CHAPTER III

RESEARCH DESIGN, MATERIALS AND METHODS

1. Research design

1.1 Preparation of standard controls

A known amount of HCMV DNA (standard strain, AD169) and human genomic DNA control were prepared for use as controls in all experiments.

The HCMV genomic DNA was kindly provided by Dr. Krauvan Balachandra of the Department of Medical Sciences, National Institute of Health, Ministry of Public Health, Thailand. The MIE gene fragment of HCMV DNA was amplified by conventional PCR assay[95]. To get enough MIE gene fragments, they were multiplied by the gene cloning technique.

The human genomic DNA was prepared from whole blood by using a DNA isolation kit. This DNA served as a template for beta-globin gene amplification. The yield of human genomic DNA and plasmid containing HCMV MIE gene fragment were measured quantitatively by fluorescence assay and spectrophotometry, respectively.

Serial ten-fold dilutions of HCMV plasmid DNA containing 0.1 to 10^{10} HCMV DNA copies/μl and a series of human genomic DNA concentrations (1, 10, 20, 30, 40, 50, 100, and 500 ng) were prepared for use in all optimization experiments.

1.2 Optimization of nested PCR and comparison of PCR product detection methods

The conventional nested PCR assay, described by Arai *et al.*[96] was optimized to reach better sensitivity and make conditions suitable for the PCR facilities presented in the laboratory. The PCR conditions were critical for a successfully optimized PCR assay. Those conditions included the annealing temperature, annealing time, dNTP concentration, $MgCl_2$ concentration, control DNA concentration, primer concentration and some additional co-solvents.

Another step of the experiment was to optimize the duplex PCR conditions in order to detect both HCMV DNA and human beta-globin gene in a single tube reaction. Finally, the PCR conditions that provided the best result of HCMV DNA and human beta-globin gene detection were selected.

Comparison of the PCR product detection methods was also performed. The sensitivity of the agarose gel electrophoresis with ethidium bromide staining and the dot blot hybridization assay was compared. The lowest copy number of HCMV DNA and lowest concentration of human genomic DNA, which could be detected by both methods, were observed.

1.3 HCMV diagnosis in retinitis and transplantation patients by optimized duplex nested PCR

The optimized duplex nested PCR was used to detect the HCMV DNA in ocular specimens from HIV positive HCMV suspected retinitis and HIV negative control patients. HCMV DNA detection in plasma and the PBMCs of renal transplantation recipients, together with some donors, was performed in comparison to detecting the IgM antibody by the ELISA method. The data of HCMV infection in both groups of patients were analyzed.

The steps of the entire experiment are summarized in Figure 3.

