CHAPTER IV

RESULTS

1. Preparation of standard controls

1.1 Preparation of HCMV MIE gene fragment control by the PCR cloning method

The HCMV MIE gene fragments were amplified from the HCMV (AD169) infected cell lysate by the conventional first round PCR amplification using PF and PR primers. The first round PCR product was confirmed by the second round PCR using the NFP and NRP primers. The PCR products from both rounds were detected by 1.5% agarose gel electrophoresis with the ethidium bromide staining. The PCR product could be directly visualized as DNA bands of 351 bp for the first round PCR (Figure 6) and 170 bp for the second round PCR (Figure 7).

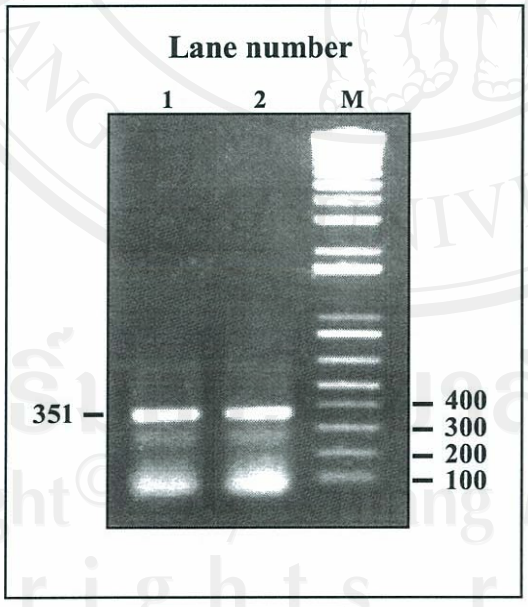

Figure 6 The amplification of the HCMV MIE gene fragment by the conventional first round PCR. Lane M shows the DNA marker, lane 1 and 2 show the 351 bp PCR product of the HCMV MIE gene.

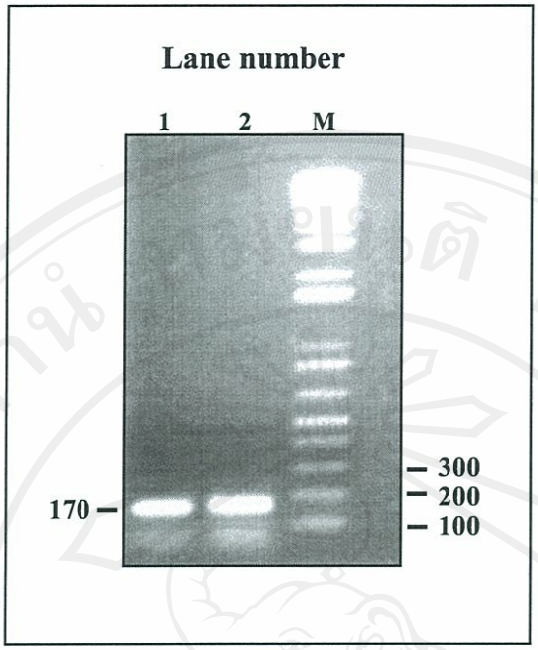

Figure 7 The amplification of the HCMV MIE gene fragment by the conventional second round PCR. Lane M shows the DNA marker, lane 1 and 2 show the 170 bp PCR product of the HCMV MIE gene.

The amplified MIE gene fragment of the first round PCR was purified and cloned into the pGEM®-T Easy vector plasmid (Promega corporation, USA). These plasmids were transformed to the competent *E. coli* and the transformed colonies were selected by using the PCR assay. A part of the well-isolated transformed colony was added directly to the PCR for an amplification of the MIE gene fragment by the conventional first round PCR. The successfully transformed cells provided an approximately 351 bp PCR product, as shown in Figure 8.

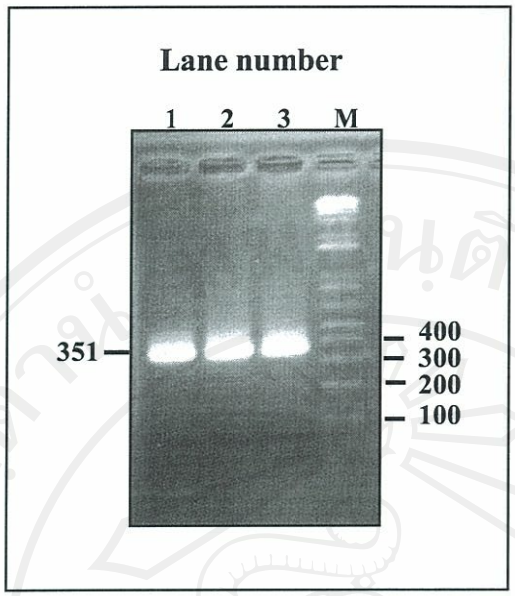

Figure 8 The amplification of the HCMV MIE gene fragment from colonies of transformed *E. coli* by the conventional first round PCR. Lane M shows the DNA marker, lane 1, 2, and 3 show the 351 bp PCR product of the HCMV MIE gene from different colonies.

The plasmid DNA was also purified from the recombinant *E. coli* by the miniprep method. The plasmid DNA with the MIE DNA fragment was detected by 1.0% agarose gel electrophoresis with ethidium bromide staining. The pGEM® -T Easy vector plasmid had a length of approximately 3,015 bp. After insertion of the MIE gene fragment, it became a circular plasmid of approximately 3,366 bp, as shown in Figure 9. The presence of the MIE gene fragment insert was confirmed by the conventional first round PCR, in which a 351 bp PCR product was detected, as shown in Figure 10.

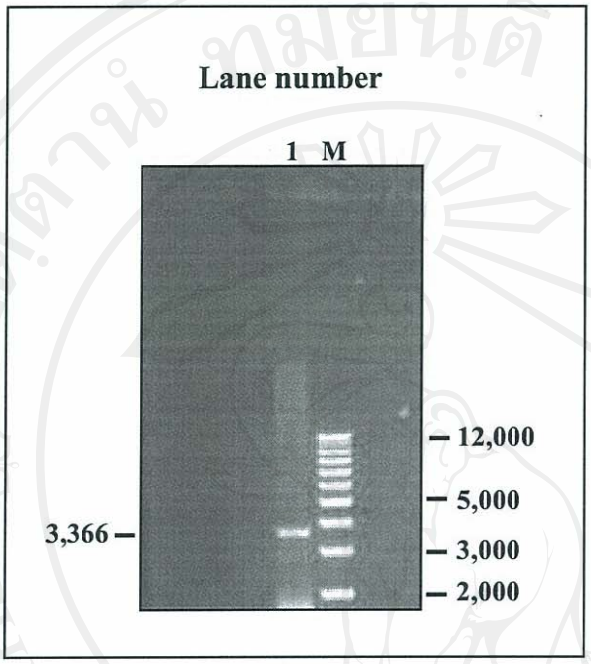

Figure 9 The circular plasmid with the HCMV MIE gene fragment insert was purified by the miniprep technique. Lane M shows the DNA marker, lane 1 shows a 3,366 bp of the pGEM ®-T Easy vector plasmid (3,015 bp) with the HCMV MIE gene fragment (351 bp) insertion.

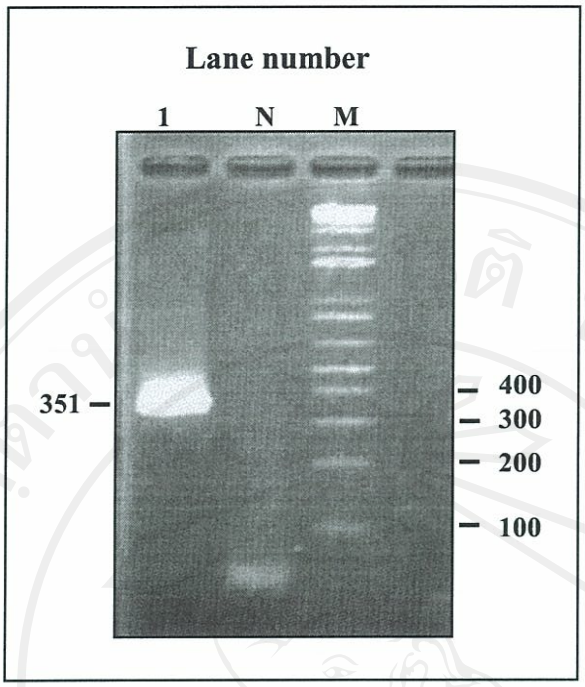

Figure 10 The amplification of the HCMV MIE gene fragment from miniprep plasmid DNA by the conventional first round PCR. Lane M shows the DNA marker, lane N shows a negative control, and lane 1 shows the 351 bp of an amplified HCMV MIE gene fragment.

