# CHAPTER III

# MATERIALS AND METHODS

## Materials

### 1. Tissue of New Zealand White (NZW) rabbits

Abdominal aorta and liver samples of rabbits were provided by Associate Professor Dr. Laddawal Phivthong-ngam, Department of Pharmacology, Faculty of Medicine, Srinakharinwirot University. The information of rabbits care and treatment are shown in appendix C

### 2. Instruments

The following instruments were used in the experiment.

1) Analytical balance [Precisa, Switzerland]
2) Autopipettes 10, 20, 200 and 1000 μL [Gilson, France]
3) Chemi genius bioimaging system [Syngene, UK]
4) iQ™5 Multicolor Real-Time PCR Detection System [Bio-Rad, USA]
5) Micro centrifuge (mini) GMC-260 [Labtech, Korea]
6) Mikro 22R centrifuge [Hettich, Germany]
7) Mini-sub® cell GT system (casting gates, gel caster, UVTP tray 7x10 cm, combs 8-well, combs 15-well) [Bio-Rad, USA]
8) Orion Model 420A pH meter [Therma, Canada]
9) PTC-200 DNA engine cycler [Bio-Rad, USA]
10) UV-2450 (UV-spectrophotometer) [Shimadzu, Japan]
11) Vortex mixer [Clay Adams, USA]

### 3. Chemicals

The following chemicals were used in the experiment.

**Chemicals used in tissue preparation**

- Di-potassium hydrogen phosphate [Fluka, Switzerland]
- Di-sodium hydrogen phosphate [Merck, Germany]

- Potassium chloride [Merck, Germany]
- Potassium dihydrogen phosphate [Fluka, Switzerland]
- Sodium chloride [Merck, Germany]

**Chemicals used in RNA extraction**

- Chloroform [Merck, Germany]
- Diethyl pyrocarbonate (DEPC) [Sigma, USA]
- 99.5% ethanol [Sigma, USA]
- Isopropanol [Merck, Germany]
- TRIzol® reagent [Invitrogen, USA]

**Chemicals used in reverse transcription**

- iScript™ Select cDNA Synthsis Kit [Bio-Rad, USA] contained
  - GSP enhancer solution
  - iScript reverse transcriptase
  - 5x iScript select reaction mix
  - nuclease-free water
  - oligo$(dT)_{20}$ primer mix
  - random primer mix

**Chemicals used in polymerase chain reaction (PCR)**

- 10 mM dNTP mix [Fermentas, Canada]
- 100 pmole/μL forward/reverse primer [Eurogentec Ait, Singapore]
- 25 mM magnesium chloride [Fermentas, Canada]
- 10x Taq buffer with ammonium sulfate [Fermentas, Canada]
- Taq DNA polymerase [Fermentas, Canada]

**Chemicals used in DNA electrophoresis**

- agarose [Bioexpress, UT]
- boric acid [Sigma, USA]
- 0.25% bromophenol blue [Sigma, USA]
- ethylenediaminetetraacetic acid (EDTA) [Merck, Germany]
- GeneRuler™ 100bp DNA Ladder [Fermentas, Canada]

- 50% glycerol [Sigma, USA]
- Tris(hydroxymethyl)aminomethane hydrochloride (Tris base) [Sigma, USA]
- 0.25% xylene cyanol [Sigma, USA]

**Chemicals used in real-time polymerase chain reaction (real-time PCR)**

- 10 mM dNTP mix [Fermentas, Canada]
- 100 pmole/μL forward/reverse primer [Eurogentec Ait, Singapore]
- 25 mM magnesium chloride [Fermentas, Canada]
- 10x Taq buffer [Fermentas, Canada]
- Taq DNA polymerase [Fermentas, Canada]
- SYBR green I sDNA-nucleic acids gel stain dye [Bio Basic Inc., Canada]

**4. Water**

Autoclaved 18.2 Ω deionized water was used for preparation of reagents in all experiments.