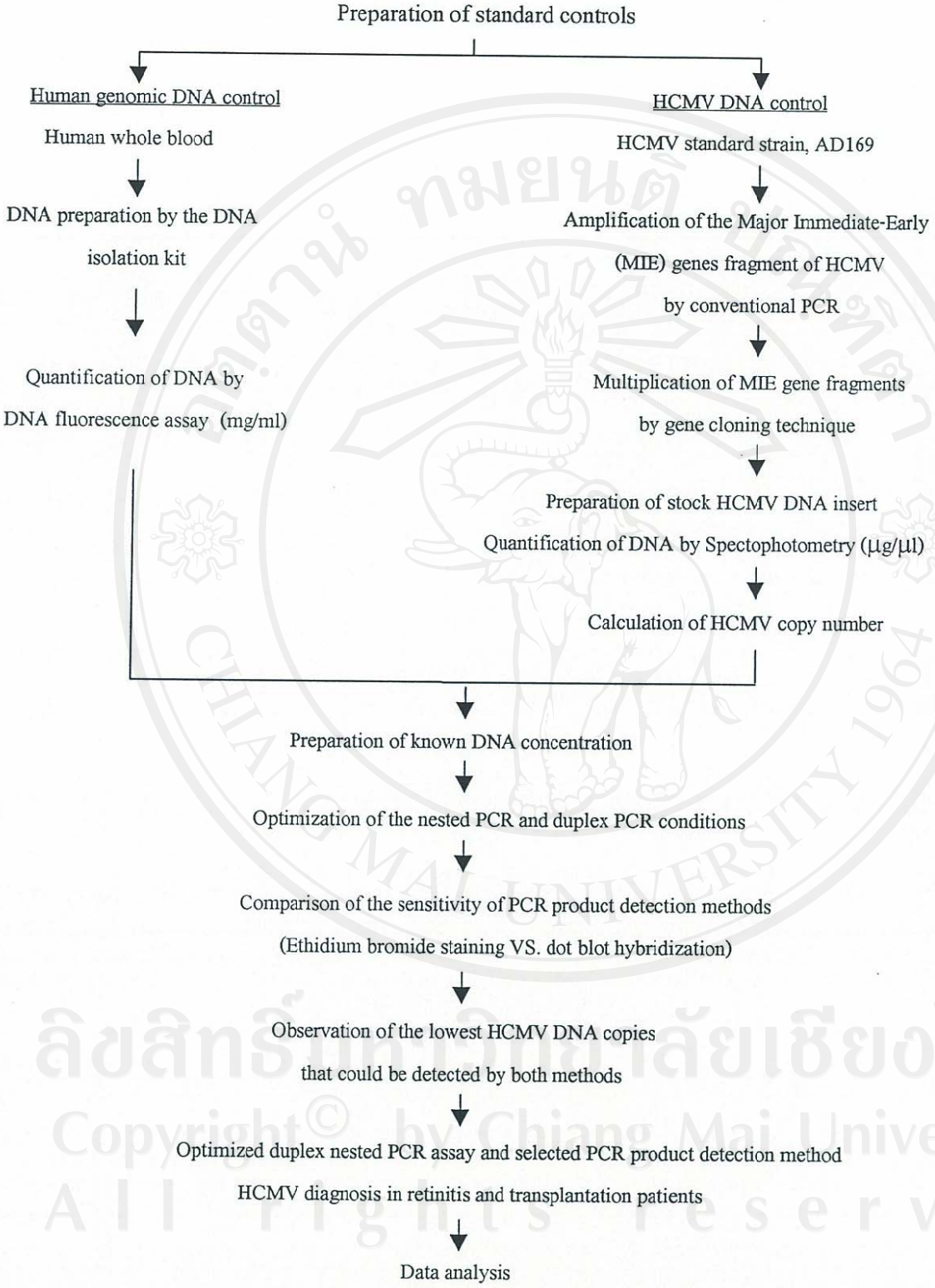

Figure 3 The schematic diagram of the research design in this study

2. Materials and methods

2.1 Subjects

The ocular and blood specimens were collected from patients attending Maharaj Nakorn Chiang Mai Hospital. The ocular specimens were collected from suspected HCMV retinitis patients with AIDS. The control patients were HIV negative with other signs that required an eye operation. Specimens were collected by the Ophthalmologist, with informed consent of the patients, from February 2002 to January 2003. A total of 74 samples (24 aqueous humors, 24 vitreous humors and 26 conjunctival scrapings) were obtained from 13 suspected HCMV retinitis patients with AIDS and 13 control patients. The location of specimen collection was shown in Figure 4.

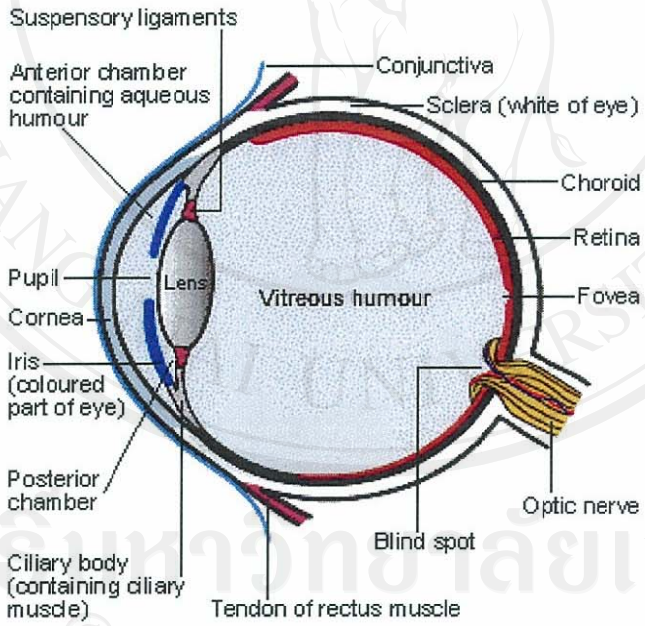

Figure 4 Structure of the human eye[97]

A total of 46 EDTA-anticoagulated blood samples were obtained from 11 renal transplant recipients (age range 13 to 58 years) and 4 renal transplant donors at the Kidney Transplantation Unit from January 2003 to September 2003. The blood samples of the recipients were collected before transplantation and every two weeks for 8 weeks after transplantation.

2.2 Oligonucleotide primers and probe

2.2.1 HCMV primers

Two sets of primer pairs corresponding to the second and third exons of the HCMV major immediate-early gene (MIE) (Figure 2), were selected from the report of Arai *et al.*[96] The first set of forward and reverse primers, which amplified a 351 bp product were 5'-GTCCTCTGCCA AGAGAAAGATGGAC – 3' (FP) and 5' – ACATCTTTCTCGGGGTTC TCGTTGC – 3' (RP), respectively. The nested forward and reverse primers, which amplified a 170 bp product were 5' –TCTCCTGTATGTGACCCATGTGCTT – 3' (NFP) and 5' – TTGAGGGATTCTTCGGCCA ACTCTG – 3' (NRP), respectively.

2.2.2 HCMV probe

An oligonucleotide probe was also synthesized, complementary to the MIE gene fragment of HCMV genomic DNA. Twenty bases of the probe, which hybridized to the PCR product of the MIE gene fragment amplification of 2.2.1, were 5' – TGCAGACTATGTTGAGGAAG – 3'.

The summary of the HCMV primers and probe is shown in Table 5. The locations of the HCMV primers and probe at the MIE gene fragment are shown in Figure 5.

Table 5 HCMV MIE PCR Primers and oligonucleotide probe information

Name	Sequence (5' to 3')	Length (bp)	Product length (bp)
FP	5'-GTCCTCTGCCAAGAGAAAGATGGAC–3'	25	351
RP	5'–ACATCTTTCTCGGGGTTCTCGTTGC–3'	25	
NFP	5'–TCTCCTGTATGTGACCCATGTGCTT–3'	25	170
NRP	5'-TTGAGGGATTCTTCGGCCAACTCTG–3'	25	
MIE-Probe	5'-TGCAGACTATGTTGAGGAAG-3'	20	-