The recombinant plasmid DNA with the confirmed MIE gene fragment insert was prepared from 50 ml of recombinant *E. coli* by the QIAGEN Plasmid Midi Kit (Qiagen, Germany). This purified DNA was used as the standard HCMV MIE gene fragment control throughout this study.

The quantity of the standard HCMV MIE gene fragment control was measured using the spectrophotometry method and it was found to be 176 ng/µl. The calculation was shown in the following:

The absorbance at 260 nm = 0.0704

The absorbance at 280 nm = 0.0385

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

= 0.0704 x 50 x 50

= 176 ng/µl

The purity of DNA could be determined by the ratio of the O.D. at 260 and 280. The O.D. ratio of DNA with good purity should not be lower than 1.65. Hence, the purity of this DNA preparation was acceptable, as the O.D. ratio was found to be 1.83.

The quantity of the pGEM® -T Easy vector plasmid with HCMV MIE DNA insert, in terms of a copy number, was calculated. The amount of this plasmid DNA, at a concentration of 176 ng/µl, was found to be approximately 5.0×10^{10}/µl. The following mathematical correlation and formulas were used.

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

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

1 mol = 6×10^{23} molecules (copies)

Then;

$$\frac{6 \times 10^{23} \times 176 \times 10^{-9}}{3{,}366 \times 660} = 4.75 \times 10^{10} \text{ copies/µl}$$

$$\approx 5.0 \times 10^{10} \text{ copies/µl}$$

1.2 Human genomic DNA control

The human genomic DNA was purified from 10 ml of whole blood. It was used as a specimen extraction control. The yield of human genomic DNA measured by the fluorescence assay was 210 ug/1.5 ml (140 ng/µl).

The successful detection of beta-globin genes was an indicator of the presence of human genomic DNA in the PCR mixture. Therefore, to evaluate the efficiency of the beta-globin gene primers, various concentrations of human genomic DNA were tested.

The human genomic DNA was prepared in various concentrations of 1, 10, 100, and 500 ng. The beta-globin gene from these human genomic DNA was amplified by the

conventional PCR assay using BF and BR primers. The 251 bp PCR product was detected by 1.5% agarose gel electrophoresis with ethidium bromide staining. It was found that a minimum of 10 ng of human genomic DNA could be detected by this method (Figure 11).

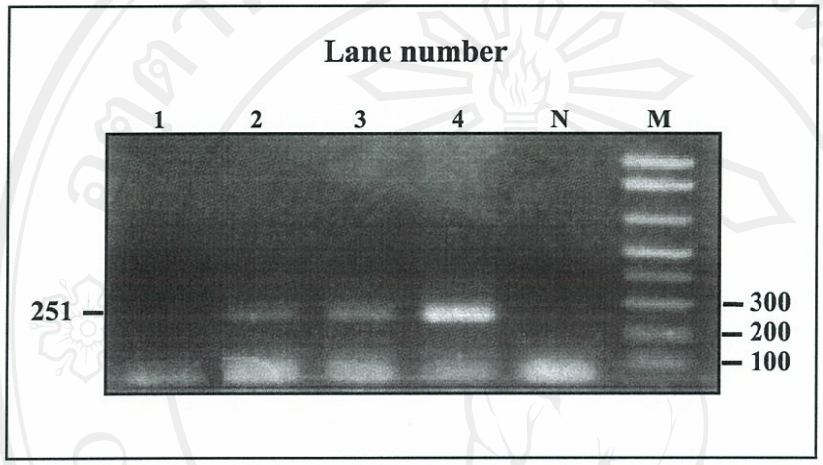

Figure 11 The amplification of a beta-globin gene from 1 to 500 ng of human genomic DNA controls by the conventional PCR assay. Lane 1-4 show the results of 1, 10, 100 and 500 ng beta-globin gene amplification. Lane 2, 3, and 4 show the 251 bp beta-globin gene PCR product from 10, 100 and 500 ng of human genomic DNA controls, respectively. Lane M shows the DNA marker and lane N shows a negative control.

2. Determination of conventional PCR assay sensitivity

The sensitivity of the first round conventional PCR assay was found to be 10^5 copies. The detection of the 351 bp PCR product by agarose gel electrophoresis and ethidium bromide staining was possible only at the lowest amount of 10^5 copies, as shown in Figure 12.

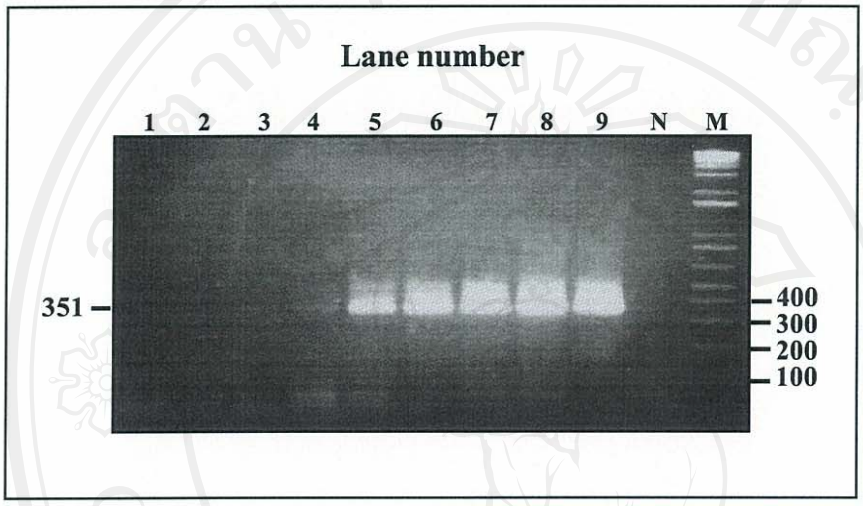

Figure 12 The sensitivity of the conventional first round PCR assay. Ten-fold concentration of 10^2 to 10^{10} copies of the HCMV MIE gene were amplified by the conventional first round PCR. Lanes 4-9 show the 351 bp PCR products from 10^5-10^{10} copies of the HCMV MIE gene, respectively. Lane M shows the DNA marker, and lane N shows a negative control.

The sensitivity of the second round conventional PCR assay was found to be 10^2 copies. The detection of the 170 bp PCR product by agarose gel electrophoresis and ethidium bromide staining was possible only at the lowest amount of 10^2 copies, as shown in Figure 13.

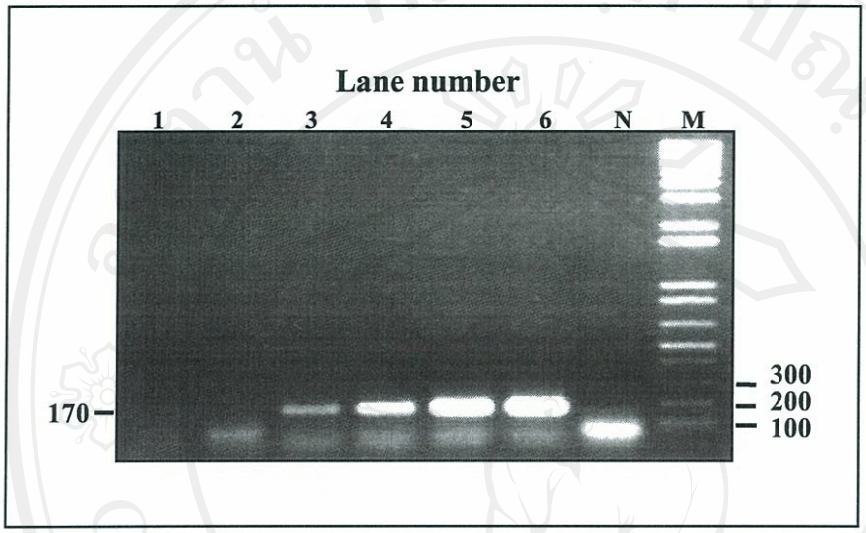

Figure 13 The sensitivity of the conventional second round PCR assay. Ten-fold concentration of 1 to 10^5 copies of the HCMV MIE gene were amplified by the conventional second round PCR. Lanes 3-6 show the 170 bp PCR products from 10^2-10^5 copies of the HCMV MIE gene, respectively. Lane M shows the DNA marker, and lane N shows a negative control.

3. Optimization of the PCR conditions

The results found in the optimization experiments were as follows.

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

Serial 10-fold dilutions covering 0.1-10^7 copies of HCMV control were used to define the sensitivity achieved.

3.1.1 Optimization of the annealing temperature

Among 4 temperatures tested (56, 58, 60, and 65 °C), every one showed the PCR products of a minimal detection at 10^5 and 10^2 copies in the first and second round PCR, respectively. The lower temperature did not show a marked difference, therefore, the annealing temperature at 65 °C, which was similar to the conventional PCR assay, was selected for use in the next step.

3.1.2 Optimization of the annealing time

The comparison between a conventional annealing time of 2 min and a shorten time of 1 minute was performed. It was found that a shorten time of 1 min gave the same sensitivity. Therefore, to minimize the time consuming of the PCR assay, an annealing time of 1 minute was selected.

