# CHAPTER III

# MATERIAL AND METHOD

## 3.1 Materials

### 3.1.1 Isolates of *Plasmodium falciparum*

- clone T9/94RC17 (from isolate T9 collected from Tak)
- isolate MH20 – collected from Mae Hong Son (Thai-Myanmar border)
- isolate TD12 – collected from Trad (Thai-Cambodia border)
- isolate K160 – collected from Kanchanaburi (Thai-Myanmar border)

All of the parasites were obtained by *in vitro* cultivation from Division of Malaria Research Centre, Institute of Health Science, and Chulalongkorn University. Parasites were cultured by the candle-jar method (Trager & Jensen, 1976).

### 3.1.2 Enzyme and Buffers

*Taq* DNA polymerase used for DNA amplification was purchased from commercial source (Promega Biotech Co., Ltd.) and the buffers, 10 x magnesium free buffers and 25 mM $MgCl_2$ for this enzyme were provided by the manufacture.

### 3.1.3 Commercial Kits

In this study, commercial kits were used for DNA and RNA extraction (Perfect RNA Eukaryotic Mini kit and Perfect gDNA Blood Mini; Eppendorf and RT-PCR (cMaster $RT_{plus}$PCR System; Eppendorf AG).

### 3.1.4 Reagents and Chemicals

All reagents were sterilized by either autoclaving or filtration.

#### 3.1.4.1 Media for *Plasmodium falciparum* culture and transport

Media of *Plasmodium falciparum* was composed of 50% haematocrit uninfected red blood cells and complete RPMI-1640 media. All medium were filtered to sterile. Compositions of media are listed below.

| Media | Compositions |
|---|---|
| Stock RPMI 1640 Medium | per liter<br>RPMI Medium 1640 powder with L-glutamine without $NaHCO_3$ (Sigma) 10.41 g<br>Sterile $ddH_2O$ 960 ml<br>Hepes H-3375 (Sigma) 5.94 g<br>Gentamicin sulphate 1 ml |
| Incomplete RPMI 1640 Medium | Stock RPMI 1640 Medium 100 ml<br>5% $NaHCO_3$ 4.2 ml |
| Complete RPMI 1640 Medium | Stock RPMI 1640 Medium 100 ml<br>5% $NaHCO_3$ 4.2 ml<br>pool serum 10.4 ml |
| Transporting medium | Stock RPMI 1640 Medium 100 ml<br>5% $NaHCO_3$ 4 ml<br>Heparin added to 10 unit/ml<br>Make fresh and store at 4°C |

### 3.1.4.2 Antimalarial drugs for drug susceptibility test

In this study, four antimalarial drugs: pyrimethamine, chloroquine, mefloquine, and quinine were used for drug susceptibility test. Compositions of drugs are listed below.

| Drugs | Compositions |
|---|---|
| Stock $10^{-2}$ M pyrimethamine(Sigma) | per 100 ml<br>Pyrimethamine powder 0.2487 g<br>Dimethysulfoxide 100 ml |
| Stock $10^{-4}$ M chloroquine (Sigma) | per 100 ml<br>Chloroquine diphosphate salt 0.0052 g<br>Sterile $ddH_2O$ 100 ml |
| Stock $10^{-3}$ M mefloquine (WHO) | stock solution $10^{-3}$ M |
| Stock $10^{-3}$ M quinine (WHO) | stock solution $10^{-3}$ M |

### 3.1.4.3 Genomic DNA preparation reagents

| Reagents | Compositions |
|---|---|
| Lysis buffer | 40 mM Tris-Cl, pH 8.0<br>80 mM EDTA, pH 8.0<br>2% SDS |
| Proteinase K<br>(Roche) | 20 mg/ml Proteinase K<br>(added into lysis buffer before use) |
| PBS | 137 mM NaCl<br>2.7 mM KCl<br>10 mM $Na_2HPO_4$<br>2 mM $KH_2PO_4$<br>adjusted pH to 7.4 with HCl |
| TE buffer | 100 mM Tris-Cl, pH 8.0<br>10 mM EDTA, pH 8.0 |
| Phenol: Chloroform | 50% phenol, saturated solution(AMRESCO®)<br>48% chloroform<br>2% isoamyl alcohol |