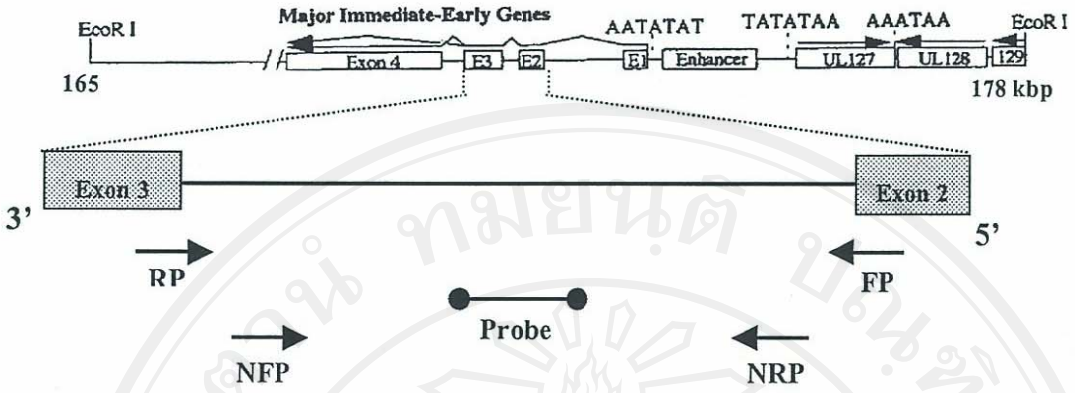

Figure 5 The locations of primers (FP, RP, NFP, NRP) and probe on the HCMV MIE gene.

2.2.3 Human beta-globin gene primers

The primers directed against the human beta-globin gene were used as specimen extraction control in the PCR reaction. These primers were selected from a report by Pan *et al.*[98] The forward and reverse primers, which amplified a 251 bp product were 5' - ACACAAC TGTGTTCACTAGC – 3' (BF) and 5' – GGAAAATAGACCAATAGGCTG – 3' (BR), respectively.

2.3 Specimen collection and preparation

2.3.1 Ocular samples

From each patient, 50 to 200 microliters of aqueous and vitreous humors were kept in a sterile 1.5 ml microcentrifuge tube, whereas conjunctiva scrapings were kept in a sterile 1.5 ml microcentrifuge tube containing 1.0 ml of the minimal essential medium (MEM). All samples were stored at –70 °C until tested.

2.3.2 EDTA-anticoagulated blood samples

Three milliliters of peripheral blood was drawn by venipuncture from each subject and put into ethylene diamine tetraacetic acid (EDTA) tubes (Becton Dickinson). The tubes were mixed well and kept at room temperature or in a refrigerator (for not more than 24 hours) until further processing. The whole blood was centrifuged at 700 x g for 20 min. Plasma was separated by using a sterile pipette and put into a sterile 1.5 ml microcentrifuge tube before storing at –70 °C for further investigations. The peripheral blood mononuclear cells (PBMCs) were collected from the white blood cell layer (buffy coat) by Ficall-Hypaque gradient centrifugation.

2.3.3 DNA preparation from the patient's specimen

2.3.3.1 Preparation of the ocular specimen

The conjunctiva scraping was put in 1.0 ml of the minimal essential medium (MEM) and centrifuged at 7,000 x g for 30 min. The supernatant was removed by using an autopipette leaving approximately 100 µl of the precipitate for further examination.

The aqueous and vitreous humor, and the conjunctiva scraping specimens were incubated at 100 °C for 10 min, and the tubes were returned immediately to an ice bath for at least 2 min before applying into the PCR reaction mixture.

2.3.3.2 Isolation of PBMCs from whole blood

One and a half millilitres of whole blood were diluted with a threefold volume of phosphate-buffered saline (PBS). The mixture was carefully layered over the Ficall-Hypaque (density = 1.077 g/ml) using an approximately equal volume of whole blood in 15 ml conical tubes. The layered mixture was centrifuged at 1,500 x g for 20 min at room temperature. PBMCs were collected and a maximum of 5 x 10^6 cells in 200 µl of PBS were used in the DNA isolation steps.

2.3.3.3 DNA isolation from PBMCs and plasma

The DNA was isolated by using the QIAamp® DNA Blood Mini Kit (QIAGEN, USA). The procedure of isolation was performed as recommended in the manufacturer's instruction manual.

Twenty microliters of QIAGEN Protease was pipetted into a 1.5 ml microcentrifuge tube containing 200 µl of sample (PBMCs solution or plasma). The mixture was added to 200 µl of Buffer AL, mixed by pulse-vortexing for 15 seconds, and incubated at 56 °C for 10 min. The microcentrifuge tube was briefly centrifuged to removed drops from the lid. The mixture was then carefully applied to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed and the mixture centrifuged at 6,000 x g for 1 min. The QIAamp spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 500 µl of Buffer AW1 were added, without wetting the rim, and centrifuged at 6,000 x g for 1 min. Then, the column was placed in a new clean 2 ml collection tube, carefully opened and 500 µl of Buffer AW2 were added, without wetting the rim, and centrifuged at full speed (20,000 x g) for 3 min. The column was then placed in a new 2 ml collection tube and centrifuged at full speed for 1 min. Finally, the QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube. To elute the PBMCs DNA, 100 µl of Buffer AE were added to the column, whereas 50 µl of Buffer AE were used to elute the DNA from plasma. The column was incubated at room temperature for 5 min, and then centrifuged at 6,000 x g for 1 min. DNA solution was stored at 20 °C until further examination.

2.4 Cloning of the HCMV MIE gene fragment

2.4.1 Amplification of the MIE gene fragment by polymerase chain reaction (PCR)

An approximate 351 bp fragment of the MIE gene at positions 1,484 to 18,345 (GenBank accession number M21295) was amplified by conventional PCR using the first primer set. The conventional reaction mixture comprised 0.2 mM of each deoxynucleotide triphosphate

(dNTP: dATP, dTTP, dGTP, and dCTP), 1.25 units (U) of *Taq* DNA Polymerase (Promega, USA), PCR buffer (10 mM Tris-HCl, pH 9.0 at 25 °C, 50 mM KCl and 0.1% Triton X-100), 1.5 mM of $MgCl_2$, 0.4 µM of primer, genomic HCMV DNA (AD169), and distilled water that was added to a final volume of 50 µl.