3.1.3 Optimization of the PCR step

The initial step of 94 °C (5 min), 65 °C (30 seconds) and 72 °C (30 seconds), prior to the 35 HCMV PCR cycles, was omitted in this experiment. However, it was found that the HCMV PCR assay with or without an initial step provided the same sensitivity. Therefore, to minimize time consumption, the PCR assay without an initial step was selected.

3.1.4 Optimization of the $MgCl_2$ concentration

Among the $MgCl_2$ concentrations tested (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM/reaction), the concentration of 3.0 mM $MgCl_2$ was found to provide the best result for both the first round PCR (Figure 14) and second round PCR (Figure 15) and it was therefore selected.

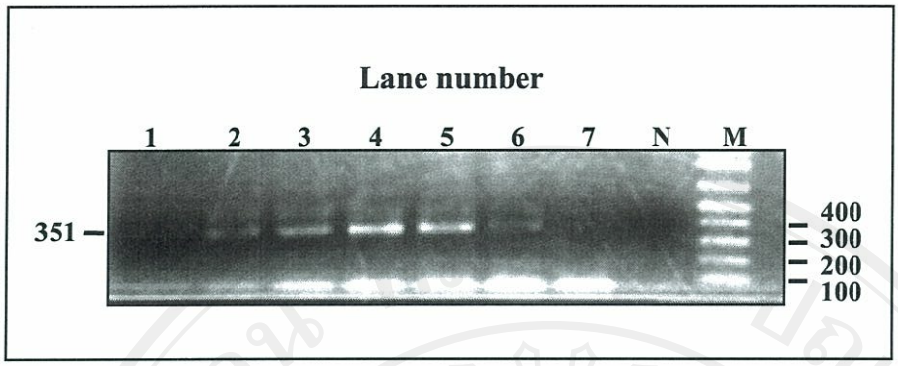

Figure 14 The amplification of 10^6 copies of the HCMV MIE gene fragment (351 bp) in the presence of various $MgCl_2$ concentrations by the first round PCR. Lanes 1-7 show $MgCl_2$ concentrations of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM, respectively. Lane M shows the DNA marker, lane N shows a negative control.

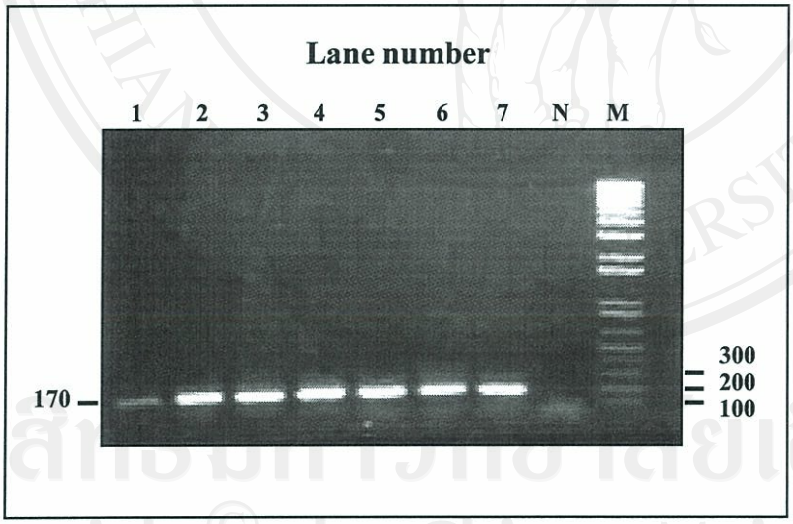

Figure 15 The amplification of the HCMV MIE gene fragment (170 bp) in the presence of various $MgCl_2$ concentrations by the second round PCR. Lanes 1-7 show $MgCl_2$ concentrations of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM, respectively. Lane M shows the DNA marker, lane N shows a negative control.

3.1.5 Optimization of the dNTP concentration

Among various dNTP concentrations tested (0.1, 0.2, 0.3, and 0.4 mM/reaction), the presence of the 0.3 mM dNTP in the first and second round PCR provided the highest yield of the PCR product. The strong bands of PCR products were observed after the first (Figure 16) and second round PCR (Figure 17), when the PCR mixture contained 0.3 mM dNTP, which was therefore selected.

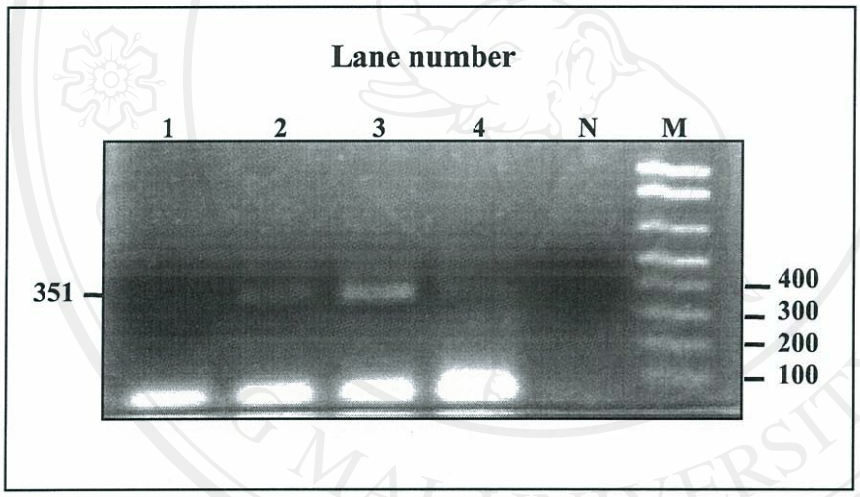

Figure 16 Comparisons in the first round HCMV MIE gene (10^6 copies) amplification efficiency after adding various dNTP concentrations. Lanes 1-4 show dNTP concentrations of 0.1, 0.2, 0.3, and 0.4 mM, respectively. Lane M shows the DNA marker, lane N shows a negative control.

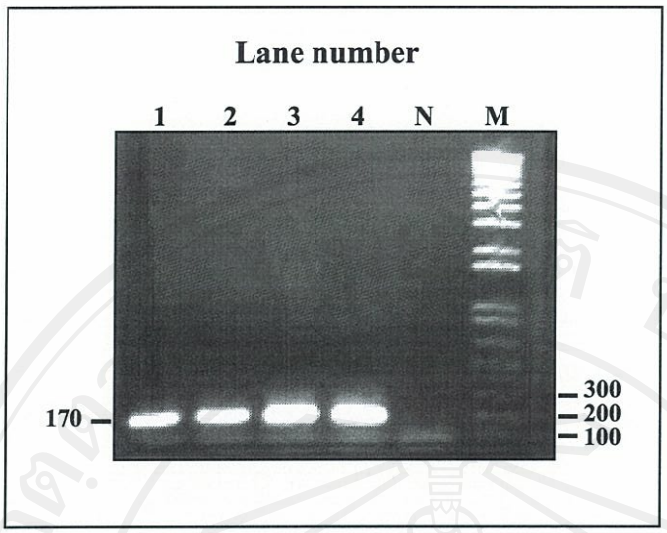

Figure 17 Comparisons in the second round HCMV MIE gene (10^6 copies) amplification efficiency after adding various dNTP concentrations. Lanes 1-4 show dNTP concentrations of 0.1, 0.2, 0.3, and 0.4 mM, respectively. Lane M shows the DNA marker, lane N shows a negative control.

3.1.6 Determination of optimized nested PCR assay sensitivity

The serial 10-fold dilution covering 0.1 to 10^7 copies of the HCMV MIE gene were used to define the sensitivity of the HCMV MIE gene fragment amplification.

The optimized PCR mixture consisted of 0.3 mM of dNTP, 3.0 mM of $MgCl_2$, 0.4 µM of primer, PCR buffer (10 mM Tris-HCl, pH 9.0 at 25 °C, 50 mM KCl and 0.1% Triton X-100), 1.25 U of *Taq* DNA polymerase, DNA template, and distilled water that was added to make a final volume of 50 µl. The DNA amplification was performed in 35 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min; followed by a final cycle at 72 °C for 5 min.

It was found that the optimized first round PCR assay could detect a minimum amount of HCMV MIE gene fragment at 10^5 copies (Figure 18), whereas the optimized nested PCR assay could detected a minimum amount of 10 copies (Figure 19). The PCR products were detected by the agarose gel electrophoresis and ethidium bromide staining.