### 3.1.4.4 DNA amplification stock reagents

| Reagents | Concentration |
|---|---|
| dNTPs<br>(Promega) | 5 mM dATP<br>5 mM dTTP<br>5 mM dGTP<br>5 mM dCTP |
| Primers (Proligo Primer & Probe)<br>***rap-1*** (Pongchai, 1993)<br>• FRAP-1<br>• RAP-1R<br>***ssrRNA*** (Snounou *et al.*, 1993)<br>• rPLU5<br>• rPLU6 | <br><br>0.2 µM<br>0.2 µM<br><br>0.2 µM<br>0.2 µM |

### 3.1.4.5 Electrophoresis reagents

| Reagents | Compositions |
|---|---|
| 6X Loading buffer | 0.25% Bromophenol blue<br>0.25% Xylene cyanol FF<br>40% sucrose |
| 1kb Ladder marker(New England Biolabs) | 0.1 µg/µl 1 kb ladder<br>1X loading buffer |
| 5X TBE buffer | per liter<br>Tris base 54 g<br>Boric acid 27.5 g<br>0.5 M EDTA pH 8.0 20 µl<br>adjusted volume to 1 L with $ddH_2O$<br>filtered with filter paper |
| Staining Solution | 0.2 µg/ml ethidium bromide |

## 3.2. Methods

### 3.2.1 *Plasmodium falciparum* Cultural Technique

#### 3.2.1.1 Collection of *Plasmodium falciparum* (Thaithong and Beale, 1992)

50-100 µl of infected blood was collected by finger-prick technique and immediately put into a sterile 1.5 microcentifuge tube containing 0.5 ml transport medium then gently mixed by inverting. The samples should be taken to cultural laboratory on the day of collection and centrifuge at 1500 rpm for 10 minutes. The plasma and buffy coat were discarded. 0.4 ml of complete RPMI was added and gently mixed. 100 µl of the suspension was pipetted into each well of a sterile 96 micro well plate. Each isolate was cultured in at least 4 wells (Figure 3.1).

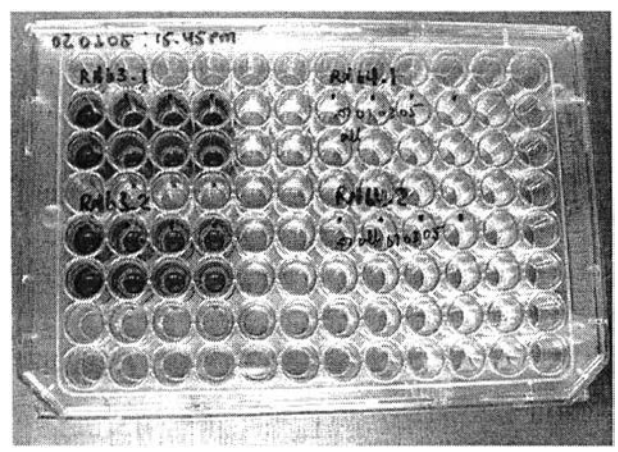

**Figure 3.1** *P. falciparum* isolates collected from patients were cultured in 96 micro well plate

#### 3.2.1.2 Continuous culture of *Plasmodium falciparum*

*Plasmodium falciparum* isolates were cultured by the candle-jar method (Trager & Jensen, 1976). Infected red blood cells were added into culture dishes containing 5- 6 ml complete RPMI media and uninfected red blood cell. Parasite cultures were placed into a dessicator with lighted candle (Figure. 3.2). Once the dessicator lid was closed, the lighted candle went off and 5-8% $CO_2$ was maintained inside. The dessicator was incubated at 37°c. The complete medium was changed every 24 hours and fresh uninfected red blood cells were added every 4 day. To observed quantity and morphology of parasite, thin blood films were prepared from the cultures and stained with Giemsa. Percent parasitaemia was examined by microscopy at 1000 times magnification.