The amplification was performed in an automated thermal cycler (GeneAmp PCR system 2700, Applied Biosystems, USA). As an initial step, single incubation cycles of 94 ° C for 5 min, 65 °C for 30 seconds, and 72 °C for 30 seconds, were followed by 35 cycles of 94 °C for 1 min, 65 °C for 2 min and 72 °C for 1 min. These were followed by a final cycle of 72 °C for 5 min.

Fifteen microliters of the amplification products were electrophoresed on 1.5% agarose gel in 0.5x TAE (Tris-Acetate-EDTA, pH 8.0) buffer at 100 volts for 35 min. The amplified fragments were stained by 2 µg/ml of ethidium bromide for 15 minutes then destained with distilled water for 10 minutes. They were then photographed under ultraviolet light using an ultraviolet transilluminator. The DNA fragment size was determined by using the standard DNA size marker, 1 Kb Plus DNA Ladder (Gibco BRL, USA).

2.4.2 Purification of the PCR product

The MinElute Gel Extraction Kit (Qiagen, Germany) was used to purify the amplified product of the MIE gene fragment from 2.4.1. The procedure of purification was performed as recommended in the manufacturer's instruction manual. Briefly, the amplified DNA fragment was separated on 1.5% agarose gel electrophoresis and the DNA band was excised by using a sterile scalpel. The gel slice was incubated in 3 gel volumes (weight by volume) of Buffer QG at 50 °C for 10 min. After the gel slice was completely dissolved, one gel volume of isopropanol was added and the mixture was then applied to the MinElute column. The column was centrifuged at 10,000 x *g* for 1 min and the supernatant was discarded. Then, 500 µl of Buffer QG was added into the column and centrifuged for 1 min before the supernatant was removed, as before. The DNA was adsorbed into the silica-gel membrane of the MinElute column in the presence of high salt, while contaminants passed through the column. After that, the other

impurities were washed out with 750 µl of Buffer PE. Finally, 10 µl of Buffer EB was added to elute the DNA.

2.4.3 Ligation of the purified HCMV MIE gene fragment into a vector

The purified HCMV MIE gene fragment was cloned into a vector by using the pGEM®-T Easy Vector Kit (Promega, USA). The ligation of the MIE gene fragment into the vector was performed according to the protocol recommended by the manufacture. Briefly, 60 ng of the purified DNA was added to the ligation reaction mixture containing the Rapid Ligation Buffer (60 mM Tris-HCl,pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% Polyethylene glycol), 50 ng of the pGEM®-T Easy Vector and 3 Weiss units of T4 DNA ligase at a final volume of 10 µl. After mixing, the reaction was incubated at 4 °C overnight.

2.4.4 Transformation of the recombinant plasmid DNA

The recombinant plasmid from 2.4.3 was transformed into the competent *E.coli* cells (JM109 strain; Promega, USA). The transformation was carried out in accordance with the protocol recommended by the manufacturer. Briefly, 2 µl of each ligation reaction were added into 50 µl of competent cells in a sterile 1.5 ml microcentrifuge tube on ice. The tube was flicked to mix the content and placed on ice for 20 min. Then, the cells were heat shocked at exactly 42 ° C in a water bath for 50 seconds, and the tubes were returned immediately to the ice bath for 2 min. Nine hundred and fifty microlitres of room temperature SOC medium were added into the reaction tube, containing cells transformed with ligation reaction, and incubated for 90 min at 37 ° C while slowly shaking (~150 rpm). One hundred microlitres of transformation culture were placed onto a LB plate containing 100 µg/ml of ampicillin and incubated at 37 °C overnight. After incubation, the bacterial colonies were screened for the presence of recombinant plasmid.

2.4.5 Screening for the recombinant plasmid DNA in the transformed bacteria

The transformed colonies from 2.4.4 were screened for the presence of recombinant plasmid DNA. The colony, selected by random, was either subjected directly to the HCMV PCR assay or processed through the plasmid DNA preparation. The plasmid DNA was extracted by using the method described below. Then 3,366 bp recombinant plasmid DNA containing 3,015 bp pGEM®-T Easy vector and 351 bp HCMV MIE gene fragment was demonstrated in 1% agarose gel electrophoresis. The presence of the inserted HCMV MIE gene fragment was confirmed by PCR assay using the first primer set. Only colonies that showed PCR products specific to the HCMV MIE gene were confirmed as successfully transformed.

For plasmid DNA preparation, the plasmid DNA was extracted from the bacterial colony by a mini-preparation of the alkaline lysis method (Samdrook et al., 1987). Briefly, a single bacterial colony was cultured in 2 ml of LB medium containing 100 µg/ml of ampicillin in a loosely capped 15 ml tube, and incubated at 37 °C for 18-24 hours with vigorous shaking. After 24 hours incubation, the culture was transferred into a sterile 1.5 ml microcentrifuge tube and centrifuged at 12,000 x g for 30 seconds at 4 °C. The supernatant was removed and the bacterial pellet suspended in 100 µl of ice-cold Solution I and vortexed quickly. Then, 200 µl of Solution II were added and the contents mixed by inverting the tube and incubating on ice for 5 min. One hundred and fifty microliters of ice-cold Solution III were added and mixed by gently vortexing, and the mixture was then incubated on ice for 5 min. After centrifugation at 12,000 x g for 5 min at 4 °C, the supernatant was collected and the DNA precipitated with 2 volumes of 95% ethanol before the same centrifugation process was repeated. The supernatant was removed by gentle aspiration. To ensure that impurities were removed, a 0.5 volume of 7.5 M ammonium acetate was added, stored at –70 °C for 10 min, and centrifuged for supernatant collection. Two volumes of 95% ethanol were added again to precipitate the DNA. The DNA pellet was rinsed with 1 ml of 70% ethanol and allowed to dry in air for 30 min. Finally, the DNA was dissolved in 10 µl of distilled water and stored at –20 °C until further examination.