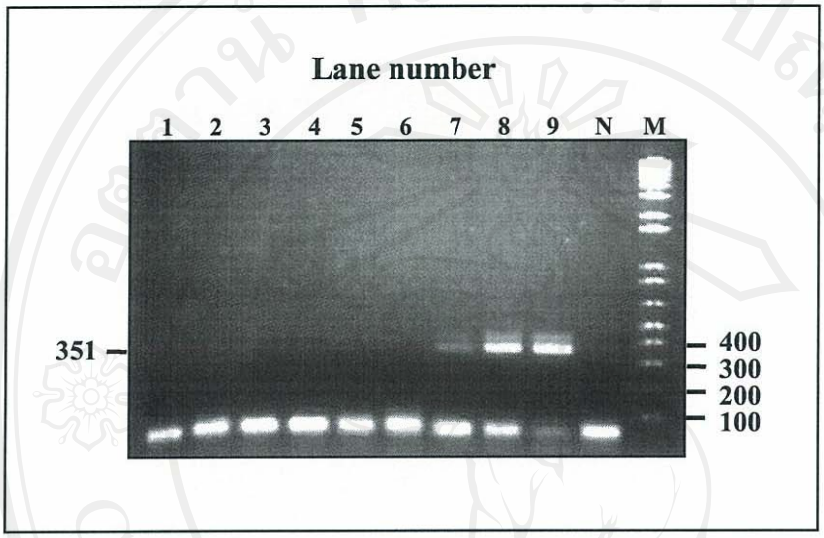

Figure 18 The amplification of a ten-fold concentration of 0.1 to 10^7 copies of the HCMV MIE gene fragment by the optimized first round PCR. Lanes 7-9 show the 351 bp PCR product of 10^5-10^7 copies of the HCMV MIE gene fragment, respectively. A minimum of 10^5 copies (lane 7) of the HCMV MIE gene could be detected. Lane M shows the DNA marker, and lane N shows a negative control.

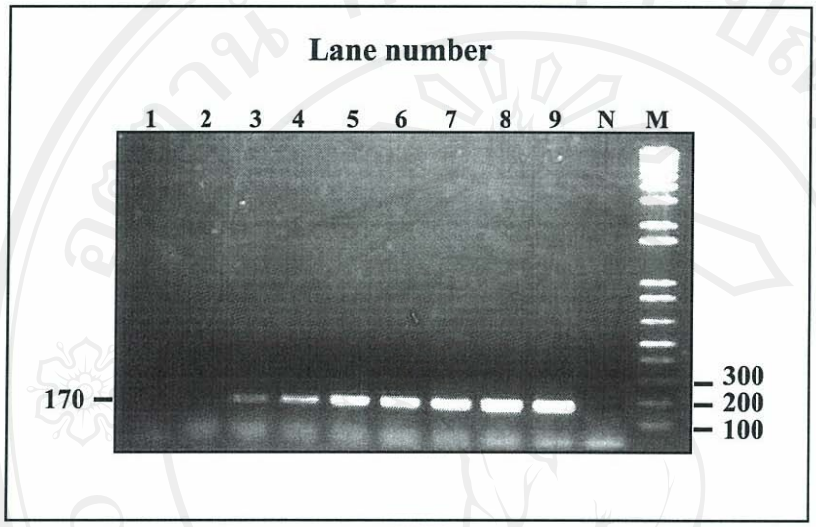

Figure 19 The amplification of a ten-fold concentration of 0.1 to 10^7 copies of the HCMV MIE gene fragment by the optimized second round PCR. Lanes 3-9 show the 170 bp PCR product of 10-10^7 copies of the HCMV MIE gene fragment, respectively. A minimum of 10 copies (lane 3) of the HCMV MIE gene could be detected. Lane M shows the DNA marker, and lane N shows a negative control.

3.1.7 Comparison of the methods for PCR product detection

The nested PCR products from 3.1.6 were detected by 2 methods; agarose gel electrophoresis with ethidium bromide staining and dot blot hybridization. It was found that a minimum of 10 HCMV MIE gene copies could be detected by dot blot hybridization (Figure 20), which is comparable to the method of gel electrophoresis and ethidium bromide staining. Therefore, to minimize time consumption and cost, 1.5% agarose gel electrophoresis and ethidium bromide staining was selected as the PCR product detection method throughout this study.

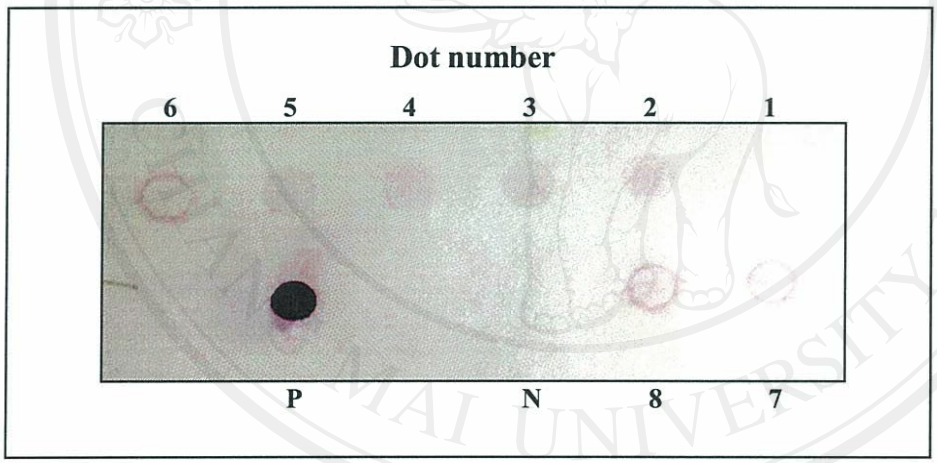

Figure 20 The detection of optimized second round PCR product of 1 to 10^7 HCMV DNA by dot blot hybridization. Dots 1-8 show the results of PCR product detection that correspond to ten-fold concentration of 1-10^7 HCMV DNA copies. N and P show the negative control and standard positive control, respectively.

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

Serial 10-fold dilution, covering 0.1-10^7 copies of the HCMV MIE gene fragment and amounts of 1, 10, 50, 100, and 500 ng of human genomic DNA controls, were used to define the sensitivity achieved.

3.2.1 Optimization of the annealing temperature for amplification of the HCMV MIE gene fragment and beta-globin gene

Human genomic DNA of 100 ng and 100 copies of the HCMV MIE gene fragment were used to optimize the annealing temperature.

Among 4 annealing temperatures tested (56 °, 58 °, 60 °, and 65 °C), only 56 °C could amplify the beta-globin gene. However, all 4 temperatures tested gave a result of HCMV MIE gene fragment amplification that was similar to a single nested PCR.

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

The fixed concentration of human genomic DNA (50, 100, 500 ng) was separately combined to 1-10^8 copies of the HCMV MIE gene fragment and subjected to the first round duplex PCR assay. It was found that 50, 100 and 500 ng concentrations of human genomic DNA in the presence of 10^5, 10^6 and 10^7 copies of the HCMV MIE gene fragment, respectively, provided a balanced amplification of both templates, as shown in Figure 21 (lane 2), Figure 22 (lane 7) and Figure 23 (lane 8), respectively. Therefore, the 10^5 copies of the HCMV MIE gene was selected for use in further optimization steps.

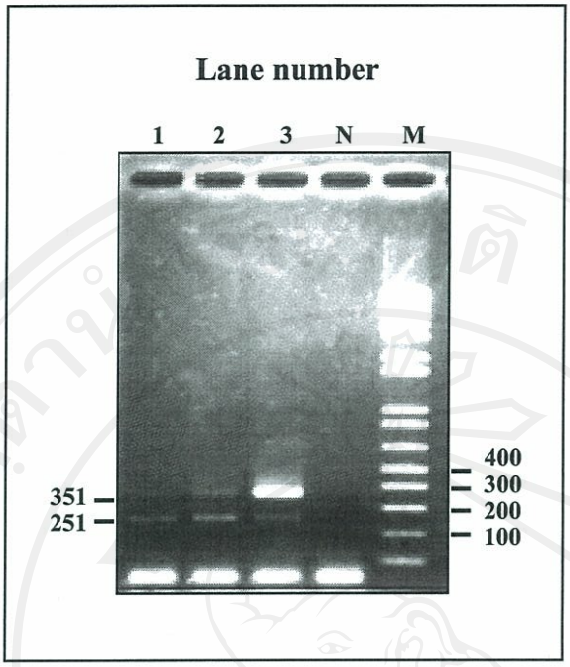

Figure 21 The amplification of 50 ng of human genomic DNA combined with 10^4, 10^5 and 10^6 copies of the HCMV MIE gene fragment in the optimized first round duplex PCR. Lane 2 and 3 show PCR products of the beta-globin (251 bp) and HCMV MIE gene (351 bp), and the duplex PCR composed of 10^5 and 10^6 copies of the HCMV MIE gene fragment, respectively. Lane M shows the DNA marker, and lane N shows a negative control. The amplification balance is shown in lane 2.

