CHAPTER II

MATERIALS AND METHODS

2.1. Chemicals

100 Base pair +1.5 KB DNA ladder (SibEnzyme, Russia)

Absolute ethanol (Merck, Germany)

Agarose gel

: MetaPhor Agarose (FMC Bioproducts, USA)

: Seakem LE Agarose (FMC Bioproducts, USA)

Boric acid (Merck, Germany)

Bovine serum albumin; BSA (Sigma, USA)

Bromophenoal blue (Merck, Germany)

Chloroform (Merck, Germany)

Deoxynucleotide triphosphate: dNTPs (Pacific Science, USA)

Diethyl pyrocarbonate: DEPC (Sigma, USA)

Ethidium bromide (Sigma Chemical Company, USA)

Ethylene diamine tetra-acetic acid di-sodium; Na2EDTA (Fluka, Switzerland)

Ficoll type 400 (Sigma, USA)

Glycine (Sigma, USA)

Hydrochloric acid (Merck, Germany)

Isoamyl alcohol (Merck, Germany)

Isopropanol (Merck, Germany))

Liquid nitrogen

Omniscript RT Kit (QIAGEN, Germany)

Phenol crytal (BDH, England)

QIAquick Gel Extraction kit (QIAGEN, Germany)

Sodium acetate (Merck, Germany)

Sodium chloride (BDH, England)

Sodium dodecyl sulfate: SDS (Sigma, USA)

Sodium hydroxide (Carlo Erba Reagenti, Italy)

TriZol Reagent (Invitrogen life Technologies, UK)

2.2. Equipments

- 20 °C Freezer (Krungthai Ltd., Thailand)

- 80 °C Freezer (Bara laboratory Co. Ltd., Thailand)

Autoclave Model # LS-2D (Rexall Industries Co. Ltd., Taiwan)

Automatic micropipette:pipetman P2, P20, P100, P200, P1000 (Gilson Medical Electronics S.A., France)

Centrifuge:J2-21 (Beckman Instrument Inc.,USA)

Gel Documentation System (SynGene, UK)

Gelmate2000 Electrophoresis system (TOYOBO, Taiwan)

High speed microcentrifuge: MC-15A (Tomy-Seiko Co. Ltd., Japan)

Incubator water bath: M20S (Lauda, Germany)

Incubator: BM-600 (Memmert GambH, Germany)

Magnetic stirrer and heater (Fisher Scientific, USA)

Microcentrifuge tubes 0.6 ml and 1.5 ml (Bio-RAD Laboratories, USA)

Microwave Oven: TRX1500 (Tarbora International Co. Ltd., Korea)

Minicentrifuge (Costar, USA)

PCR workstation Model # P-036 (Scientific, USA)

pH meter: PHM 95 (Radiometer, Denmark)

Power supply: POWERPAC 300 (BioRad Laboratories, USA)

RNase inhibitor (Bio Basic Inc., Canada)

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen, Germany)

Spectrophotometer DU650 (Beckman, USA)

Thermocycler: Mastercycler (Eppendorf AG, Germany)

Ultrasonic: 28H (Ney Dental Inc., USA)

UV transilluminator: 2001microwave (San Gabriel California, USA)

Vortex: K-550-GE (Scientific Industries, USA)

2.3. Inventory Supplies

PCR thin wall microcentrifuge tubes 0.2 ml (Axygen® Scientific, USA)

Pipette tips 10, 20, 100 and 1000 μl (Axygen® Scientific, USA)

2.4. Enzymes

DNase I (Promaga, USA)

DyNazyme™II DNA Polymerase (Finnzymes, Finland)

Proteinase K (Life Technologies, Inc., USA)

Restriction endonucleases

:*Hinf* I, *Rsa* I, *Sau*3A I (New England Biolab, England)

2.5. Primers

Oligonucleotides (Bio Basic Inc., Canada)

Oligo$(dT)_{15}$ primers (Promega Coperation, USA)

2.6. .Sample Preparations

2.6.1. Honeybee Samples

The honeybee workers, *Apis cerana*, at various stages as newly emerged, 5-10 -day-old nurse, 11-15 -day-old nurse and forager bees were collected from manage beekeeping colonies at bee research center (Chumporn province) which were the same sample group as in Puttarat's master thesis. Newly emerged bees were collected when they bit the brood cell covers. Nurse bees were collected when color marked newly emerged bees were 5-10 -day-old and 11-15 -day-old. Forager bees were collected near the hive entrance when they returned to the hive with pollen on their pollen baskets or with on extended abdomen that estimate their age to vary from 20-30 -day-old. Honeybee samples were immediately frozen in liquid nitrogen and stored at –80 °C until further used.

2.6.2. Hypopharyngeal Gland Samples

A hypopharyngeal gland was dissected out from the head of each of the above-mentioned honeybee sample under a binocular microscope at 4°C. A knife was used to cut through the wall of the mask, across the vertex, round the margins of the compound eyes, and round the edges of the mask. The mask was then taken off. A hypopharyngeal gland located in front of the brain was removed and placed in a tube containing pre-chilled buffer constituting of guanidium thiocyanate and *N*-lauroyl sarcosine (supply with TriZol Reagent) and stored in liquid nitrogen. Total RNA were extracted from hypopharyngeal glands and used to prepare the first stranded cDNA.