2.5 Preparation of standard DNA control

2.5.1 HCMV MIE gene fragment control

To obtain enough standard DNA, the HCMV MIE gene fragment was prepared from transformed colonies by using the QIAGEN Plasmid Midi Kit (Qiagen, Germany). The purification procedure was performed as recommended in the manufacturer's instruction manual. Briefly, the confirmed colony from 2.4.5 was picked from a freshly streaked selective plate and inoculated into a starter culture of 2-5 ml LB medium containing ampicillin. The starter culture was poured into 50 ml of the selective LB medium, and grown at 37 °C for 12-16 h with vigorous shaking (~ 150 rpm). The bacterial cells were harvested by centrifugation at 6,000 x g for 15 min at 4 °C. The bacterial pellet was resuspended in 4 ml of Buffer P1 (resuspension buffer, 50 mM Tris·Cl, pH 8.0; 10 mM EDTA; 100 µg/ml Rnase A). The mixture was added to 4 ml of Buffer P2 (lysis buffer, 200 mM NaOH, 1% SDS (w/v)), mixed gently but thoroughly by inverting 4-6 times, and incubated at room temperature for 5 min. The lysate was added to 4 ml of chilled Buffer P3 (neutralization buffer, 3.0 M potassium acetate, pH 5.5), and mixed immediately but gently by inverting 4-6 times. The lysate was poured into the barrel of the QIAfilter Cartridge, and incubated at room temperature for 10 min. The QIAGEN-tip 100 was equilibrated by applying 4 ml of Buffer QBT (equilibration buffer, 750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® x-100 (v/v)) and the column allowed to empty by gravity flow. The cap was removed from the QIAfilter outlet nozzle, the plunger gently inserted into the QIAfilter Midi Cartridge and the cell lysate filtered into the QIAGEN-tip previously equilibrated. The cleared lysate was allowed to enter the resin by gravity flow. The QIAGEN-tip was rinsed with 2 x 10 ml of Buffer QC (wash buffer, 1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)). The DNA was eluted with 5 ml of Buffer QF (elution buffer, 1.25 M NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol (v/v)). The eluted DNA was precipitated by adding 3.5 ml (0.7 volumes) of isopropanol, mixed and centrifuged immediately at ≥ 15,000 x g for 30 min at 4 °C. The supernatant was then carefully decanted. The pellet was rinsed with 2 ml of 70% ethanol and centrifuged at ≥ 15,000 x g for 10 min, and the supernatant was carefully decanted without disturbing the pellet. The pellet was air-dried for 5-10 min, and the DNA redissolved in 200 µl of

TE buffer. Finally, the purified DNA containing the HCMV MIE gene fragment was confirmed by nested PCR.

2.5.2 Human genomic DNA control

The human genomic DNA was extracted from PBMCs by the Pure Gene DNA isolation kit (Gentra systems, USA). The DNA extraction procedure was performed as recommended by the manufacturer's instruction manual. Briefly, to prepare the WBC from whole blood, 10 ml of whole blood was diluted with a threefold volume of phosphate-buffered saline (PBS). The mixture was carefully layered over the 10 ml of Ficall-Hypaque (density = 1.077 g/ml) in a 50 ml conical tube. The tube was centrifuged at 700 x g for 20 min at room temperature. PBMCs were collected and added to a 15 ml conical tube containing 3 parts of RBC lysis solution. The tube was mixed by inverting and incubating for 10 min at room temperature. The tube was inverted at least once during the incubation. The mixture was centrifuged at 2,000 x g for 10 min. The pellet was added to 10 ml of cell lysis solution. The protein was precipitated by 3.33 ml of protein precipitation solution, and centrifuged at 2,000 x g for 10 min. The precipitated proteins were expected to form a tight brown to white pellet. The supernatant containing the DNA was poured into a clean 15 ml conical tube containing 10 ml of 100% isopropanol for DNA precipitation, and centrifuged at 2,000 x g for 10 min. The DNA pellet was rinsed with 10 ml of 70% ethanol and allowed to dry in air for 60 min. Finally, the DNA was dissolved in 1,500 µl of DNA hydration solution and incubated at 65 °C for 1 hour or overnight at room temperature. The DNA concentration was measured by using the spectrophotometry method, and DNA was stored at –20 °C until further examination.

The purified human DNA was confirmed by the detection of a beta-globin gene using the PCR technique. The approximate 251 bp fragment of the beta-globin gene was amplified by conventional PCR using BF and BR primers. The reaction mixture consisted of 0.2 mM of each deoxynucleotide triphosphate (dNTP: dATP, dTTP, dGTP, and dCTP), 1.25 units (U) of *Taq* DNA Polymerase (Promega, USA), PCR buffer (10 mM Tris-HCl, pH 9.0 at 25 °C, 50 mM KCl and 0.1% Triton X-100), 1.5 mM of $MgCl_2$, 1 µl of human DNA control, and distilled water that was added to make a final volume of 50 µl.

The amplification was performed in an automated thermal cycler (GeneAmp PCR system 2700, Applied Biosystems, USA). An initial denaturation step of 5 min at 94 °C was followed by 35 cycles of 1 min at 94 °C, 2 min at 56 °C and 1 min at 72 °C, followed by a final cycle of 5 min at 72 °C.