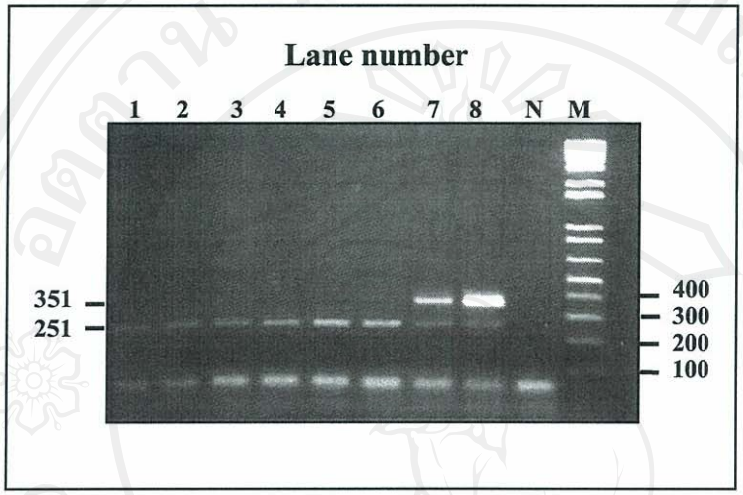

Figure 22 The amplification of 100 ng human genomic DNA combined with ten-fold concentration of 1 to 10^7 copies of HCMV MIE gene fragment in the optimized first round duplex PCR. Lane 1-8 show 251 bp of beta-globin gene PCR product. Lane 7 and 8 show 351 bp of HCMV MIE gene PCR product when the duplex PCR composed of 10^6 and 10^7 copies of HCMV MIE gene fragment, respectively. Lane M shows the DNA marker, and lane N shows a negative control. The amplification balance is shown in lane 7.

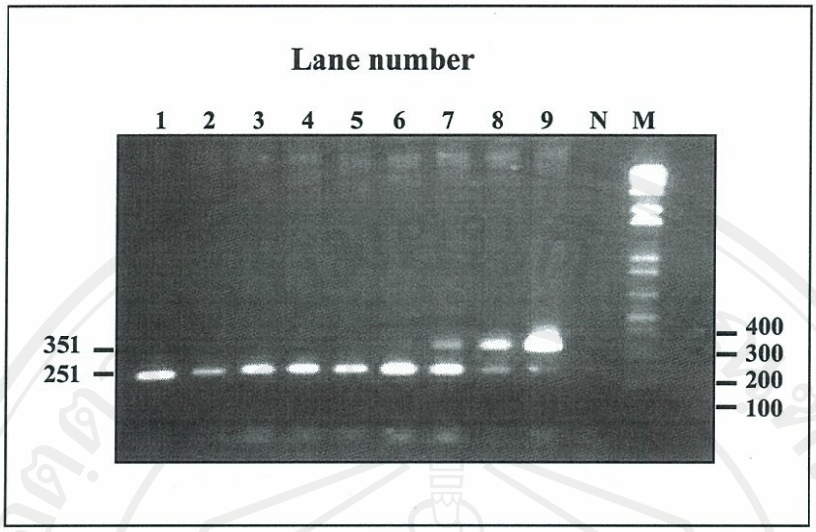

Figure 23 The amplification of 500 ng of human genomic DNA combined with a ten fold concentration of 1 to 10^8 copies of the HCMV MIE gene fragment in the optimized first round duplex PCR. Lanes 1-9 show PCR products of the beta-globin gene (251 bp). Lane 7, 8 and 9 show 351 bp HCMV MIE gene product when the duplex PCR composed of 10^6 to 10^8 copies of HCMV MIE gene fragment, respectively. Lane M shows the DNA marker, and lane N shows a negative control. The amplification balance is shown in lane 8.

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

The fixed concentration of the HCMV MIE gene (10^5 copies) was combined with 1, 10, 20, 30, 40 and 50 ng of human genomic DNA and subjected to the first round duplex PCR assay. It was found that concentrations of 40 ng and 50 ng of human genomic DNA in the presence of 10^5 copies of the HCMV MIE gene provided the highest yield of both PCR amplifications (Figure 24).

The duplex PCR assay could detect a minimum of 20 ng of human genomic DNA, but it provided a very faint band. A tiny band was also found after amplification of 30 ng of human genomic DNA.

Therefore, 10^5 copies of the HCMV MIE gene and 40 ng of human genomic DNA were selected to place into the positive control tube of the duplex PCR assay throughout this study.

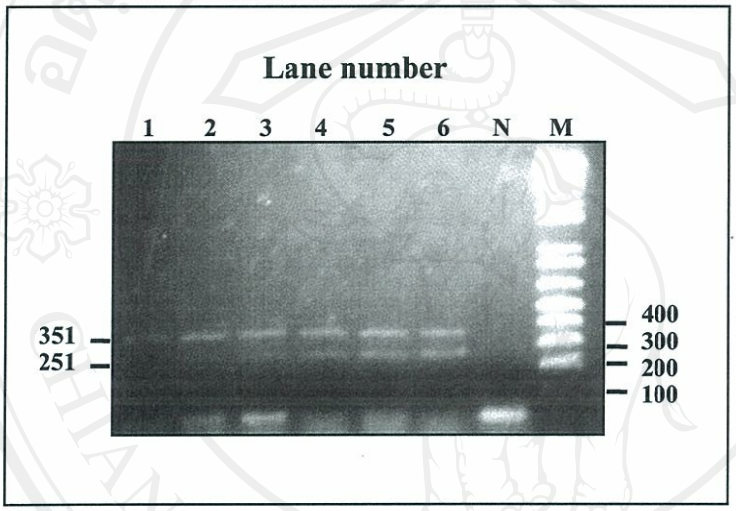

Figure 24 The optimized first round PCR amplification of 1, 10, 20, 30, 40 and 50 ng of the human genomic DNA in the presence of 10^5 copies of the HCMV MIE gene fragment. Lanes 1-6 show PCR products of 1, 10, 20, 30, 40, and 50 ng beta-globin (251 bp) of human genomic DNA and 10^5HCMV MIE gene (351 bp) , respectively. Lane M shows the DNA marker, and lane N shows a negative control. The amplification balance is shown in lane 5 and 6.

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

Among the primer concentrations tested (0.2 μM, 0.4 μM and 0.6 μM), 0.4 μM and 0.6 μM of both primer sets were found to provide the best result for both templates (Figure

25), whereas a 0.2 μM concentration of both primer sets provided a lower yield of PCR product. However, a concentration of 0.6 μM increased the primer-dimer artifact.

Therefore, a 0.4 μM concentration of each primer was selected for use in the duplex PCR throughout this study.

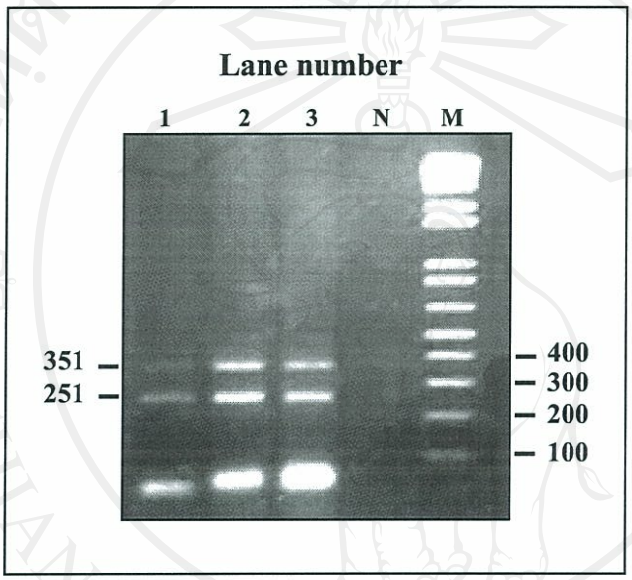

Figure 25 PCR products achieved when different primer concentration of beta-globin gene and HCMV MIE gene primers were used in the duplex PCR assay. Lane 1, 2 and 3 show concentrations of 0.2 μM, 0.4 μM and 0.6 μM for both primer sets, respectively. Lane M shows the DNA marker and lane N shows a negative control.

3.2.5 Determination of cosolvent enhancement of PCR product

The 0.1, 1, 10, and 100 copies of the HCMV MIE gene and 40 ng of human genomic DNA were used to define the ability of 10% glycerol, and 2.5% and 5% formamide in enhancing optimized duplex PCR. The result revealed that 10% glycerol and 2.5% formamide provided no benefit to the PCR, whereas 5% formamide inhibited DNA amplification. Therefore, cosolvent was not added to the PCR mixture.

Finally, the optimized duplex nested PCR was used to detect HCMV infection in retinitis and transplantation patients. The optimized duplex PCR consisted of 0.3 mM of dNTP, 3.0 mM of $MgCl_2$, 0.4 µM of each primer, PCR buffer (10 mM Tris-HCl, pH 9.0 at 25 °C, 50 mM KCl and 0.1% Triton X-100), 1.25 U of *Taq* DNA polymerase, DNA template, and distilled water that was added to make a final volume of 50 µl. The DNA amplification was performed in 35 cycles of 1 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C; followed by a final cycle of 5 min at 72 °C. PCR product was detected by agarose gel electrophoresis and ethidium bromide staining.