2.7. Total RNA Extraction

Total RNA extractions from hypopharyngeal glands of *A.cerana* honeybees were carried out with the TriZol Reagent (Invitrogen life Technologies, UK), according to the manufacturer's instructions. After hypopharyngeal glands were dissected out and placed into a microcentrifuge tube containing 1 ml of the Trizol reagent composed with guanidium thiocyanate and *N*-lauroyl sarcosine as in 2.6.2, each sample was homogenized with a sterilized micropestle for a few times and incubated for 5 minutes at room temperature. After incubation, 0.2 ml chloroform was added and tightly covered, then vigorously shake for 15 seconds. The mixture was then incubated at room temperature for 15 minutes and centrifuged at 12,000 xg for 15 minutes at 4 °C. After centrifugation, the collected upper aqueous phase containing total RNA was transferred to a new tube. RNA in the aqueous layer was precipitated with 0.5 ml of isopropanol at -20 °C. The mixture was stored at room temperature for 10 minutes and centrifuged at 12,000 xg for 8 minutes at 4 °C. The supernatant was removed and a white RNA pellet was washed twice with 1 ml of 75% ethanol and subsequently centrifuged at 7,500 g for 5 minutes at 4 °C. The ethanol wash was removed and briefly air-dried for 5 minutes. RNA pellets were resuspended in diethyl pyrocarbonate (DEPC) -treated sterile water and then incubated the solution for 10-15 minutes at 60°C. The RNA was stored at -80 °C until used.

DNA contamination in total RNA solution was eliminated by DNase I. Twenty five micrograms of each total RNA sample was incubated in the reaction mixture of 50 µl containing 10 unit of RNase-free DNase I, 0.5 M Tris-HCl, pH 7.5, 0.5 M $MgCl_2$ and 10 units of RNase inhibitor. After gently mixed and incubated at 37°C for 1 hour, the reaction was stopped by addition of 2.5 µl of 0.2 M EDTA and 2

μl of 3 M sodium acetate pH 5.2. The total RNA was extracted from the reaction mixture using TriZol reagent as mention above.

Quantity and quality of total RNA were then spectrophometrically determined and electrophoretically analyzed through 1% denaturing agarose gel electrophoresis. The concentration of total RNA was determined by measuring the absorbance at 260 nm in a UV spectrophotometer and estimated in μg/ml using the following equation,

[Total RNA] = A_{260} x 40 μg/ml* x dilution factor

*An absorbance of 1 OD unit at 260 nm corresponds to approximately 40 μg of RNA per ml (Sambrook *et al.*, 1989)

For qualify of total RNA, the ratio of the absorbance at 260 nm and 280 nm was performed to estimate the purity of RNA with respect to contaminant that absorb in the UV such as protein and phenol. Pure RNA ratio of absorbance at 260 nm and 280 nm in 10 mM Tris-HCl, pH 7.5 must be 1.9-2.1. In addition, the integrity of extracted total RNA was analyzed by denaturing formaldehyde agarose gel electrophoresis following by ethidium bromide staining. A 1.2% formaldehyde agarose gel was prepared using 1x FA gel buffer which diluted from a 10x FA gel buffer (200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid), 50 mM sodium acetate, 10 mM EDTA, pH to 7.0 with NaOH). The gel slurry was boiled until complete solubilization, and allowed to cool to 60 °C. Formaldehyde (0.66 M final concentration) were added to the gel and poured into a chamber set. The comb was then inserted.

Twenty micrograms of total RNA in 3.5 μl of DECP-treated H_2O, 5 μl of formamide, 1.5 μl of 10x FA gel buffer and 2 μl of formaldehyde were combined,

mixed well and incubated at 65 °C for 15 minutes. The mixture was immediately placed on ice. One-forth volume of the gel-loading buffer (50%, v/v, glycerol; 1mM EDTA, pH 8.0, 0.5%, w/v bromophenol blue) was added to each sample. The sample was loaded to the 1.0 % agarose gel containing formaldehyde. Electrophoresis was carried out in 1x FA gel buffer at 5 volts/cm, until bromophenol blue was migrated approximately three-forth of the gel length. The gel was stained with a 2.5 µg/ml ethidium bromide (EtBr) solution for 5 minutes and destained in deionized water for 15 minutes. RNA band in the EtBr stained gel was visualized as fluorescent bands by a UV transilluminator (UVP Inc.).When needed, extracted total RNA was reverse transcribed to the first stranded cDNA template.

2.8. First Stranded cDNA Synthesis

The first stranded cDNA was synthesized from the RNA using an Omniscript RT (QIAGEN, Germany). Template RNA, the primers solution, 10x Buffer RT, dNTP Mix and RNase-free water was thawed and immediately stored on ice after thawing. A fresh master mix in a final reaction volume of 20 µl containing 1x RT buffer (25 mM Tris–HCl, pH 8.3, 37.2 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT) and 0.5 mM of each dNTP, 1 µM Oligo-dT primer, 10 units of RNase inhibitor, and 4 units of Omniscript Reverse Transcriptase was prepared and aliquot into individual reaction tubes on ice. Next, 1 µl of RNA template (2.0 µg) that was diluted from extracted total RNA in DEPC water of various honeybee stages was added to each reaction tubes. The mixture was mixed thoroughly and carefully by vortexing for no more than 5 second. After centrifuged briefly to collect residual liquid from the walls of the tube, reactions were incubated at 37°C for 60 min following by 93°C for 5 min. The

synthesized first stranded cDNA was stored at -80 °C until used to be template for quantification of mRNA expression levels of major royal jelly proteins and Apisimin from *A. cerana* (AcMRJPs and AcApisimin) at various stages of worker bees using semi-quantitative PCR.

