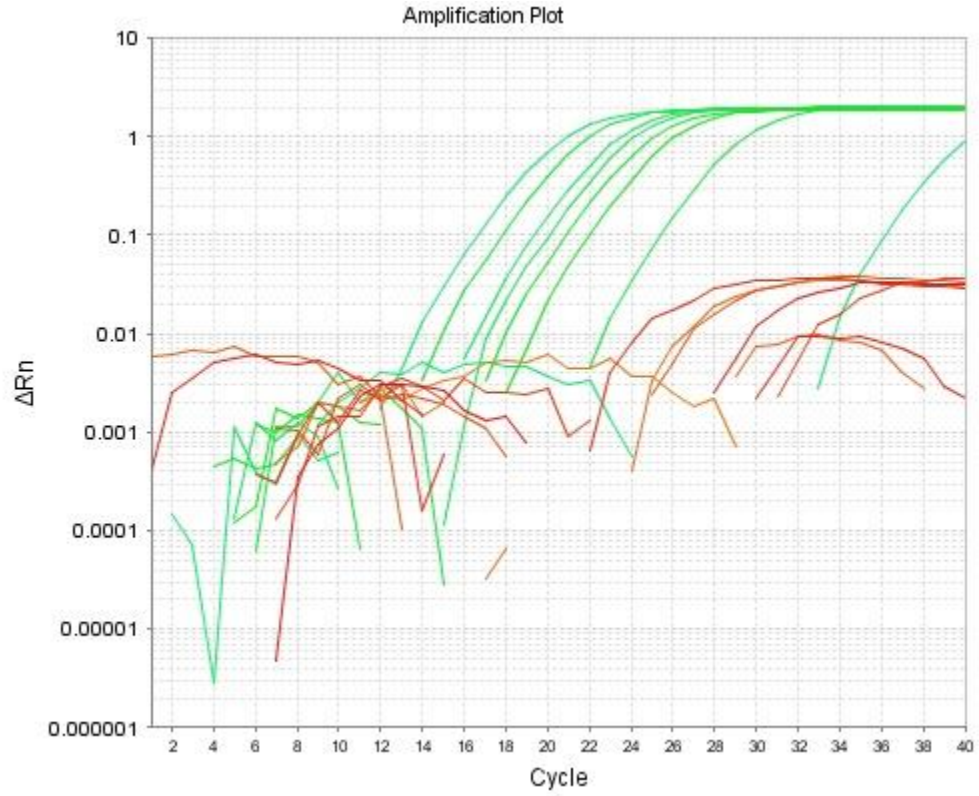
Sample name	*Pfmdr1*(Target gene)		*β-actin* (Refference gene)	
	Start cycle	C_T	Start cycle	C_T
3D7	17	18.97	10	16.84
TD523	20	20.86	13	19.81
TD525	25	25.78	17	22.93
TD526	20	22.64	13	20.08



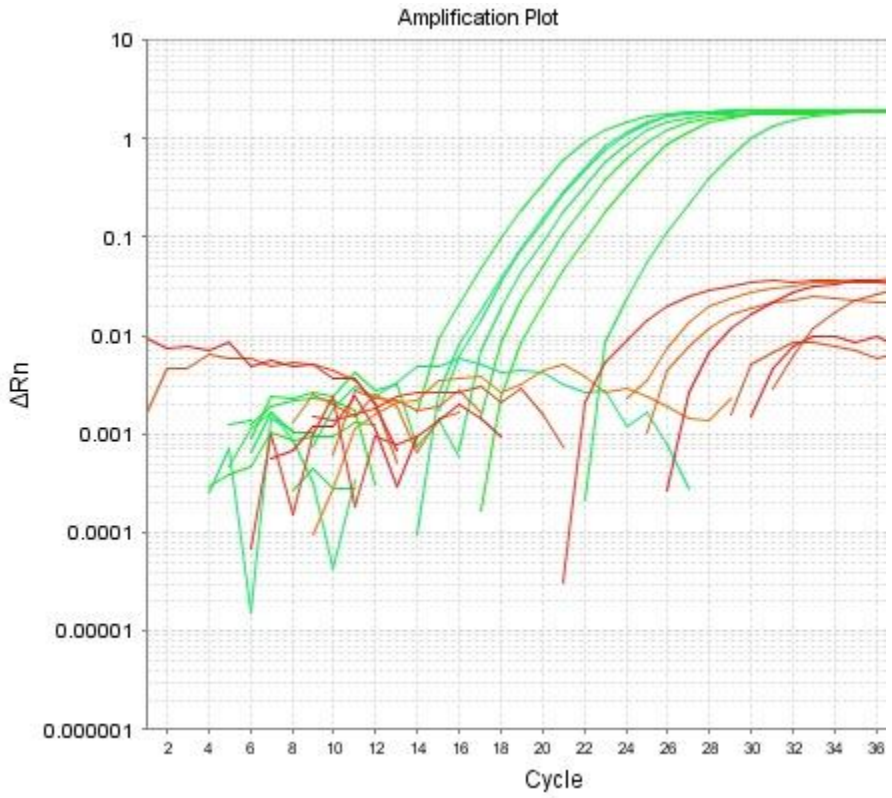
Sample name	*Pfmdr1*(Target gene)		*β-actin* (Refference gene)	
	Start cycle	C_T	Start cycle	C_T
3D7	24	25.44	17	19.71
TD433	22	23.29	16	19.45
TD502	21	21.95	13	17.68
TD529	21	21.94	13	16.34
TD530	23	23.20	15	17.94
TD533	21	22.60	13	16.36