The PCR conditions of the conventional and optimized duplex nested PCR are summarized in Table 6.

Table 6 The conditions of conventional and optimized nested PCR for amplification of the HCMV MIE gene fragment and beta-globin gene

Reagent/Condition	Conventional PCR	Beta-globin gene	Optimized condition
	MIE gene		for duplex PCR
Initial denaturation step	Done	Done	**Not done**
Annealing temperature (°C)	65	56	**56**
Annealing time (min)	2	2	**1**
Incubation cycles	35	35	35
Denaturation	94 °C for 1 min	94 °C for 1 min	94 °C for 1 min
Extension	72 °C for 1 min	72 °C for 1 min	72 °C for 1 min
Final cycle	72 °C for 5 min	72 °C for 5 min	72 °C for 5 min
$MgCl_2$ (mM)	1.5	1.5	**3.0**
dNTP (mM)	0.2	0.2	**0.3**
Primer (µM)	0.4	0.4	0.4 of each
Taq DNA polymerase (U)	1.25	1.25	1.25
PCR buffer (µl)	5	5	5

3.2.6 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 assay.

It was found that the optimized duplex first round PCR assay could detect a minimum amount of HCMV MIE gene fragment at 10^5 copies whereas the optimized duplex nested PCR assay could detected a minimum amount of 10 copies (Figure 26) which was comparable to the sensitivity of the optimized nested PCR assay.

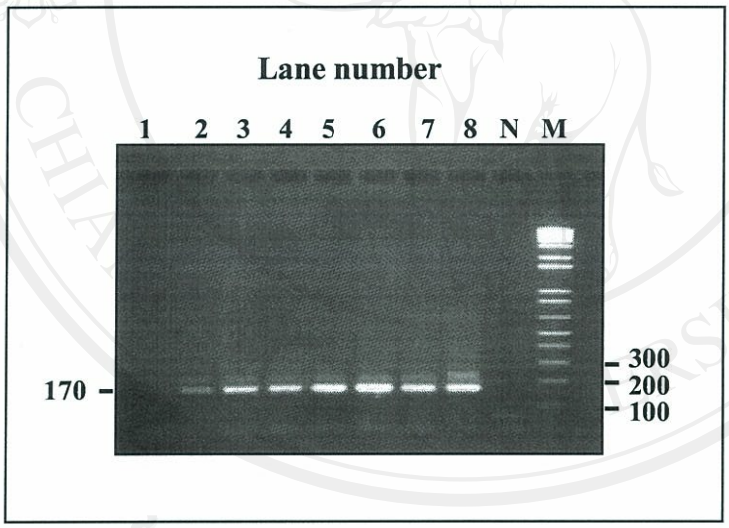

Figure 26 The amplification of a ten-fold concentration of 1 to 10^7 copies of the HCMV MIE gene fragment by the optimized second round duplex nested PCR. Lanes 2-8 show the 170 bp PCR product of 10-10^7 copies of the HCMV MIE gene fragment, respectively. A minimum of 10 copies (lane 2) of the HCMV MIE gene could be detected. Lane M shows the DNA marker, and lane N shows a negative control.

4. Detection of HCMV DNA in patients

4.1 Retinitis patients

4.1.1 Detection of HCMV DNA in ocular samples by conventional and optimized duplex nested PCR

A total of 74 ocular specimens from 13 HCMV suspected retinitis patients and 13 control patients were tested for the presence of HCMV DNA by both conventional and optimized duplex nested PCR.

4.1.1.1 Detection of HCMV DNA in ocular samples by conventional nested PCR

Among 74 ocular samples, 2 vitreous humor specimens showed positive results after the first round of conventional PCR, which meant that those specimens contained a high amount of HCMV DNA. (Figure 27, lane 6 and 7).

However, results from the second round of conventional PCR showed that 1 of 12 (8.3%), 7 of 12 (58.3%) and 12 of 13 (92.3%) samples from conjunctival scraping, aqueous humor , and vitreous humor were positive for HCMV DNA.

An example of the positive and negative result of the HCMV DNA fragment from the second round of conventional PCR is shown in Figure 28.

The results of HCMV DNA detection by conventional nested PCR in each sample of HCMV suspected retinitis patients are shown in Table 7.

Among the control patients, none of 37 samples was positive for HCMV DNA, which showed the high specificity of the test.

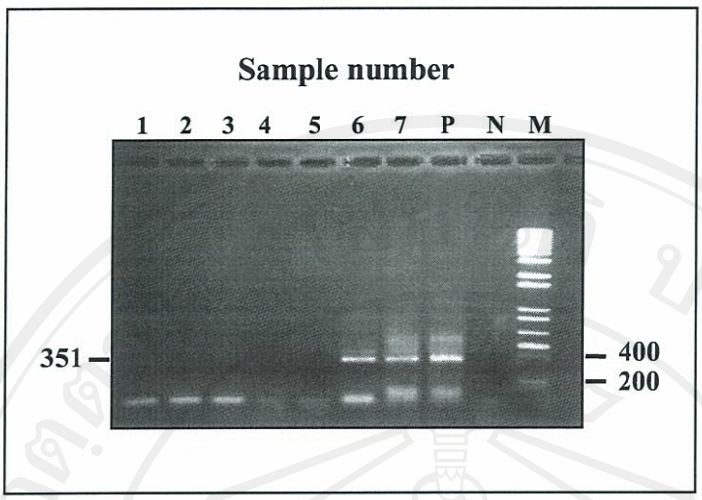

Figure 27 The amplification of HCMV DNA in ocular samples by the first round of conventional PCR. Lanes 1-5 show negative results, whereas lane 6 and 7 show a 351 bp HCMV DNA PCR product. Lane M shows the DNA marker, lane N shows a negative control, and lane P shows a positive control.

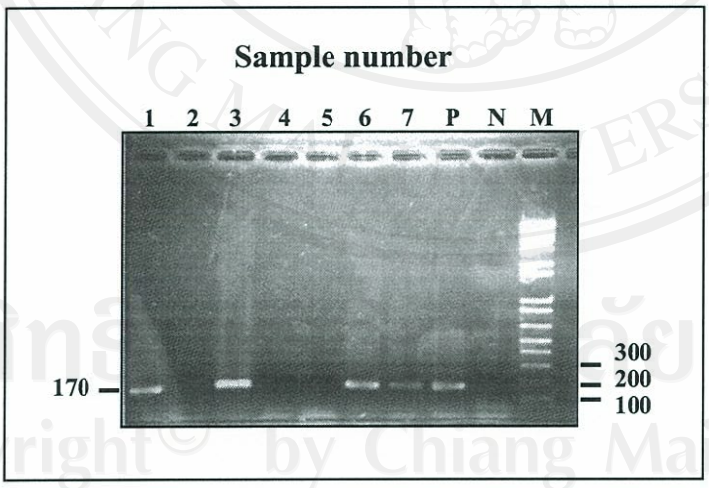

Figure 28 The amplification of HCMV DNA in ocular samples by the second round of conventional PCR. Lane 1, 3, 6, and 7 show 170 bp HCMV DNA PCR products with different intensity. Lane 2, 4 and 5 show negative results. Lane M shows the DNA marker, lane N shows a negative control, and lane P shows a positive control.

4.1.1.2 Detection of HCMV DNA and the beta-globin gene in ocular samples by optimized duplex nested PCR

Among 74 ocular samples, 3 vitreous humor specimens showed positive results after the first round of optimized nested duplex PCR (Figure 29, lane 3, 6 and 7). The conventional nested PCR failed to detect HCMV DNA in a sample in lane 3. However, by using the optimized PCR a faint band at 351 bp could be detected. The 251 bp PCR product of the beta globin gene control was probably seen as a faint band only in the sample in lane 7.

However, the results from the second round of optimized duplex nested PCR showed that 7 of 12 (58.3%), 10 of 12 (83.3%) and 12 of 13 (92.3%) samples from conjunctival scraping, aqueous humor, and vitreous humor were positive for HCMV DNA.

The examples of a positive and negative result of HCMV DNA by the optimized second round duplex nested PCR are shown in Figure 30.

The result of HCMV DNA detection by optimized second round duplex nested PCR in each sample is shown in Table 7. Comparison of positive results after HCMV DNA detection by conventional and optimized duplex nested PCR is shown in Table 8 and Figure 31.

Among the control patients, none of 37 samples was positive for HCMV DNA, which also showed a high specificity of the optimized duplex nested PCR assay.