There are several methods for using quantitative PCR to determine relative initial levels of target mRNAs. Nonetheless, in all of these methods, the complication of results interpretation from variations in amplification efficiency was found. In order to limit tube-to-tube variation of PCR-amplification efficiency in quantitative analysis of mRNA level, the internal standard is usually included in the amplification reaction. The internal standards could be not only an endogenous sequence or gene transcript that normally present in the sample, but also an exogenous fragment added to the amplification reaction. Here in this study, both of internal standard types was tried to use for semi-quantification which was shown in the experiment work flow (Figure 2.1).

2.9. Quantification mRNA Level of AcMRJP and AcApisimin by Using Internal Standard as Endogenous Sequence

One of internal standard used in the quantitative PCR was 28S rRNA gene endogenous sequence. In the PCR reaction, a second pair of gene-specific primers was included in the same reaction as the target sequence. The ratios of the amount of two PCR products generated by target and endogenous standard sequences in the different samples were then determined and quantified for mRNA level which was shown in the experiment workflow (Figure 2.2).

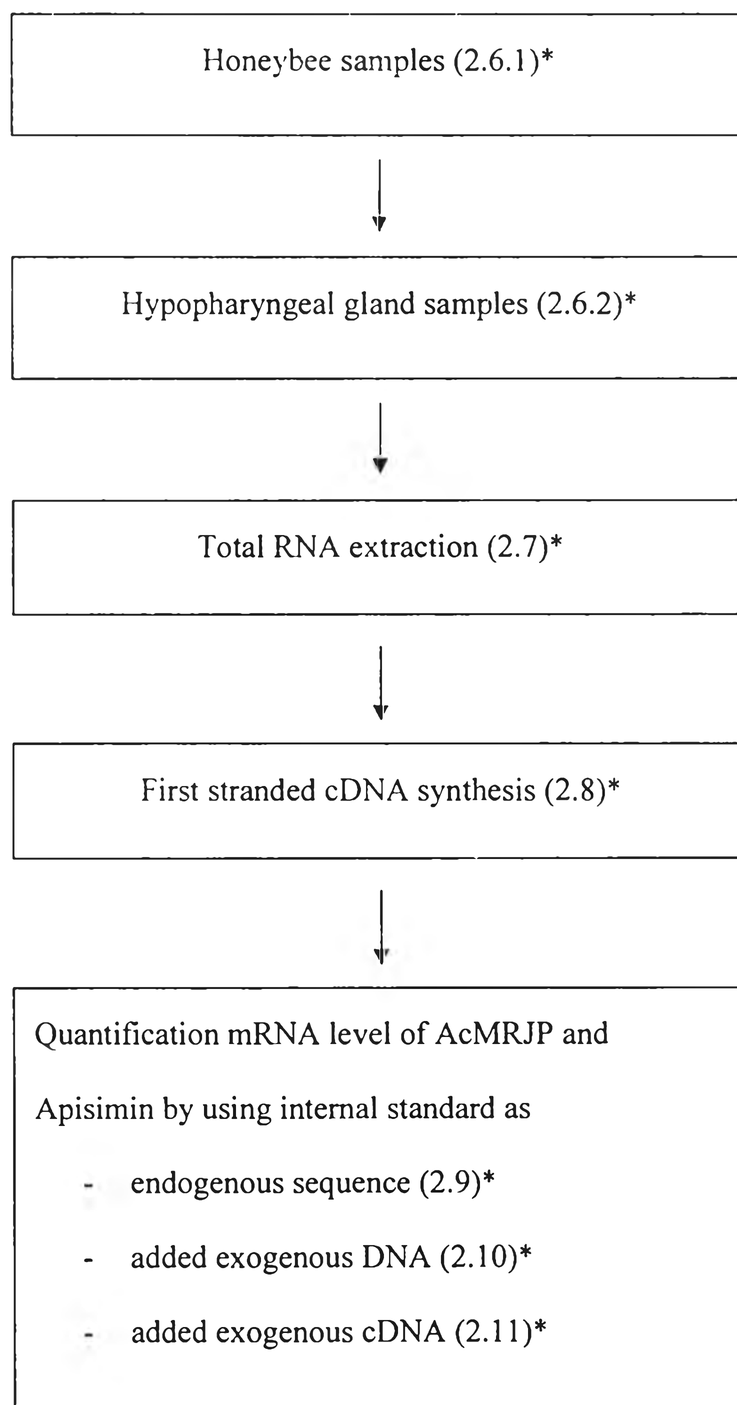


Figure 2.1 The workflow for the quantification of mRNA level using semi-quantitative PCR.