The number of red blood cells which consistently distributed (around 250-300 red blood cells per microscopic field) were repeatedly counted for 3 times. The red blood cell numbers from 3 microscopic fields were used to calculate the number of microscopic fields which contain 10,000 red blood cells as followed (1) and (2).

$$\frac{A+B+C}{3} = X \quad \text{..... (1)}$$

$$\frac{10,000}{X} = Z \quad \text{..... (2)}$$

A, B and C = red blood cell numbers from 3 microscopic field

X = average red blood cell number per microscopic field

Z = numbers of microscopic fields which contain red blood cell for 10,000 cells

The numbers of infected red blood cells in Z microscopic fields were determined. The parasitemia was calculated in percentage as followed (3)

$$\frac{Y \times 100}{10,000} = \text{parasitemia} \quad \text{..... (3)}$$

Y = the number of parasites in 10,000 red blood cells

**Figure 3.2** Dessicator was used for growing cultures of malaria parasite

*Serial dilutions of P. falciparum*

Parasites were diluted with uninfected red blood cells at different percent parasitaemia: 10%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, and 0.0005%. Serial dilutions of parasite were used for primers sensitivity test. Actual percent parasitaemia of each concentration was measured by counting from thin blood films stained with Giemsa.

**3.2.1.3 *in vitro* Drug Susceptibility** (Thaithong et al., 1983)

Three replicates of all experiment were performed. 10 µl of parasite was dispensed into TC 96 micro-well NUNCLON® containing complete RPMI media with 10% serum (Figure 3.3). 100 µl of the antimalarial drug was added into each well at different concentrations (Table 3.1).

| **Drugs** | **Concentrations(M)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Pyrimethamine | 0 | $5x10^{-9}$ | $10^{-8}$ | $5x10^{-8}$ | $10^{-7}$ | $5x10^{-7}$ | $10^{-6}$ | $5x10^{-6}$ |
| Chloroquine | 0 | $10^{-8}$ | $5x10^{-8}$ | $10^{-7}$ | $2x10^{-7}$ | $5x10^{-7}$ | $10^{-6}$ | |
| Mefloquine | 0 | $10^{-8}$ | $3x10^{-8}$ | $5x10^{-8}$ | $7x10^{-8}$ | $10^{-7}$ | $2x10^{-7}$ | $5x10^{-7}$ |
| Quinine | 0 | $10^{-8}$ | $5x10^{-8}$ | $10^{-7}$ | $3x10^{-7}$ | $5x10^{-7}$ | $10^{-6}$ | |

**Table 3.1** Different drug concentrations were used during drug susceptibility test.

The starting cultures had an initial parasitaemia between 0.2 and 0.5 with 5% haematocrit. During the test, the culture medium (with or without drug) was changed every 24 hours. After 72 hours thin blood films were prepared from each well and stained with Giemsa. The minimum drug concentration which the malaria parasite cannot be found in the blood films was reported as the MIC value.

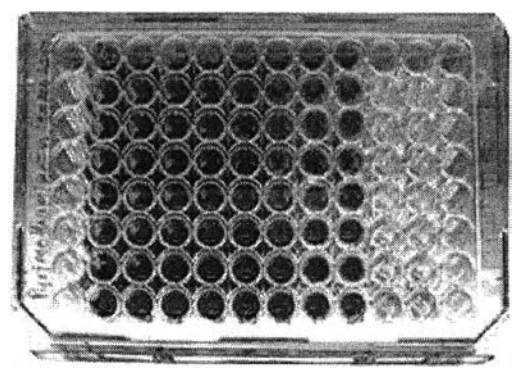

**Figure 3.3** A TC 96 micro-well was used for testing of drug susceptibility.

### 3.2.2 Molecular Technique

#### 3.2.2.1 DNA preparation

In this study, three DNA extraction methods: rapid boiling method, phenol chloroform extraction, and Perfect gDNA blood mini were used to extract *P. falciparum* DNA.

**3.2.2.1.1 Rapid Boiling Method** (Foley *et al,* 1992)

Parasites were washed twice with 200 µl ice-cold PBS and 200 µl ice-cold 5 mM sodium phosphate buffer pH 8.0 ($Na_2HPO_4.12H_2O$, adjusted pH with 1M HCl) was added. The solution was vortexed and centrifuged for 2 minutes at 10,000 rpm. The supernatant was discarded and the process was repeated until the supernatant was clear. The pellet was dissolved with 10 µl of sterile water, and boiled for 10 minutes. Finally, the samples were centrifuged for 10 minutes at 13,000 rpm and the supernatant was removed to a fresh tube. 4 µl of the solution were used for PCR amplification. The DNA samples can be stored at -20°C until needed.