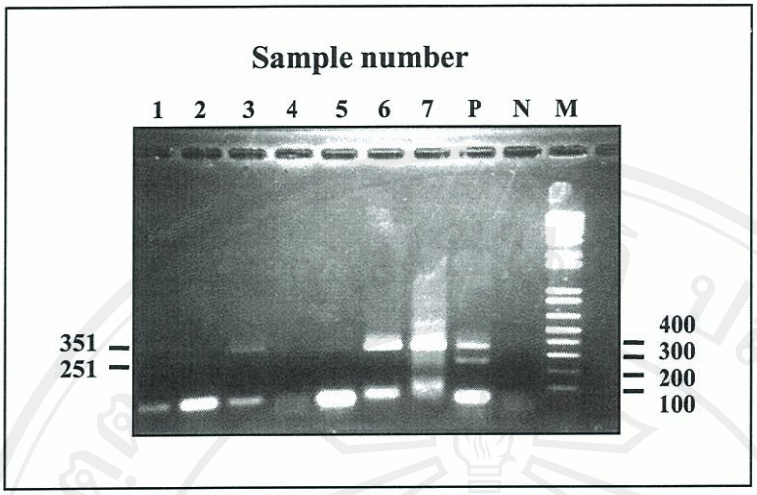

Figure 29 The amplification of HCMV DNA in ocular samples by the optimized first round duplex nested PCR. Lane 3, 6 and 7 show the positive result of a 351 bp HCMV DNA PCR product. Lane 7 probably shows the faint band of a 251 bp beta-globin gene PCR product. Lane 1,2, 4 and 5 show negative results. Lane M shows the DNA marker, lane N shows a negative control, and lane P shows positive controls with 351bp and 251 bp PCR products of the HCMV and beta-globin gene, respectively.

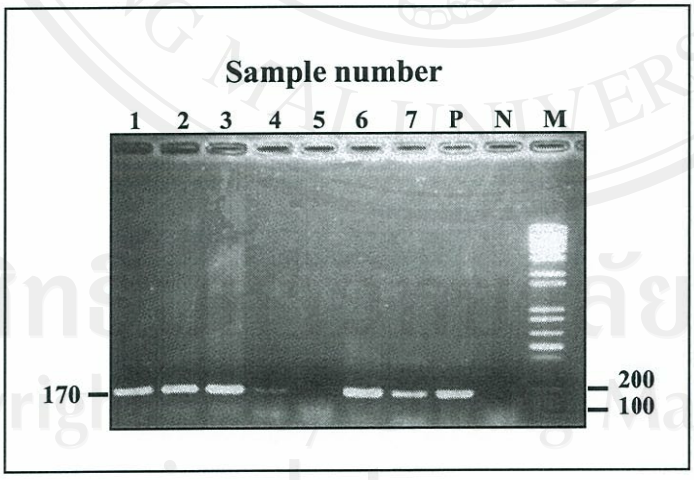

Figure 30 The amplification of HCMV DNA in ocular samples by the optimized second round duplex nested PCR. Lane 1, 2, 3, 4, 6 and 7 show positive results. Lane 5 shows negative results. Lane M shows the DNA marker, lane N shows a negative control, and lane P shows a positive control.

Table 7 The results of HCMV DNA detection by conventional and optimized duplex nested PCR in HCMV suspected retinitis patients

Results / Patient numbers	Conventional nested PCR			Optimized duplex nested PCR		
	Conjunctiva	Aqueous	Vitreous	Conjunctiva	Aqueous	Vitreous
1	neg	neg	**pos**	neg	neg	**pos**
2	neg	**pos**	**pos**	neg	**pos**	**pos**
3	neg	neg	**pos**	**pos**	**pos**	**pos**
4	neg	**pos**	**pos**	neg	**pos**	**pos**
5	neg	**pos**	**pos**	**pos**	**pos**	**pos**
6	neg	**pos**	**pos**	**pos**	**pos**	**pos**
7	neg	**pos**	**pos**	neg	**pos**	**pos**
8	neg	neg	**pos**	**pos**	**pos**	**pos**
9	neg	neg	neg	neg	neg	neg
10	neg	**pos**	**pos**	**pos**	**pos**	**pos**
11	**pos**	**pos**	**pos**	**pos**	**pos**	**pos**
12	neg	neg	**pos**	**pos**	**pos**	**pos**
13	ND	ND	**pos**	ND	ND	**pos**
Total positive	1	7	12	7	10	12
N	12	12	13	12	12	13

ND, not done (no sample available); pos, positive; neg, negative, N; total number.

Table 8 Comparison of positive results in conjunctival scraping, aqueous and vitreous humor samples after HCMV DNA detection by conventional and optimized duplex nested PCR.

Test method	Number of positive/total number (%)		
	Conjunctival scraping	Aqueous humor	Vitreous humor
Conventional nested PCR	1/12(8.3)	7/12 (58.3)	12/13 (92.3)
Optimized duplex nested PCR	7/12 (58.3)	10/12 (83.3)	12/13 (92.3)

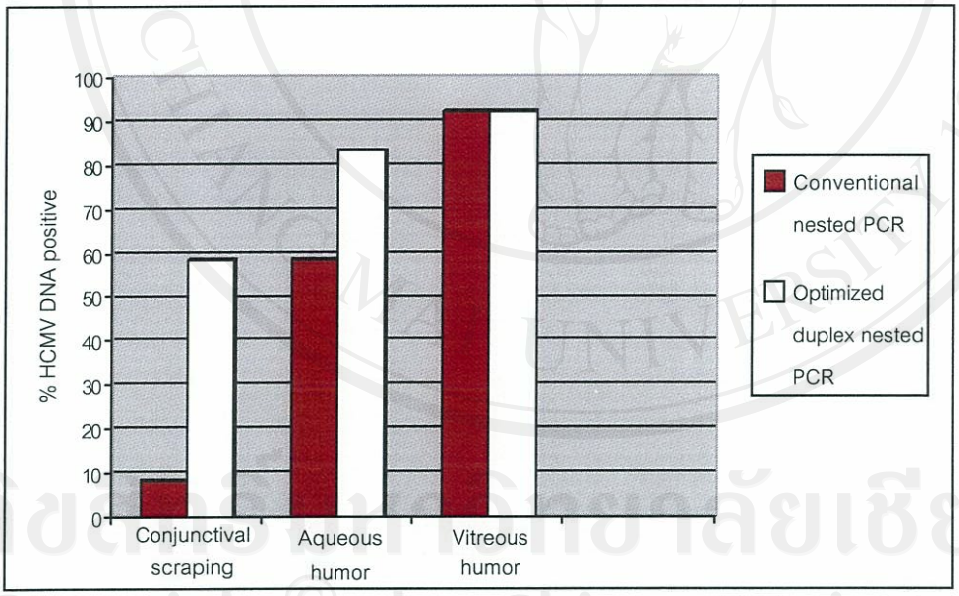

Figure 31 Comparison of the positive results of HCMV DNA amplification in ocular samples by conventional and optimized duplex nested PCR.

4.1.2 Comparison of efficiency of the ocular specimens

When using the conventional method, conjunctival scraping, aqueous humor and vitreous humor from the HCMV suspected patients showed positive results of 8.3, 58.3 and 92.3%, respectively. These results revealed that conjunctival scraping was not suitable for HCMV diagnosis by PCR assay. However, to determine the difference between percentages of HCMV DNA positive samples in aqueous humor and vitreous humor, the Z test was used at a level of significance, (α) = 0.05. It was found that aqueous humor specimens provided a significantly lower percentage of positive results when compared to the vitreous humor specimen ($p<0.05$). Therefore, aqueous humor could not replace the use of vitreous humor when HCMV DNA detection by conventional nested PCR was needed.

With the same samples using the optimized duplex nested PCR method, conjunctival scraping, aqueous humor and vitreous humor showed the positive results of 58.3, 83.3 and 92.3%, respectively. These results revealed that conjunctival scraping still showed a low positive rate in contrast to an aqueous humor, which showed a higher positive percentage. Therefore, to determine the difference between the percentages of HCMV DNA positive in aqueous humor and vitreous humor, the Z test was used at a level of significance, (α) = 0.05. It was found that the positive percentage of the aqueous humor specimen was significantly comparable to the vitreous humor specimen ($p>0.05$). Therefore, the aqueous humor could replace the use of the vitreous humor when the optimized duplex nested PCR was used to detect the HCMV DNA.

4.2 Renal transplant patients

Detection of HCMV infection in transplant donors and pre- and post- recipients

Donors

Eleven renal transplant recipients received kidneys from 7 living donors and 4 fresh cadavers. All donors were previously exposed to HCMV, as they contained anti-HCMV

IgG. However, none of them showed anti-HCMV IgM. Four donors were tested for the presence of HCMV DNA in their PBMCs and plasma. It was found that only one of them was positive for HCMV DNA in PBMCs, but all were found negative for HCMV DNA in plasma. The specimens from 3 living donors and 4 cadavers were not available for HCMV DNA detection.