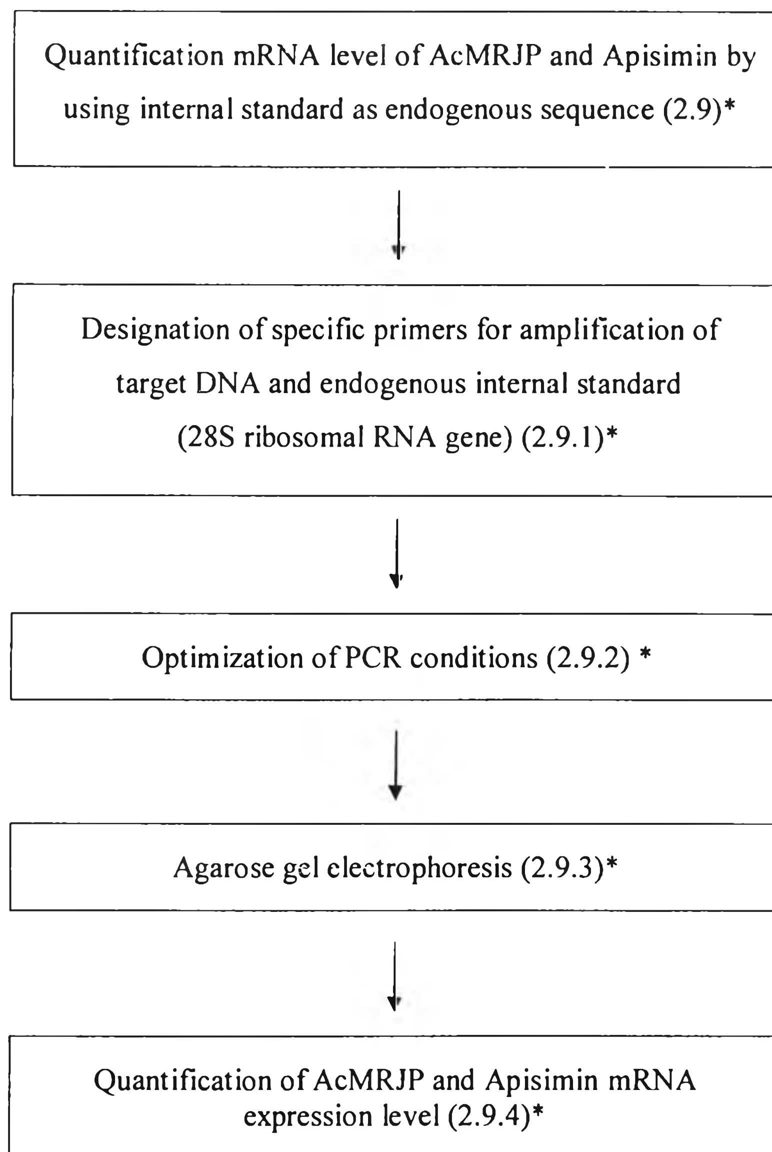


Figure 2.2 The workflow for the quantification of mRNA level using endogenous internal standard.

2.9.1. Designation of Specific Primer

For amplification of target cDNA (MRJPs and AcApisimin), specific primers for target amplification of AcMRJPs and AcApisimin were designed from *A. cerena* major royal jelly proteins (AcMRJPs) 1-6 and AcApisimin sequences (Srisuparbh *et al.*, 2003, Imjongjairak *et al.*, 2005 and Cenphakdee, 2003). These sequences were aligned using Clustal X and searched for the specific primer sites whereas internal standard were desingned from 28S ribosomal RNA gene sequence (deposited in the GenBank accession number AF181590). A pair of primers of each AcMRJPs family, AcApisimin and 28S ribosomal RNA was designed using the primer3 program (http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi) and the Oligo version 4 program. Each pair of upstream and downstream primers was checked for minimal self-priming and upper/lower dimer formation. Furthermore Tm values and product size should be similar.

2.9.2. Optimization of PCR Conditions

For optimization of PCR conditions, appropriate concentrations of each pair of primers and $MgCl_2$ were adjusted. PCR was carried out in the total volume of 20 µl composing 20 ng of the first stranded cDNA template, 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 100 mM of each dNTP, 1.0-2.0 mM $MgCl_2$, 0.05 - 0.20 µM of each primer and 1.5 units of DyNazyme™ II DNA Polymerase (Finnzymes). The temperature profiles were predenatured at 94 °C for 3 minutes followed by 30 cycles of denaturation at 94 °C for 50 seconds, annealing at 54 °C for 50 seconds and extension at 72 °C for 1 minute. The post-extension was performed at 72 °C for 10 minutes. After amplification, an aliquot of 5 µl of PCR product was

electrophoretically analyzed on 3.0 % agarose gel. Additionally, PCR product was also digested with restriction endonucleases to verify specificity of primers. Digested DNA fragment was electrophoretically analyzed as above.

In addition, different numbers of the amplification cycles (2 cycles increment between 10-24 cycles) were carried out to know the plateau phase of amplification reaction. A PCR reaction was stopped according to described cycle numbers and further incubated at 72°C for 10 minutes. PCR products were electrophoretically analyzed on 1.8% Metaphor agarose gel.

2.9.3. Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to size-fractionate the amplification product. Different concentrations of agarose gel were prepared depending on sizes of DNA fragments. An appropriate amount of agarose was weighted out and dissolved in the appropriate volume of 1X TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA pH 8.0). The gel slurry was heated until completed solubilization. The agarose gel solution was incubated at 65°C before poured into the electrophoretic gel mould. The comb was inserted. For Metaphor agarose, solidified gel are required to pre-chilled at 4 °C for overnight to achieve the sieving ability of the gels. After agarose was completely set, the comb was carefully removed. The gel was placed in the chamber. An enough volume of 1X TBE was poured to cover the gel for 2-3 mm. One-fifth volume of loading dye (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 15 % Ficoll 400) was added into the sample and loaded into the gel. Electrophoresis was usually operated at 12 volts/cm for 2.5-3.0 % agarose gel and 1.8% Metaphor agarose gel until bromophenol blue reached approximately 1 cm from

the end of gel. The gel was stained with ethidium bromide solution (2.5 μg/ml) for 5 minutes and destained in deionized water for 30 minutes. The DNA was visualized under a UV transilluminator and photographed with Gel Documentation System (GeneCam FLEX1, SynGene).

2.9.4. Quantification of mRNA Level Using Endogenous Internal Standard

Amplification reaction was carried out in a 20 μl reaction volume containing 1 μl of the first stranded cDNA and specific primer for a target gene and 28S rRNA in optimal condition derived from 2.9.2. After gel electrophoresis, The DNA was stained with a 2.5 μg/ml ethidium bromide solution and visualized under a UV transilluminator and photographed with Gel Documentation System (GeneCam FLEX1, SynGene).