**Methods**

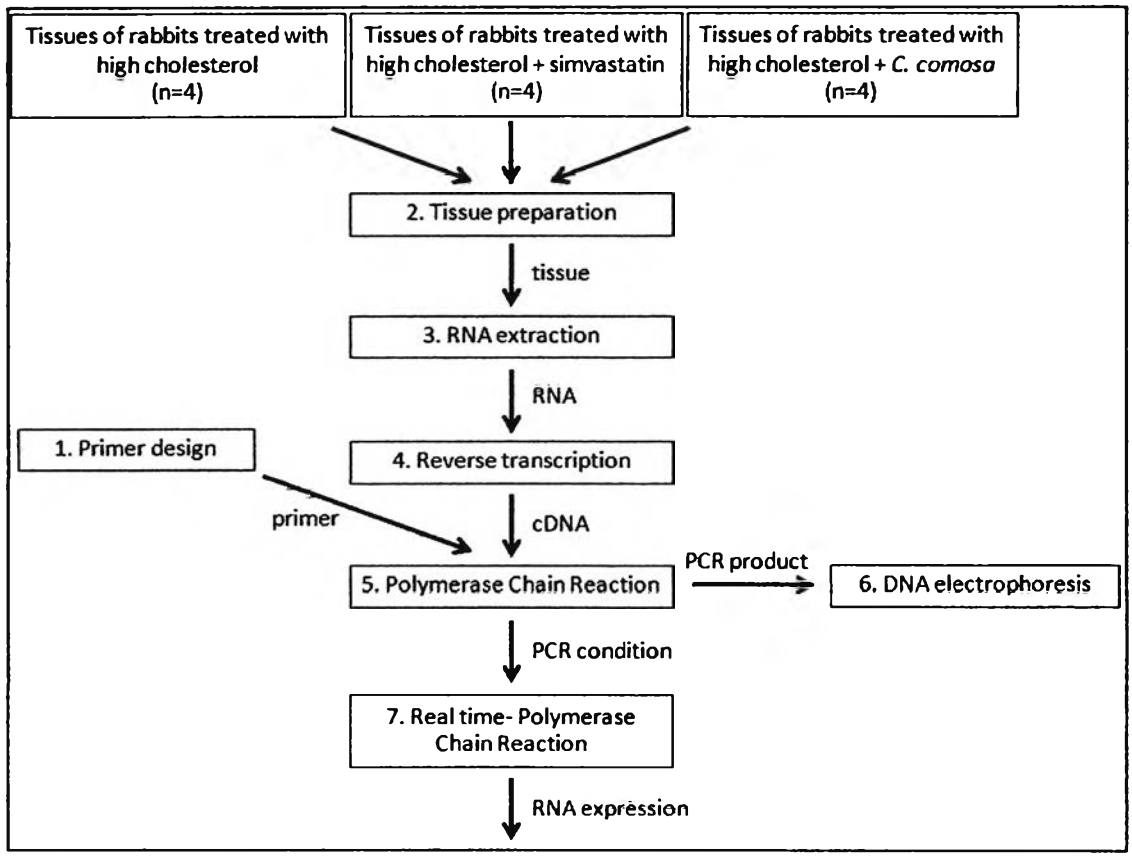


**Figure 9** The flow chart of experimental study.

**1. Primer design**

In order to perform PCR, couples of different sequence that anneal to complementary strands of the template DNA are needed. The primers were designed according to the criteria as following (McPherson and Møller, 2006):

- A primer should be 16 – 30 nucleotides long, which provides good specificity for a unique target sequence.
- A primer should contain approximately equal numbers of each nucleotide.
- A primer should be avoided repetitive sequences or regions containing stretches of the same nucleotide as this can lead to slipping of the primer on the template.
- A primer should be avoided runs of three or more G or Cs at the 3'-end as this can lead to mispriming at GC-rich regions.
- A primer should not be able to form secondary structures due to internal complementarity.

- A primer should not contain sequences at the 3'-ends that will allow base pairing with itself or any other primer that it may be coupled with in a PCR; otherwise this can lead to the formation of primer-dimers.

Procedure

mRNA sequences retrieval: The data of cytokine mRNA sequences were retrieved from Pubmed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed) by indicating cytokine and species of animal and transferred the data in FASTA format. The mismatch of amplification between primer and contaminated DNA was avoided by tracking the location of exon-exon junction.

Application of Primer design's program: The programs used in this study included PerlPrimer version 1.1.18 and AmplifX version 1.5.4. PerlPrimer were input with substantial parameters such as melting temperature (Tm), Tm difference between forward and reverse primer, PCR product size, and location of PCR product on cDNA template. After program calculation, each primer parameter such as percentage of GC (GC%), dG°37 were checked. Thereafter, primer sequence, position on cDNA template, 3' end stability, polyX, self dimer and self end dimer were checked by using AmplifX program. To ensure of specification of primer design, primer sequence was blast back to Basic Local Alignment Search Tool (BLAST) N and NCBI/Primer-BLAST by using NCBI/BLAST.

**2. Tissue preparation**

Reagent

One litre of Phosphate Buffer Saline (PBS), pH 7.4 containing 0.12 g of $KH_2PO_4$, 0.72 g of $Na_2HPO_4$, 0.1 g of KCl and 4.0 g of NaCl. The solution was adjusted to pH 7.4 with NaOH or HCl.