Recipients

Pre-transplant specimens; Among 11 transplant recipients, 10 of them were found positive for anti-HCMV IgG. It was noted that one did not give positive results for anti-HCMV IgG and none was found positive for anti-HCMV IgM. At a time before transplantation, 7 pre-transplant specimens were tested for HCMV DNA in both PBMCs and plasma. It was found that none of them showed a positive result.

Post-transplant specimens; Specimens were collected at 2-week intervals for as long as 8 weeks after transplantation.

Specimens at 2 weeks post-transplantation could be collected from 7 recipients. Among these 7 post-renal transplant recipients, only one, who received a kidney from the donor with HCMV positive PBMCs, showed positive results for HCMV DNA in both PBMCs and plasma. At the time, this recipient was negative for anti-HCMV IgM. Two recipients showed positive results for HCMV DNA in their PBMCs, but not in plasma. HCMV DNA could not be found in the other 4 recipients.

Specimens at 4 weeks post-transplantation could be collected from all 11 recipients. Four of these recipients were found to be HCMV DNA positive in both plasma and PBMCs, whereas another four recipients were found HCMV DNA positive in only their PBMCs. The other 3 recipients remained negative. Therefore, by 4 weeks, 8 of 11 recipients showed signs of HCMV reactivation.

Specimens at 6 weeks post-transplantation could be collected from 10 recipients. One recipient was lost to follow up. Four of the remaining recipients, who previously showed positive results for HCMV DNA in both PBMCs and plasma at 4 weeks, remained positive. Two of the four recipients, who previously showed HCMV DNA in only their PBMCs, appeared to harbor HCMV DNA in both PBMCs and plasma by this time. One of the 2 in the HCMV DNA negative group became positive for HCMV DNA in both PBMCs and plasma, whereas one remained negative. The remaining one was lost to follow up. Among the 10 recipients, the one who did not showed anti-HCMV IgG/IgM at pre-transplantation, eventually developed anti-HCMV IgM, which was 2 weeks after HCMV DNA was detected. Therefore, by 6 weeks, 9 of 10 recipients showed signs of HCMV reactivation, with one recipient confirmed as having primary HCMV infection.

Specimens at 8 weeks post-transplantation could be collected from 7 recipients. One recipient, who was positive for HCMV DNA in PBMCs at week 4, had became positive for both PBMCs and plasma by this time. However, 5 recipients remained positive for HCMV DNA in both PBMCs and plasma. The one who had been negative for HCMV DNA detection since transplantation, remained negative for 8 weeks.

A percentage of HCMV DNA positive specimens in plasma and PBMCs among post-renal transplant recipients at 2- week intervals is shown in Figure 32.

Examples of HCMV DNA detection in PBMCs and plasma by the optimized duplex nested PCR are shown in Figure 33 (first round PCR) and Figure 34 (second round PCR), respectively.

When the results were HCMV DNA positive in plasma, they were compared to the presence of anti-HCMV IgM in post-transplant recipients, and it was found that only one recipient, who experienced a primary infection, showed anti-HCMV IgM positive at 6 weeks after transplantation. The HCMV DNA in the PBMCs of this recipient was found 2 weeks earlier. However, at the time of monitoring, none of the recipients showed any symptoms related to HCMV infection.

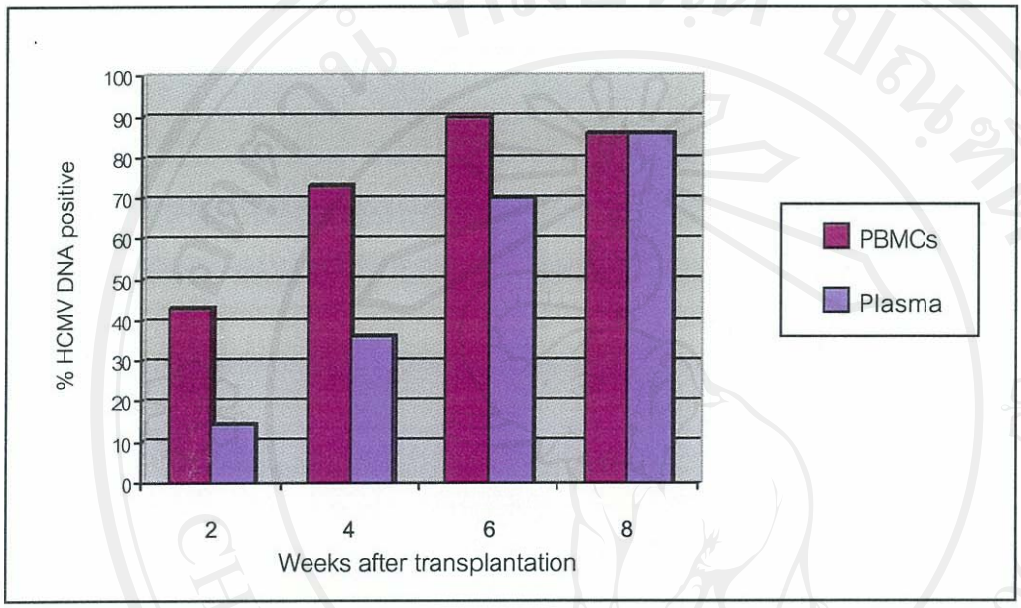

Figure 32 Percentage of HCMV DNA positive specimens in plasma and PBMCs at 2-week intervals after transplantation.

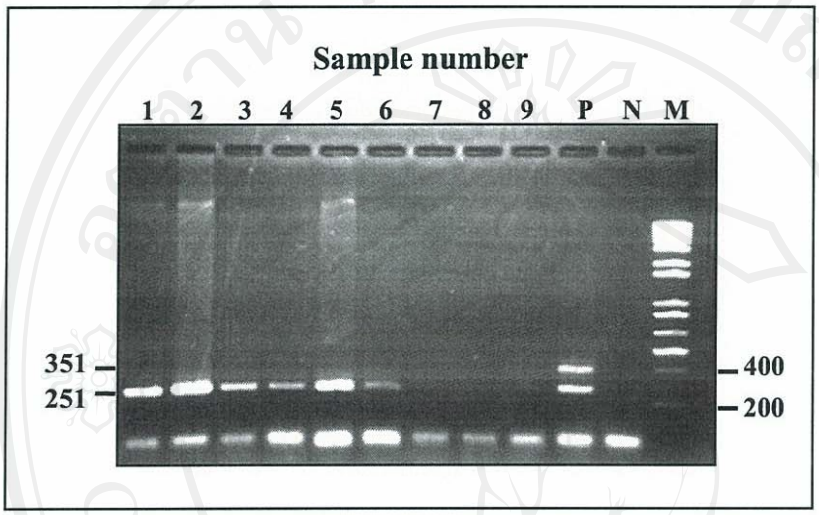

Figure 33 The amplification of HCMV DNA from PBMCs and plasma samples in transplantation patients by the optimized first round duplex nested PCR. Lane 1-6 (PBMCs samples) show 251 bp beta-globin gene PCR products with different intensity. Lane 7, 8 and 9 (plasma samples) show negative result. Lane M shows the DNA marker, lane N shows a negative control, and P shows positive control.

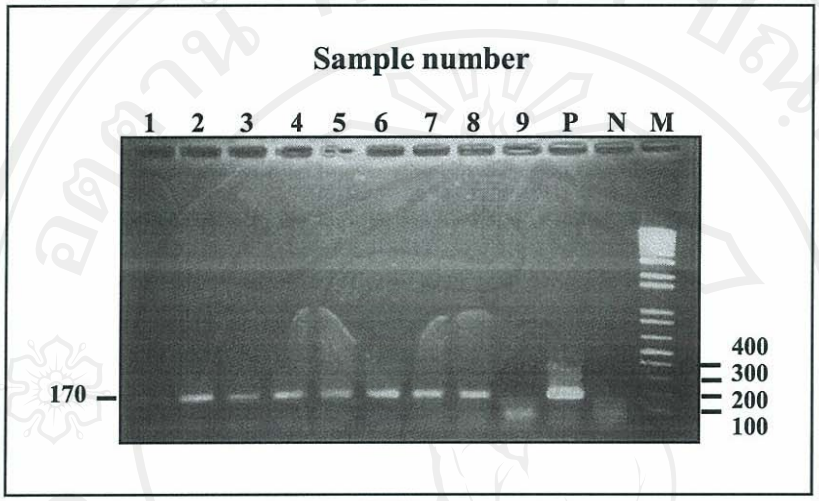

Figure 34 The amplification of HCMV DNA from PBMCs (lane 1-6) and plasma (lane 7-9) samples in transplantation patients by the optimized second round duplex nested PCR. Lane 2-8 shows 170 bp HCMV DNA PCR products. Lane 1 and 9 show negative result. Lane M shows the DNA marker, lane N shows a negative control, and P shows positive control.