The intensity (exhibited as the absorbance unit) of PCR product band was quantified from the photographs in a secure file format (SynGene Gel document or .sgd file) using GeneTools analysis software (Synoptics). The ratios of the amount of PCR products generated by the internal standard DNA ([S]) and the target cDNA ([T]) which obtained before the amplification reactions reach the plateau phase were determined and compared for each individual reaction. The relative initial amounts of a target sequence and the endogenous standard was determined from this equation:

$$N_{0t} / N_{0s} = Nt\,(1 + Es)n / Ns\,(1 + Et)n$$

Where N_{0t} = the initial number of target molecules

N_{0s} = the initial number of standard molecules

Nt = the number of amplified target molecules

Ns = the number of amplified standard molecules

Et = amplification efficiency of the target

Es = amplification efficiency of the standard

n = the number of amplification cycles

Even without a full mathematical analysis- and even in cases where Es does not equal Et- it was shown empirically that endogenous mRNAs could be used to normalize target mRNA levels between samples to be compared. Thus, instead of determining the ratio of the intial absolute amounts of target and standard using linear regression, the relative amounts of PCR products generated by the target and standard templates in different samples was simply compared. In other words, if the internal standard mRNA was expressed at the same level in two samples, the ratio of PCR products generated from the target and standard should indicate the relative level of expression of the target mRNA on those samples. As with this method , the relative amount of mRNA level of AcMRJP and AcApisimin at various stages could be detemined and compared.

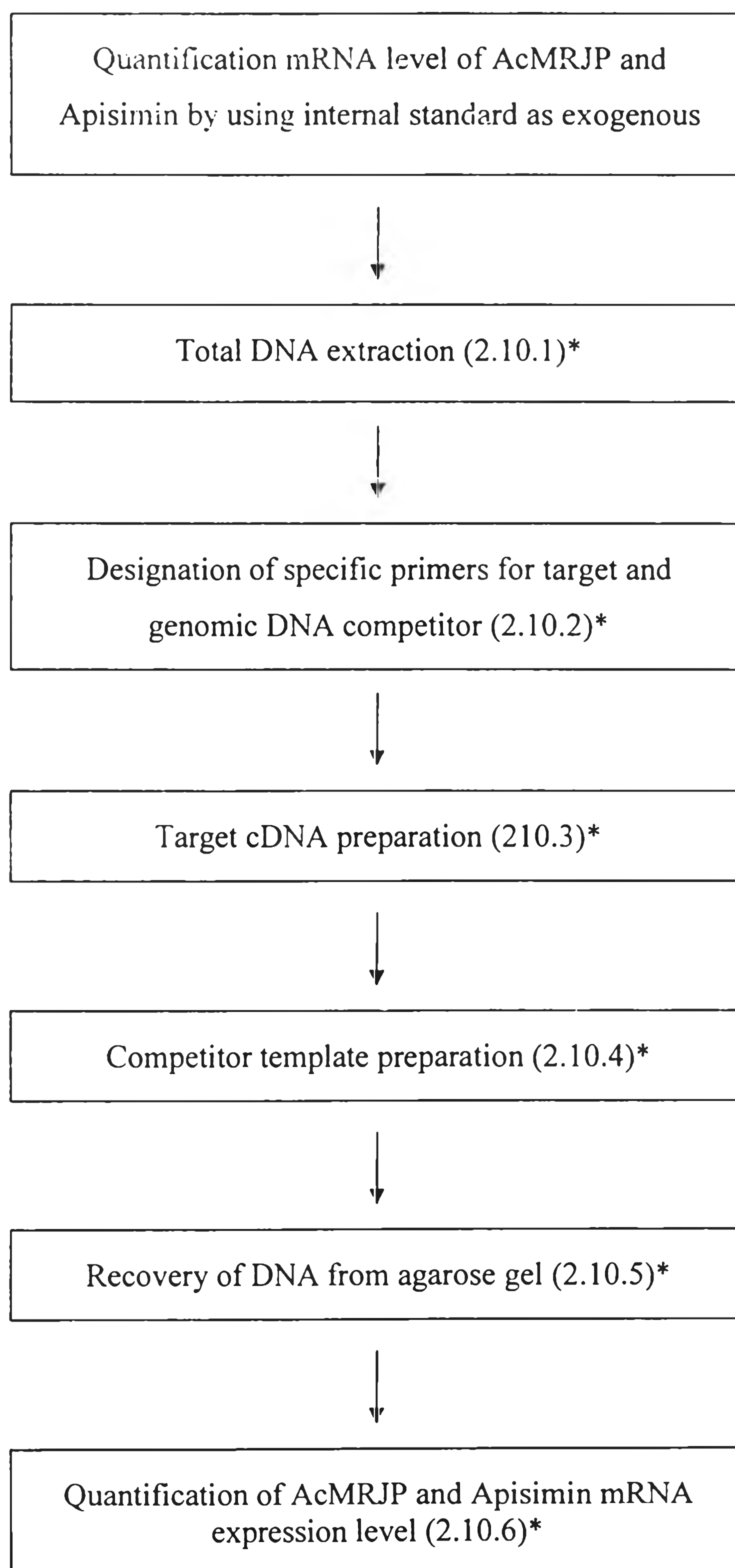
2.10. Quantification of mRNA Level of AcMRJP and Apisimin Using Internal Standard as Added Exogenous DNA

Exogenous sequences could also be used as internal PCR standard DNA. In this approach, an exogenous DNA standard was added and amplified simultaneously with the target transcrit in a single PCR reaction mixture using the same primers. This kind of semi-quantification are termed competitive PCR. The reaction was performed

in a truly competitive fashion by the same primers in amplification of target and competitor (internal standard DNA which is usually amplified from chromosomal DNA). Therefore, the specific primers for target and competitor of each gene were designed. Exogenous internal standards that share the same primer annealing sequences with the target but differ in size from target sequences was used. In variety of method for synthesis these standard, the amplification of exogenous DNA that differ only by the presence of small introns was chosen. The workflow for this method was shown in figure 2.3.