**3.2.2.1.2 Phenol chloroform extraction** (Snounou, 1994)

Parasite samples were washed twice by 200 µl ice-cold PBS. 500 µl of 0.05% saponin in PBS was mixed to each sample. The mixture was placed at room temperature for 5 minutes or until lysis was observed. The samples were centrifuged at 10,000 rpm for 5 minutes and the supernatant was removed. Pellet was washed 2-3 times with ice-cold PBS and 12.5 µl of lysis buffer (40 mM Tris-HCl, pH 8.0; 80 mM EDTA, pH 8.0; 2% SDS) with 2 mg/ml proteinase K was added. To dilute the mixture, 50 µl of sterile water was added and gently mixed. The mixture was incubated overnight at 37°C. At the end of the incubation period, the mixture was adjusted to 300 µl by adding sterile $ddH_2O$ and mixed. DNA was extracted with an equal volume of saturated phenol (Tris-equilibrated phenol pH 8.0) and, again, with the mixed solution of phenol: chloroform (1:1). To precipitate the DNA 1: 10 volume of 3 M sodium acetate (pH 5.2) and 2 volume of cold absolute ethanol were added to the extract solution. After incubation for overnight at -20°C. DNA was pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C and the supernatant was carefully removed. The pellet was washed with 1 ml cold 70% ethanol and dissolved in 20 µl of TE buffer (100 mM Tris-HCl pH 8.0; 10 mM EDTA pH 8.0). 1 µl of dissolved DNA was used for PCR amplification.

**3.2.2.1.3 Perfect gDNA Blood mini** (Eppendorf, Germany)

The kit was used according to the supplier's instruction. Briefly, 10 µl of parasite samples were added with 20 µl reconstituted proteinase K (20 mg/ml) and 350 µl solution G1 (provided by the kit) and mixed by vortexing for 5 seconds. The sample was incubated in a heat block at 70 °C for 10 minutes. To pellet the cell debris, centrifugation was performed for 3 minutes at 13,000 rpm. The supernatant was transferred into a fresh tube and mixed with 200 µl of solution G2 (provided by the kit) by vortexing for 5 seconds. The sample was transferred to the spin column assembly. After incubating for 1 minute at room temperature, the column was centrifuged for 2 minutes at 13,000 rpm. The DNA bound column was washed for 2 times with 600 µl and 400 µl of diluted wash buffer (provided by the kit) by centrifugation for 3 minute at 13,000 rpm. After incubating for 3 minute at 70°C, DNA was eluted by 20 µl of elution buffer (provided by the kit) into a fresh tube by centrifugation for 1 minute at 13,000 rpm. 2 µl of DNA were used for PCR amplification.

**3.2.2.2 RNA preparation** (Sambrook *et al.*, 1989; Eppendorf, Germany)

In this study, Perfect RNA Eukaryotic Mini kit was used for RNA preparation. The method is recommended by the supplier. To break cell, 350 µl of Lysis solution (provided by the kit) was added to 10 µl of parasite samples and mixed by vigorous vortexing and pipetting several times. The homogenized sample was transferred to a 1.5 ml tube, and centrifuged for 5 minutes at 13,000 rpm. The supernatant was transferred to a fresh 1.5 ml tube and mixed with 350 µl of 70% ethanol with gentle inversion. Perfect RNA Binding Matrix Solution was mixed thoroughly in order to completely resuspense the matrix. 200 µl of Perfect RNA Binding Matrix Solution (provided by the kit) was mixed to the lysate/ethanol mixture and completely mixed by gently inversion. Lysate/Binding Matrix mixture was pipetted into the Spin Column evenly and centrifuged for 60 seconds at 13,000 rpm then filtrate was discarded. 700 µl of Wash Solution I (provided by the kit) was added to the column and was removed by centrifugation for 30 seconds at 13,000 rpm. Filtrate was discarded and the column was centrifuged for 30 seconds at 13,000 rpm to completely remove Wash Solution I. Washed Spin Column was transferred to a fresh collection tube. 500 µl of diluted Wash Solution II (provided by the kit) was added and the column was centrifuged for 15 seconds at 13,000 rpm, the filtrate was discarded. To completely remove Wash Solution II, the column was centrifuged for, another 2 minutes, at 13,000 rpm. Spin Column was

transferred to a fresh collection tube and 20 µl of "Molecular Biology Grade Water" (supplied in the kit) was added to elute RNA bounded to the Binding Matrix. To moisten the entire dried binding matrix, Spin Column assembly was vortexed for 1 second and was incubated at 50°C for 5 minute. After the incubation, the spin column was vortexed for 5 seconds and centrifuged for 2 minutes at 13,000 rpm to collect RNA. The obtained RNA was stored at -20°C. 1 µl of RNA was used for RT-PCR amplification.