Procedures

Each tissue of abdominal aorta and liver was weighed, cut into pieces and then rinsed with ice cold PBS buffer pH 7.4 for 1 time before transferred to 1.5-ml microtube for further RNA extraction process.

**3. RNA extraction by TRIzol® reagent**

TRIzol® reagent was used for the isolation of total RNA from cells and tissues. The reagent was a mono-phasic solution of phenol and guanidine isothiocyanate. During sample homogenization or lysis, TRIzol® reagent maintained the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separated the solution into an aqueous phase and an organic phase. RNA remained exclusively in the aqueous phase. After transferring of the aqueous phase, the RNA was recovered by precipitation with isopropyl alcohol. Total RNA isolated by TRIzol® reagent was free of protein and DNA contamination.

Reagent

DEPC-treated water was prepared by dissolve 1 ml of DEPC in 1,000 ml of autoclaved 18.2 Ω deionized water. The solution was stirred for 18 hours for completely dissolving.

Procedures

RNA extraction was performed by using TRIzol® reagent according to the manufacturer's protocol with minor modification. A hundred-milligram of abdominal aorta or liver tissues were homogenized in 1.5 ml of TRIzol® Reagent using plastic pestle in 1.5-ml microtube. In separation phase, tissue was homogenized for 5 minutes on ice and then added with 0.3 ml of chloroform, shaked for 30 second by end-to-end and placed on ice for 15 minutes. After Centrifugation the sample at 12,000 x*g* for 15 minutes at 4°C, the mixture was separated in three parts- a lower red, phenol-chloroform phase; an interphase; and a colorless upper aqueous phase. The aqueous phase which contained most of the RNA was transferred to a new 1.5-ml microtube. In precipitation phase, RNA was precipitated by adding 0.5 ml of cold isopropyl alcohol and the tube was centrifuged at 12,000 x*g* for 20 minutes at 4 °C. The supernatant was removed by decanting and the RNA pellet was washed by 1 ml of 75% ethanol in DEPC-treated water for at least two times. At the end of the procedure, RNA pellet was briefly dried on air-dry at room temperature for 10 minutes. RNA pellet was then redissolved with DEPC-treated water, and the RNA concentration was measured using spectrophotometer at the absorbance wavelength of

260 and 280 nm. RNA was diluted to appropriate concentration, aliquoted and stored at -80 °C until used.

**4. Reverse transcription**

Reverse transcription or first strand DNA synthesis is the first step of reverse transcription polymerase chain reaction (RT-PCR). To perform PCR, this step is very important since DNA polymerase can act only on DNA templates. Reverse transcriptase enzyme, originally found in retroviruses, was added to the mRNA so as to generate complementary DNA (cDNA). Several further steps were required to generate a double-stranded cDNA copy of the original mRNA (Sambrook and Russel, 2001).

Reagent

iScript™ Select cDNA Synthsis Kit contained individual tube of iScript reverse transcriptase, 5x iScript select reaction mix, random primer mix, and nuclease-free water.

Procedures

Reverse transcription reactions were performed using iScript™ Select cDNA Synthesis Kit. One microlitre of 1 μg/μL of RNA sample, 2 μL of random primer mixtures, and 12 μL of nuclease-free water were mixed and incubated at 65 °C for 5 minutes. Afterward, the reaction was snap-chilled on ice for 60 seconds. Then, 4 μL of 5x iScript select reaction mixtures and 1 μL of iScript reverse transcriptase were added to the reaction. Reverse transcription was performed as following protocol: 25 °C for 5 minutes, 42 °C for 30 minutes, and 85 °C for 5 minutes. The cDNA product was stored at -20 °C until used.

**5. Polymerase chain reaction (PCR)**

PCR is a mean of amplifying DNA sequences. Starting with small amounts of any particular DNA sequence, the PCR can be used to generate microgram quantities of DNA. PCR allows the rapid generation of large amounts of specific DNA sequences by repeated cycles of strand separation and replication. Cycling through the PCR encompasses three main steps. The first step, denaturation step, is to separate the

strands of the template DNA by heating the template DNA up to 90 °C. In the second step called annealing step, the temperature is maintained between 50 and 60 °C, allowing the primers to anneal to the complementary sequences on the template strands. In the last step, extension step, the temperature is maintained at 72 °C to allow the thermostable polymerase to elongate new DNA strands starting from the primers. All steps are repeated around 30 – 50 cycles to amplify large amount of specific PCR product (Clark, 2005).