2.10.1. Total DNA Extraction

Total DNA was extracted from the thorax of each forager bee using a modification of the method of Smith and Hagen (1997). A thorax was homogenized in 500 µl of STE buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA). After that, 20 % SDS and 10 mg/ml proteinase K were added to final concentrations of 1 % SDS and 500 µg/ml proteinase K, respectively. The homogenate was incubated at 37 °C for 3 hours and centrifuged at 8,000xg for 5 minutes at room temperature. The supernatant was removed to a new microcentrifuge tube and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The mixture was gently mixed for 15 minutes and centrifuged at 8,000xg for 5 minutes at room temperature. After that, the upper aqueous phase was collected and transferred to a new sterile tube. The phenol/chloroform extraction was repeated as described previously. The supernatant from the secondary phenol/chloroform extraction was further extracted once with an equal volume of chloroform/isoamyl alcohol (24:1) as above. One-tenth volume of 3 M sodium acetate pH 7.5 was added. DNA was precipitated by the



* Section for detailed information

Figure 2.3 The workflow for the quantification of mRNA level using exogenous internal standard as added genomic DNA.

addition of 2 volumes of cold absolute ethanol The mixture was kept at –20°C overnight. The DNA pellet was recovered by centrifugation at 12,000xg for 15 minutes at room temperature and briefly washed with 1 ml of 70 % ethanol. The DNA pellet was air dried and dissolved in the appropriate volume of TE buffer (10 mM Tris-HCl pH 7.5 and 1 mM EDTA). DNA concentration was roughly estimated by comparing the intensity with that of a λ/*Hind* III DNA marker after electrophoresis through a 0.7 % agarose gel and staining with ethidium bromide. The DNA concentration was finally adjusted to a final concentration of 25 ng/µl and kept at 4 °C until further used.

2.10.2. Designation of Specific Primers for Target and Genomic DNA Competitor

Specific primers for this approach must be put over the small introns in the sequences. Following the sequences aligment, known introns position of AcMRJP1 and AcMRJP2 gene sequences (Imjongjairak *et al.*, 2005) were marked, in addition, the nucletide sequence of AcMRJP1-6 before intron was found in homologous. Then the the intron positions in each AcMRJPs gene were assumed with the same position and together marked. A pair of specific primer for target and genomic DNA competitor was designed over smallest intron using method mention above in 2.9.1.1.

2.10.3. Target cDNA Preparation for Competitive PCR

The second stranded cDNA was synthesized with family-specific primers. One microliter of the first stranded cDNA was used as the template in a 20 µl reaction volume performing in the optimal condition derived from 2.9.2. The reaction was denatured at 94°C for 1 minutes following by annealing at 54 °C for 50 seconds and

extension at 72 °C for 5 minutes. The amplification product (called target cDNA) was further used to simultaneously amplify with genomic DNA competitor.

2.10.4. Competitor Template Preparation

An internal standard DNA of each AcMRJPs and Apisimin was generated using genomic DNA of *A. cerana*. Size differences between cDNA target and genomic DNA of investigated genes were resulted from the existence of intervening sequences of a particular gene. To prepare the genomic DNA competitor, 25 ng of total DNA (2.10.1) was used as the template and amplified using the same primer set for amplification of the cDNA template. The PCR reaction was performed in a 20 µl reaction volume.

After amplification, 5 µl of reaction product was electrophoretically analyzed using a 2.0 % agarose gel to determine whether the amplification was successful.

2.10.5. Recovery of DNA from Agarose Gel

Following completed electrophoresis, the gel was stained with ethidium bromide (2.5 µg /ml) for 5 minutes. The position of desired DNA fragment was located and excised from the gel and placed into the pre-weight microcentrifuge tube. Six volumes of GQ buffer (w/v) were added. The gel mixture was then incubated at 50 °C for 10 minutes or until the gel slice was completely dissolved. The color of the gel mixture was yellow. Then, the gel mixture was applied in a QIAquick spin column, which was placed into a provided 2-ml collection tube and centrifuged at 10,000xg for 1 minute at room temperature. The effluent was discarded. After that, 0.75 ml of the PE buffer was added, left for 2-5 min and centrifuged at 10,000xg for 1

minute. The column was placed into a new microcentrifuge tube. Finally, DNA was eluted by adding 50 µl of the EB buffer (10 mM Tris-HCl pH 8.5) and centrifuged at 10,000xg for 1 minute.

To prepare 20 ng genomic DNA competitors, 1 µl of eluted DNA was mixed with 1 µl of 2.5 µg/ml of ethidium bromide and 1 µl of deionized water and dropped on a plastic sheet. The concentration of the eluted PCR product was estimated by comparing its florescence intensity under a long wavelength UV light with that of λ DNA (10-25 ng).