Fifteen microliters of the amplification products were electrophoresed on 1.5% agarose gel in 0.5x TAE (Tris-Acetate-EDTA, pH 8.0) buffer at 100 volts for 35 min. The amplified fragments were stained by 2 µg/ml of ethidium bromide for 15 min then destained with distilled water for 10 min, and photographed under ultraviolet light using an ultraviolet transilluminator. The DNA fragment size was determined by using the standard DNA size marker, 1 Kb Plus DNA Ladder (Gibco BRL, USA).

2.6 Quantitation of the DNA controls

2.6.1 Quantitation of the purified HCMV plasmid DNA by spectrophotometry

The concentration of the purified HCMV plasmid DNA was determined by using the spectrophotometry method. The DNA was diluted in distilled water at an appropriate dilution and the optical density (O.D.) was then measured for nucleic acid and protein at a wavelength of 260 and 280 nm, respectively, using a UV spectrophotometer (Shimadz model 1101, Japan). The quantity of DNA was calculated by using the following equation:

Quantity of DNA (ng/µl) = $O.D._{260}$ x dilution factor x 1 $O.D._{.250}$ unit of double-stranded DNA conc.

= O.D. at 260 nm x dilution factor x 50

1 $O.D._{260}$ unit of double-stranded DNA concentration (conc.) = 50 ng/µl

The purity of the HCMV plasmid DNA preparation could be determined by the O.D. 260/280 ratio, which fell between 1.7-1.9 and constituted a purified DNA. If the value had been lower, the preparation would have contained contaminants (*e.g.*, protein or phenol).

The purified HCMV plasmid DNA concentration (ng/µl) was calculated to convert into a number of HCMV copies by using the mathematical correlation and formulas as follows:

$$\frac{6 \times 10^{23} \text{ (copies/mol)} \times \text{concentration (g/µl)}}{\text{MW (g/mol)}} = \text{amount (copies/µl)}$$

MW = (number of base pairs) x (660 daltons/base pairs)

1 mol = 6×10^{23} molecules (copies)

2.6.2 Quantitation of the human genomic DNA control by the fluorescence assay

The concentration of human genomic DNA control was measured by the DNA fluorescence assay using the Hoefer DyNA Quant 200 fluorometer. Bisbenzimide, commonly known as Hoechst 33258 (H33258) dye, exhibits changes in fluorescence characeteristics in the presence of DNA that allow accurate DNA quantitation. In the absence of DNA, the excitation spectrum of H33258 peaks at 356 nm and the emission spectrum peaks weakly at 492 nm. In the cuvette well, the sample is exposed to filtered light (365±7 nm) from a mercury lamp. This light excites the DNA-dye complex, causing light that peaks at 458 nm emission. An emission filter in front of the photodetector allows only 460 nm, ± 15nm of fluorescence to register.

Two microliters of the standard (100 ng/µl) and sample DNA was diluted in 2 ml of assay solution that had H33258 dye in the component. Then, the light (458 nm) emitted from the DNA-H33258 dye complex in the mixture was measured by the Hoefer DyNA Quant 200 Fluorometer. The quatity of DNA was calculated by the Hoefer DyNA Quant 200 Fluorometer using the following equation:

$$\text{Quantity of sample DNA (ng/µl)} = \frac{\text{O.D.}_{458} \text{ of sample DNA} \times \text{Standard concentration (100 ng/µl)}}{\text{O.D.}_{458} \text{ of standard}}$$

The calculation value was shown by the Hoefer DyNA Quant 200 Fluorometer, which indicated a direct DNA quantitation (ng/µl).

2.7 Dot blot hybridization technique

2.7.1 Labeling of the oligonucleotide probe

The probe was labeled using a 3' end digoxygenin (DIG) labeling kit (Roche Diagnostics, Germany). The process was performed as recommended by the manufacture. In brief, 100 pmol oligonucleotide probe in 10 µl of sterile double distilled water was added to a reaction vial. Then, the labeling reagent consisting of a reaction buffer of 4 µl, $CoCl_2$-solution of 4 µl, DIG-dUTP/dNTP tailing mixture of 1 µl, and Terminal transferase (50U) of 1 µl, was added. The reaction vial was incubated at 37 °C for 15 min, then placed on ice. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0), and the mixture was stored at 4 or -20 °C.

2.7.2 Determination of the labeling efficiency

The labeled oligonucleotide probe and the tailed control oligonucleotide were prepared in serial dilution as 100, 30, 10, 3, and 1, 0 fmol/µl, and 100, 50, 25, 12.5, and 0 fmol/µl, respectively. One thousand five hundred microliters of each oligonucleotide concentration were dotted onto nylon membrane (Nytran supercharge nylon transfer membrane, Schleicher & Schuell Inc., USA.), and left to dry at room temperature. DNA was cross-linked to the membranes by exposure to ultraviolet light (UV, 0.51 J/cm^2) for 51 seconds, and stored at 4 °C until needed. The membrane was transferred into a plastic container with 20 ml of maleic acid buffer, incubated under shaking for 2 min at 15-25 °C, and the solution was discarded. The membrane was blocked in 10 ml of blocking solution for 30 min at room temperature. One microliter of 1:5,000 anti-digoxygenin alkaline phosphatase antibody (Boehringer Mannheim) was added into 5 ml of blocking solution, and the membrane was incubated at room temperature for 30 min. The membrane was then washed in 1x washing buffer twice for 15 min at room temperature, and equilibrated for 2 min in the detection buffer. A color development reaction was performed using

NBT/BCIP, a 1:5,000 color substrate diluted in detection buffer. This reaction was carried out at room temperature under a light-proof box for 50 min. The reaction was stopped by washing the membrane with sterile double distilled water for 30 min. The membrane was then left to air dry.