Reagents

1. 10 mM dNTPs containing 10 mM of dATP, 10 mM of dCTP, 10 mM of dGTP, and 10 mM dTTP
2. Taq DNA Polymerase (recombinant) containing 10x Taq buffer with $(NH_4)_2SO_4$ , 25 mM $MgCl_2$, 5 U/μL Taq DNA polymerase

Procedures

PCR was performed by using Taq DNA Polymerase (recombinant) according to the manufacturer's protocol (Fermentas). The components in PCR reaction contained 0.2 mM dNTPs, 1x reaction buffer, 2.08 mM $MgCl_2$, 0.05 U/μL Taq DNA polymerase, cDNA 1 μL/reaction, and each of 1.0 pmol/μL forward primer and reverse primer. The reaction mixture was mixed gently before placing the reaction in PCR thermocycler. The optimized PCR condition was carried out as showing in Table 2. The PCR product was detected by performing agarose gel electrophoresis.

**Table 2** PCR thermocycling conditions.

| Step | Temperature | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95 °C | 3 minutes | 1 cycle |
| Denaturation | 94 °C | 20 seconds | 35 cycles |
| Annealing | 50-65 °C | 15 seconds | |
| Extension | 95 °C | 3 minutes | |
| Final extension | 72 °C | 15 seconds | 1 cycle |

**6. DNA electrophoresis**

Reagents

1. 6x gel-loading buffers containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in $H_2O$
2. 5x Tris-Borate-EDTA (TBE) buffer (pH 8.3) containing 54 g of Tris base, 27.5 g of boric acid, and 20 ml of 0.5 M EDTA (pH 8.0) in 1 litre of solution.

Procedures

Agarose gel of 1.2% was prepared by heating 0.6 g of agarose suspension in 50 ml of 0.5x TBE buffer (pH 8.3) until the solution was clear. While the temperature of the agarose gel solution was approximately 50 – 60 °C, the agarose gel was transferred in a gel caster set and air bubbles must be removed. The gel was allowed to solidify at room temperature for 30 – 45 minutes. The gel was transferred to electrophoresis tank with 0.5x TBE buffer (pH 8.3) covered the gel to a depth of 1 mm. Nine microlitres of PCR product sample was mixed with 1 μL of 6x gel-loading buffer and the mixture was loaded into the slots of the submerged gel using disposable micropipette. DNA ladder of 100bp was used as DNA marker to indicate size of PCR product. The lid of the gel tank was closed and the electrophoresis was started with a voltage of 100 V for 1 hour. After electrophoresis was completed, the gel was submerged in 200 μg/ml ethidium bromide solution tank for 20 minutes and PCR product was detected under UV by using Chemi genius bioimaging system.

**7. Determination of cytokines expression by real-time polymerase chain reaction (real-time PCR)**

Real-time PCR was performed by using Taq DNA Polymerase (recombinant) according to the manufacturer's protocol with minor modification.

Reagents

1. 10 mM dNTP containing 10 mM of dATP, 10 mM of dCTP, 10 mM of dGTP, and 10 mM dTTP
2. Taq DNA Polymerase (recombinant) contains 10x Taq buffer, 25 mM $MgCl_2$, Taq DNA polymerase

3. SYBR green I sDNA-nucleic acids gel stain dye

Procedures

Real-time PCR reaction was performed in the same manner as PCR reaction with an addition of 1.25 µL of 1:10,000-diluted SYBR green I dye. The reaction mixture was mixed gently before placing the reaction in PCR thermocycler. Real-time PCR cycle steps and melt curve analysis were carried out by the multiple steps as shown in Table 3. PCR product was kept at -20 °C until checking for the PCR product size by agarose gel electrophoresis.

**Table 3** Real-time PCR thermo cycling conditions.

| Step | Temperature | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95.0 °C | 3 minutes | 1 cycle |
| Denaturing | 94.0 °C | 20 seconds | 35 cycles |
| Annealing | 50-65 °C | 15 seconds | |
| Extension | 72.0 °C | 15 seconds | |
| Melt curve analysis | 95.0 °C | 1 minutes | 1 cycle |
| | 55.0 °C | 1 minutes | 1 cycle |
| | 55.0 °C* | 1 minutes | 81 cycles |

*The temperature was increasing 0.5 °C for each following step for 81 cycles (until 95.0 °C)

**Statistical analysis**

All data were presented as mean ± standard error of the mean (SEM). Data normality was determined by the Kolmogorov-Smirnov test. Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls. $p$-value $\leq 0.05$ was characterized as statistically significance. Data were analyzed by using the software package SPSS17.0 (SPSS, Chicago, USA).