2.10.6. Quantification of AcMRJP and AcApisimin mRNA Level

To perform a competitive PCR, a dilution series of the genomic DNA competitor of each gene was prepared. One microliter of each competitor series concentration was added to a microcentrifuge tube and co-amplified with a constant amount of 1 µl of the target cDNA. The PCR reaction was performed in optimized conditions as described in 2.9.2. The PCR product was electrophoretically analyzed through 3.0-4.0 % agarose gel and stained with 2.5 µg/ml of ethidium bromide. A range of suitable competitor amount providing competitive results was chosen and further amplified using a two-fold dilution of target cDNA for reliability.

After amplified competitive PCR, quantification was achieved by distinguishing the two PCR products from each tube based on size. PCR products were analyzed through 2.5-3 % agarose gel electrophoresis. The DNA was visualized under a UV transilluminator and photographed with Gel Documentation System (GeneCam FLEX1, SynGene).

The fluorescence intensity of PCR product band was quantified using GeneTools analysis software (Synoptics) as described previously. The amount of products generated by the competitor standard DNA ([S]) and the target cDNA ([T]) were determined for each individual reaction. The logarithm of the intensity ratios between the target cDNA and the competitor DNA amplified product (log [T]/[S]) was graphed as a function of the initial concentration amount of the added competitor standard in the PCR reaction. An amount of initial target cDNA was calculated from the graph, assuming that amount was equal to the amount of the added competitor DNA standard when an equimolar ratio of the two types of products was generated, where the log of [T]/[S] = log of 1/1=0.

2.11. Quantification of mRNA Level of AcMRJP and AcApisimin Using Internal Standard as Added Exogenous cDNA

As above-mentioned, competitive PCR was a method that performed by co-amplification of known amount of a competitor or synthetic internal standard with the cDNA target. Besides using amplified genomic DNA at the same locus as target cDNA as a genomic DNA competitor, the addition of exogenous cDNA (cDNA competitor) can also be used.

For quantification of mRNA level using internal standard as added cDNA competitor, the cDNA competitor was prepared from first stranded cDNA (2.8) using specific reverse primer. This amplified cDNA product was then used as cDNA competitor for cDNA target amplification. The experiments were shown in following workflow (Figure 2.4).

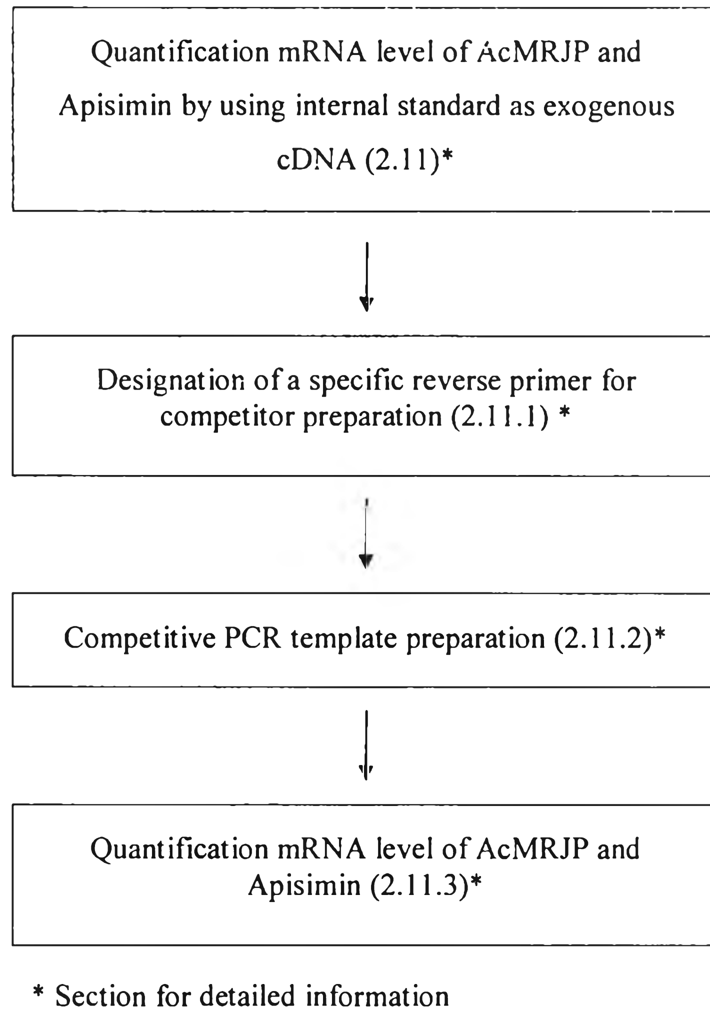


Figure 2.4 The workflow for the quantification of mRNA level using exogenous internal standard as cDNA.

2.11.1. Designation of a Specific Reverse Primer for Competitor Preparation

From primer sequences of cDNA target (forward primer A and reverse primer C in Figure 2.5 and Table 3.4) (2.9.1.1), the specific reverse primer (primer BC) was designed for generated cDNA competitor with forward primer A by PCR. The reverse primer (primer BC in Figure 2.5) is approximately 36-44 nucleotides in length composed with segment B and C. Segment B of primer BC was 18-22 nucleotides sequence at the 3' end correspond to the complementary strand of the target sequence, and segment C contained 18-22 nucleotides at the 5' end of the reverse primer BC. Segment C corresponded to the complementary strand of the target sequence n (19-44) nucleotides upstream from segment B.

Amplification with primer A and primer BC using first stranded cDNA as template would give only one PCR product called cDNA competitor. Size of their cDNA competitor is Δn smaller than cDNA target (Table 3.4).