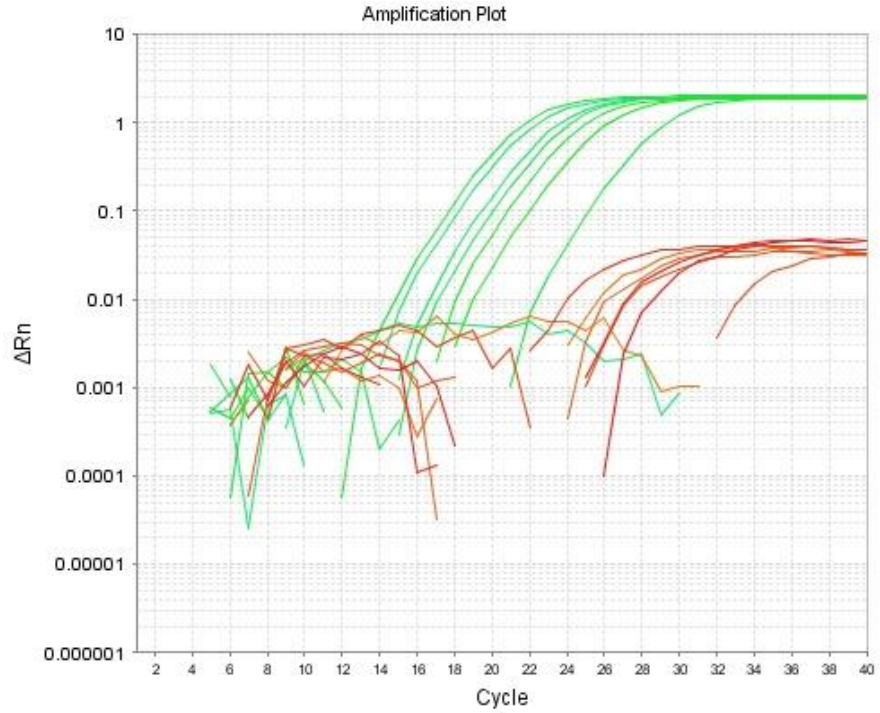
Sample name	*Pfmdr1*(Target gene)		*β-actin* (Refference gene)	
	Start cycle	C_T	Start cycle	C_T
3D7	24	24.14	15	21.43
CH13	28	27.19	18	24.25
CH15	26	25.75	16	22.96
CH18	22	21.64	13	19.92
CH25	31	31.36	22	27.54
CH28	25	25.29	16	22.36



Sample name	*Pfmdr1*(Target gene)		*β-actin* (Refference gene)	
	Start cycle	C_T	Start cycle	C_T
3D7	24	24.70	12	17.49
CH13	28	28.04	18	22.83
CH18	22	22.65	14	18.64
CH25	31	31.08	22	26.23
CH28	25	25.14	15	20.91



Sample name	*Pfmdr1*(Target gene)		*β-actin* (Refference gene)	
	Start cycle	C_T	Start cycle	C_T
3D7	24	24.61	15	21.39
CH13	26	26.91	17	24.05
CH18	21	22.22	14	19.93
CH25	31	31.12	22	27.73
CH31	25	25.57	14	21.26



Sample name	*Pfmdr1*(Target gene)		*β-actin* (Refference gene)	
	Start cycle	C_T	Start cycle	C_T
3D7	24	24.17	14	20.10
CH13	26	27.14	18	23.90
CH15	25	25.73	17	22.78
CH18	22	22.41	12	19.56
CH25	32	31.96	21	27.04
CH28	25	25.85	15	21.93
CH31	24	24.77	15	21.34

VITA

Miss Papatchaya Pa-Onta was born on 2 May1989, Thailand. Received her Bachelor's degree of Applied Thai Traditional Medicine, School of Health Science, Mae Fah Luang University. She finished her Bachelor's degree in June 2012. She continued her degree of Master Program in Public Health Sciences, College of Public Health Sciences, Chulalongkorn University.