**3.2.2.3 PCR amplification** (Mullis, 1990)

PCR amplification was performed to detect DNA of treated and non-treated parasite. During both amplification reactions, the concentrations of each component were:

- 1x *Taq* DNA polymerase buffer
- 2.5 mM $MgCl_2$
- 200 µM dNTPs
- 0.2 µM forward primer (both *ssrRNA* and *rap-1* )
- 0.2 µM reverse primer (both *ssrRNA* and *rap-1* )
- 0.05 U/µl *Taq* DNA polymerase

PCR amplification of the *ssrRNA* was performed as described previously (Snounou *et al.*, 1993) and the condition of *rap-1* amplification was as follows; pre-denaturation at 95°C for 3 minutes, Step 1: denaturation at 95°C for 45 seconds, Step 2: annealing at 50°C for 1 minute, Step 3: extension at 72°C for 2 minutes 30 seconds, step 1-3 were repeated for 30 times. At the end of the amplification cycle, the temperature was decreased to 0°C. Both primer sequences are outlined in the table below. Five microlitres of PCR product were analyzed with agarose gel electrophoresis.

| Primers name | Nucleotide sequence (*5'* → *3'*) | Annealing temp (°C) | Amplicon length (bp) |
|---|---|---|---|
| rPLU5<br>rPLU6 | CCTGTTGTTGCCTTAAACTTC<br>TTAAAATTGTTGCAGTTAAAACG | 58 | 1200 |
| FRAP-1<br>RAP-1R | ATGAGTTTCTTCTATTTGGGTAGCTTAGT<br>TTAATCTAATCTCTTGAAGGCAAAGT | 50 | 2318 |

**3.2.2.4 Reverse Transcriptase PCR amplification** (Sambrook *et al.*, 1989; Eppendorf, Germany)

RT-PCR amplification of both genes was performed to detect the presence of the parasitic RNA. Both conditions of *rap-1* and *ssrRNA* amplification were set up as the supplier instruction. The concentrations of solutions used for amplification reaction were:

- 2.5 mM $RT_{plus}$ PCR buffer with $Mg^{2+}$
- 200 µM dNTPs
- 0.15 U/µl cMaster RT enzyme
- 0.05 U/µl cMaster PCR enzyme mix
- 0.01 U/µl Prime RNase inhibitor
- 0.2 µM forward primer (both *ssrRNA* and *rap-1* )
- 0.2 µM reverse primer (both *ssrRNA* and *rap-1* )

Both reverse transcriptase amplification of the *ssrRNA* and the *rap-1* were; Step 1: reverse transcription at 50°C for 30 minutes 1 cycle, Step 2: initial denaturation at 94°C for 2 minutes 1 cycle, Step 3: template denaturation at 94°C for 15 seconds, Step 4: annealing at 50°C for 1 minute, Step 5: extension at 68°C for 2 minutes 30 seconds, step 3-5 were repeated for 30 times. At the end of the amplification cycle, the temperature was reduced to 0°C. Five microlitres of RT-PCR product were analyzed with agarose gel electrophoresis.

**3.2.2.5 Direct PCR and RT-PCR technique** (adapted from B. Mercier *et al,* 1990 and J. McCusker *et al,* 1992).

PCR and RT-PCR amplification was performed directly from 2 µl of whole blood in a 20 µl reaction. The fresh blood had been heated to 95°C for 10 minutes to lyse the cells before the *rap-1* gene was amplified. The PCR and RT-PCR amplifications of *rap-1* gene were performed in the same condition as purified DNA and RNA.

**3.2.2.6 Agarose Gel Electrophoresis** (Sambrook *et al.*, 1989)

Five microlitres of 1kb Ladder marker and five microlitres of amplification products were mixed with 1 µl of 6X Loading buffer and loaded into each well of 0.8% agarose gel. The gel was run at 110 volts for 22 minutes. The agarose gel was stained with 0.2 µg/ml ethidium bromide for 5 minutes and destained with distilled water for 2 minutes. The stained agarose gel was photographed with AutoChemi$^{TM}$System by using Labwork 4.0$^{TM}$ software (UVP Biomaging Systems).