2.11.2. Competitive PCR Template Preparation

Semi-quantitation by competitive PCR, two types of cDNA template (cDNA target and cDNA competitor) had to prepare. Target template was prepared as in 2.10.3. For cDNA competitor preparation, first stranded cDNA from 2.8 was used as the template and amplified using two primers as described in 2.11.1. For amplification of the cDNA competitor, the amplification reaction was carried out in a 20 μl reaction volume containing 1 μl of the first stranded cDNA, 1X PCR buffer (20mM Tris-HCl pH 8.4, 50 mM KCl), 100 mM of each dNTP, 1.5 mM $MgCl_2$, 0.15 μM of each primer and 1.5 unit of DyNazyme^{TM} II DNA Polymerase (Finnzymes). The reaction

was predenatured at 94°C for 3 minutes following by 30 cycles of denaturing at 94 °C for 50 seconds, annealing at 54 °C for 50 seconds and extension at 72 °C for 60 seconds. The final extension was performed at 72°C for 10 minutes. To determine whether the amplification was successful, gel electrophoresis was performed. After electrophoresis, cDNA product was recovered from agarose gel eluted as in 2.10.5. Twenty nanograms of competitors were prepared and stored at -20 °C until used for co-amplified with target cDNA.

2.11.3. Quantification mRNA Level of AcMRJP and AcApisimin

For competitive PCR, constant concentrations of cDNA target were co-amplified with various concentration of cDNA competitor. Co-amplified PCR products were analyzed by electrophoresis and the intensities of each PCR products were measured.

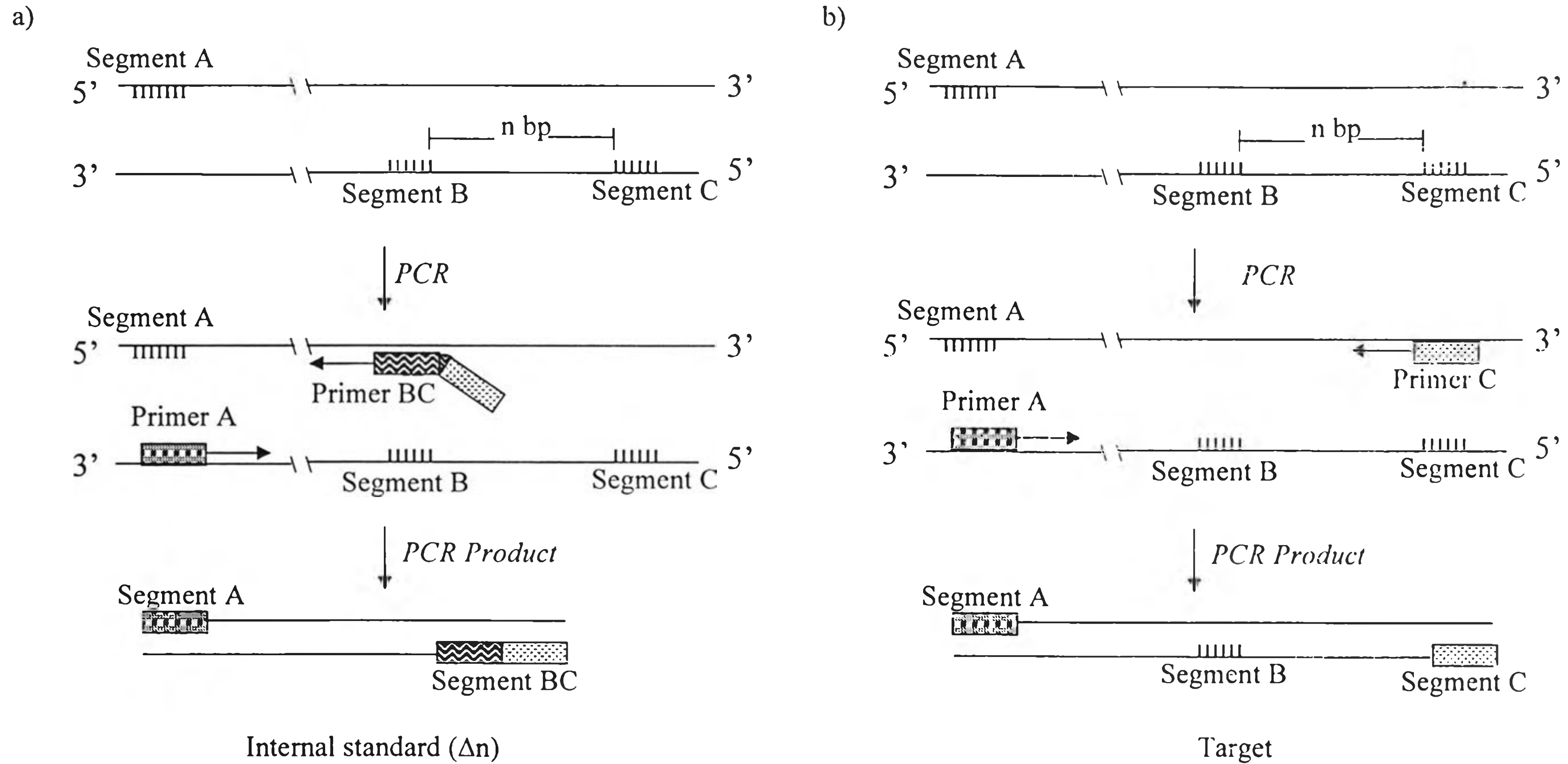


Figure 2.5 Schematic of preparation procedure for internal standard (a) and target (b)

Segment A = sequences on target sequence using for target and competitor forward primer designation.
Segment B = sequences on the opposite strand of the target sequence using for 3' reverse primer designation of competitor.
Segment C = sequences on the opposite strand of the target sequence using for reverse primer designation of target and 5' reverse primer designation of competitor.