2.7.3 Dot blotting, hybridization and detection

The nylon membrane and Whatman no.1 filter paper were cut to the size of the blotting apparatus and immersed in 10x SSC for 5 min. The filter paper was placed first in the apparatus and a nylon membrane was put on top of the paper. The lid was put on and latched into place. The blotting apparatus was hooked up to a vacuum source, and the wells were rinsed with 100 µl of 20x SSC. Fifteen microliters of the second round PCR products were mixed with 40 µl of distilled water. The mixture was heated at 100 °C for 20 min then added to the wells. Each well was rinsed with 100 µl of cold 20x SSC under vacuum for 20 min. The filter was removed. The membrane was dried on air, exposed to UV (0.51 J/cm^2) for 51 seconds, and stored at 4 °C until needed. In the hybridization steps, the membrane was pre-heated with 10 ml of hybridization solution at 42 °C for 1 hour. The pre-hybridization solution was siphoned off and the probe/hybridization solution mixture (0.1-10 pmol/ml) added immediately to the membrane. The membrane and hybridization solutions were incubated in a hybridization oven at 42 °C overnight. Then, the membrane was washed twice in 10 ml of 2x SSC with 0.1% SDS for 5 min at 15-25 °C and twice in 10 ml of 0.1x SSC with 0.1% SDS for 30 min at 50 °C in the hybridization oven. It was washed again in the hybridization oven with 10 ml of 1x washing buffer at 30 °C for 5 min. This membrane was blocked by merging in 10 ml of blocking solution for 30 min at room temperature. Then it was merged in 5 ml of blocking solution containing one microliter of 1:5,000 anti-digoxygenin alkaline phosphatase antibody (Boehringer Mannheim), and incubated at room temperature for 30 min. The membrane was washed in 10 ml of 1x washing buffer twice for 15 min at room temperature and equilibrated for 5 min in 10 ml of detection buffer. A color development reaction was performed using NBT/BCIP, a 1:5,000 color substrate diluted in detection buffer. This reaction was carried out at room temperature under a light-proof box for 50 min, and the reaction was stopped by washing the membrane for 30 min with sterile double distilled water, and air drying.

The presence of a dark brown dot on the nylon membrane was considered as positive.

2.8 Optimization of the nested PCR and duplex PCR condition

2.8.1 Optimization of the nested PCR conditions for the detection of HCMV DNA

The standard HCMV MIE gene fragment was prepared in 10-fold serial dilutions containing 1 to 10^{10} DNA copies. These various HCMV MIE gene fragment concentrations were used in all experiments. To determine the sensitivity of the conventional nested PCR assay, the first and second round PCR mixtures were performed using the 10^{2}-10^{10}copies of the HCMV MIE gene fragment for the first round PCR and the 1-10^{5} copies for the second.

The conventional nested PCR conditions were optimized to minimize time consumption and improve the sensitivity of the HCMV MIE gene fragment amplification. The processes of optimization for both the first and second round PCR were set up as follows:

2.8.1.1 Optimization of the annealing temperature

The annealing temperature was sixty-five degrees Celsius for the amplification of the HCMV MIE gene fragment by conventional nested PCR. To optimize the annealing temperature, four temperatures, 56°, 58°, 60°, and 65 °C, were tested. Then, the yield and specificity of the PCR product was observed, and the efficiency of each annealing temperature determined. The optimal annealing temperature was selected for use in the next step.

2.8.1.2 Optimization of the annealing time

The annealing time was two minutes for the amplification of the HCMV MIE gene fragment by conventional nested PCR. To reduce the annealing time, a cycle of 1 min was tested. The yields of PCR products from both assays were compared. Then, the optimal annealing time was selected for use in the next step.

2.8.1.3 Optimization of the PCR step

According to the conventional PCR condition, an initial step of one incubation cycle at 94 °C for 5 min, 65 °C for 30 seconds, and 72 °C for 30 seconds, had been performed prior to the 35 PCR amplification cycles. To minimize time consumption, the PCR program without the initial step was tested. The yield of both PCR conditions was observed and the appropriate condition was selected for use in the next step.

2.8.1.4 Optimization of the $MgCl_2$ concentration

A 1.5 mM concentration of $MgCl_2$ was used in the conventional PCR reaction. To optimize the PCR condition, various $MgCl_2$ concentrations (1.0. 1.5, 2.0, 2.5, 3.0, 3.5 and 4 mM $MgCl_2$/reaction) were tested for both the first and second round PCR. The yield of PCR product from each $MgCl_2$ concentration was observed and the optimal $MgCl_2$ concentration was selected for use in the next step.

2.8.1.5 Optimization of the dNTP concentration

The 0.2 mM of each dNTP was added to the conventional PCR. To optimize the PCR condition, four concentrations of 0.1, 0.2, 0.3, and 0.4 mM of dNTP were tested for both the first and second round PCR. The yield of PCR product from each dNTP concentration was observed and the optimal dNTP concentration was selected for use in the next step.

2.8.1.6 Determination of optimized nested PCR assay sensitivity

To determine the sensitivity of the optimized PCR assay, the first and second round PCR conditions were performed. The 0.1 to 10^7 HCMV DNA copies were amplified by optimized nested PCR conditions. The first and second round (nested) PCR products from each DNA template concentration were subjected to 1.5% agarose gel electrophoresis. The

lowest HCMV DNA copies that could be detected by ethidium bromide staining were recorded. The methods of PCR product detection, ethidium bromide staining versus dot blot hybridization assay, were also compared. The method that provided a higher sensitivity was considered.

2.8.2 Optimization of the duplex PCR conditions for detection of the HCMV MIE and beta-globin gene

The serial 10-fold concentration covering 0.1 to 10^7 copies of HCMV DNA controls, and the amounts, 1, 10, 50, 100 and 500 ng of human genomic DNA controls were used to optimize the duplex PCR conditions.

Optimization of the first round PCR conditions for the amplification of the beta-globin gene and the MIE gene fragment in one tube was set up as follows:

2.8.2.1 Optimization of the annealing temperature for amplification of the HCMV MIE and beta-globin gene in a single tube

One hundred copies of HCMV DNA combined with 100 ng of human genomic DNA were used to optimize the annealing temperature of the duplex PCR condition.

The annealing temperatures were fifty-six and sixty-five degrees Celsius for amplification of the beta-globin gene and HCMV DNA by conventional PCR assay, respectively. To optimize the duplex PCR condition, different temperatures (56 °, 58 °, 60 °, and 65 °C) were tested. Then, the yield of both the HCMV DNA and beta-globin gene from each annealing temperature was observed and the optimal annealing temperature was selected for use in the next step.

2.8.2.2 Determination of duplex PCR sensitivity in the detection of the beta-globin gene and HCMV MIE gene fragment

The fixed concentrations of human genomic DNA (50, 100 or 500 ng) were separately combined with 1-10^8 copies of the HCMV MIE gene fragments and subjected to

the first round PCR assay. The lowest detectable amount of HCMV MIE gene fragments in the presence of each human genomic DNA concentration, which provided a balanced amplification of both templates, was recorded.

2.8.2.3 Determination of the optimal amount of beta-globin gene and HCMV MIE gene fragment placed into the positive control tube of the duplex PCR assay

The lowest detectable amount of HCMV MIE gene fragments in the presence of human genomic DNA (from 2.8.2.2) was combined with various concentrations of human genomic DNA (1, 10, 20, 30, 40 or 50 ng) and then subjected to the first round duplex PCR assay. The appropriate concentration ratio was selected for use as a positive control.

2.8.2.4 Optimization of the primer concentration for the amplification of the beta-globin and HCMV MIE gene

A primer concentration of 0.4 μM per reaction was previously used in duplex PCR amplification. Since an increasing amount of DNA template might interfere with primer activity, varied primer concentrations were tested. The 10^5 copies of HCMV DNA were combined with 40 ng of human genomic DNA and subjected to the first round duplex PCR assay using an equal concentration of 0.2, 0.4 and 0.6 μM of each primer. The optimal primer concentration, which effectively amplified the beta-globin and HCMV MIE gene was selected for use in the next step.

2.8.2.5 Determination of co-solvent enhancement of the PCR product

The amount of 0.1, 1, 10 and 100 HCMV DNA copies, and a fixed concentration of 40 ng human genomic DNA, were used to define the ability of co-solvent in enhancing the optimized duplex PCR. In the optimized PCR, 10% glycerol, and 2.5% and 5% formamide were added. The yields of PCR product after adding each co-solvent were observed.

2.8.2.6 Determination of optimized duplex nested PCR assay sensitivity

The serial 10-fold concentration covering 1 to 10^7 copies of the HCMV MIE gene were used to define the sensitivity of the HCMV MIE gene fragment amplification when using the optimized duplex nested PCR conditions.

Finally, the optimized duplex nested PCR was used to detect the HCMV infection in the retinitis and transplant patients.

2.9 HCMV DNA detection in the clinical specimens by the optimized duplex PCR

The ocular specimens were studied from HCMV suspected retinitis and control patients , as well as the PBMCs and plasma of transplantation patients/donors. The HCMV DNA was detected by the optimized duplex PCR. The PCR mixture comprised 0.4 µM primers, 0.3 mM of dNTP, 3.0 mM of $MgCl_2$, an annealing temperature at 56 °C, annealing time of 1 min, 1.25 U of *Taq* DNA polymerase, DNA template, 40 ng of human genomic DNA, and distilled water that was added to make a final volume of 50 µl. The PCR product was detected by 1.5% agarose gel electrophoresis and ethidium bromide staining. The specimen that showed a PCR product of 170 bp, after the second round PCR, was considered as positive HCMV DNA detection. The specimen that showed a PCR product of 251 bp, after the first round PCR, was considered as a specimen containing a high amount of DNA.

2.10 Data analysis

2.10.1 Comparison of sensitivity and specificity of conventional nested PCR and optimized duplex nested PCR for the diagnosis of HCMV retinitis

The term of "sensitivity and specificity" in this study referred to the correlation with the clinical diagnosis. To define the sensitivity of the PCR assay, the percentage of positive HCMV DNA in vitreous humor, aqueous humor and conjunctival scraping after using

the conventional and optimized nested duplex PCR were calculated. To define the specificity of the PCR assay, the number of positive samples from the control patients was considered.

The percentages of positive samples in each type of specimen, when using the same or different PCR assays, were compared. Furthermore, the difference between the percentage of positive samples from each type of specimen, when the same PCR method was used, was statistically compared using the Z test at the level of significance, (α) = 0.05.

2.10.2 Comparisons of HCMV DNA detection in PBMCs, plasma and the presence of anti-HCMV IgM in plasma of transplantation patients

The HCMV DNA was detected in the PBMCs and plasma of transplant recipients and donors by the optimized duplex nested PCR. The anti-HCMV IgM was detected by ELISA (IgM capture technique, Organon, USA) at the Central Laboratory, Maharaj Nakorn Chiang Mai Hospital. The presence of HCMV DNA in the PBMCs and plasma of each patient and donor pair was observed before transplantation. Then, the patients were monitored after kidney transplantation by HCMV DNA and HCMV anti-IgM detection. The result of HCMV DNA detection was compared to the presence of anti-HCMV IgM. The efficiency of both methods in the monitoring of HCMV infection was analyzed from the clinical signs and symptoms.