CHAPTER IV

RESULTS

1. Preparation of pathogen DNA controls using PCR cloning.

DNA fragments of 5 focal pathogens (CMV, HSV-1, HSV-2, VZV and *T.gondii*) were separately amplified by conventional PCR using specific primers for each microorganism. As well as the chosen pathogens, a PhHV-1 DNA fragment as an internal control for the real-time PCR was also amplified. The sizes of each amplified PCR product are presented in Table 7.

Table 7 Size of amplified PCR product of interested micro-organisms.

Micro-organisms	Specific region	PCR product size (bp)
1. CMV	GP 060 Catalytic subunit of DNA polymerase	133
2. HSV-1	RL2 immediate early protein	95
3. HSV-2	RL2 immediate early protein	95
4. HSV	UL30: DNA polymerase	96
5. *T.gondii*	B1 gene	98
6. VZV	Tegument protein	82
7. PhHV-1	Glycoprotein B gene	89

The amplified product of each DNA fragment was precipitated and cloned into the pGEM®-T Easy vector plasmid (Promega, USA). The recombinant plasmids were

transformed into the competent *E.coli* grown on selected ampicillin medium. The transformed colonies were then tested using conventional PCR. A portion of a well-isolated transformed colony was added directly to the PCR mixture for amplification of each DNA fragment using specific primers for each pathogen. The PCR products were detected via 2.5% agarose gel electrophoresis with ethidium bromide staining. The successfully transformed cells provided an approximate size expected for the amplified PCR product.

Each selected transformed colony was simultaneously incubated in 5 mL of LB medium and given a code. Only the selected transformed colonies that presented the specific amplified DNA fragment were further chosen for large scale production. Plasmid DNA was then purified from the transformed *E.coli* by the miniprep method. To confirm the presence of specific target DNA fragments, each of the purified DNA products was re-amplified by conventional PCR. The resulting products were detected by 2.5% agarose gel electrophoresis with ethidium bromide staining. The presence of each specific target DNA is illustrated in Figure 5-11. These purified DNAs were used as the plasmid DNA controls throughout this study.

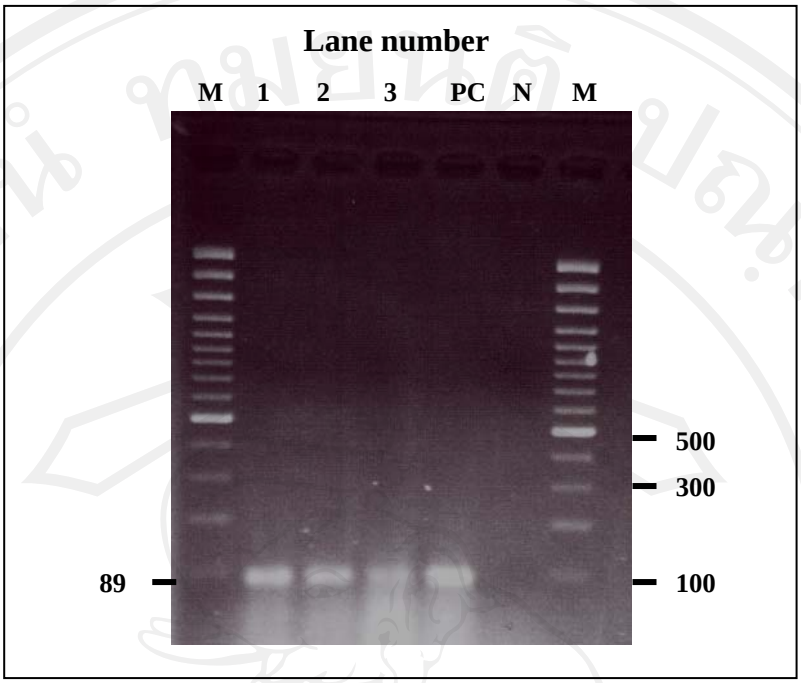

Figure 5 Confirmation of the presence of PhHV-1 DNA fragments in purified plasmid DNA after large scale production. The purified plasmid DNAs were amplified by conventional PCR. Lane M shows the 100 bp DNA ladder, lanes 1-3 show the amplified PCR products of different purified plasmid DNA, lane PC shows 89 bp of PhHV-1 DNA control and lane N shows the negative control. All purified plasmid DNAs in lane 1 to lane 3 show the 89 bp of the PhHV-1 DNA fragment.

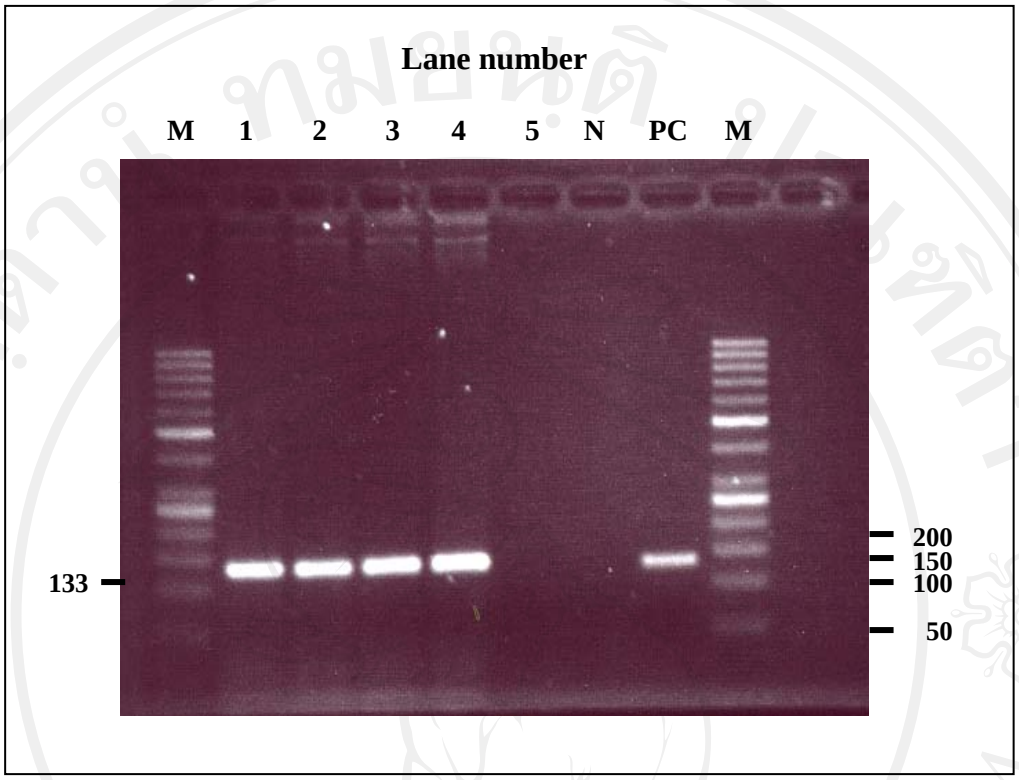

Figure 6 Confirmation of the presence of the CMV DNA fragment in purified plasmid DNA after large scale production. The purified plasmid DNAs were amplified by conventional PCR. Lane M shows the 50 bp DNA ladder, lanes 1-5 show the amplified PCR products of different purified plasmid DNAs, lane N shows the negative control and lane PC shows 133 bp of the CMV DNA control. The purified plasmid DNAs in lane 1 to lane 4 display the 133 bp of the CMV DNA fragment. Whereas, lane 5 shows the negative result representing the failure of plasmid CMV DNA control preparation from this selected transformed colony.

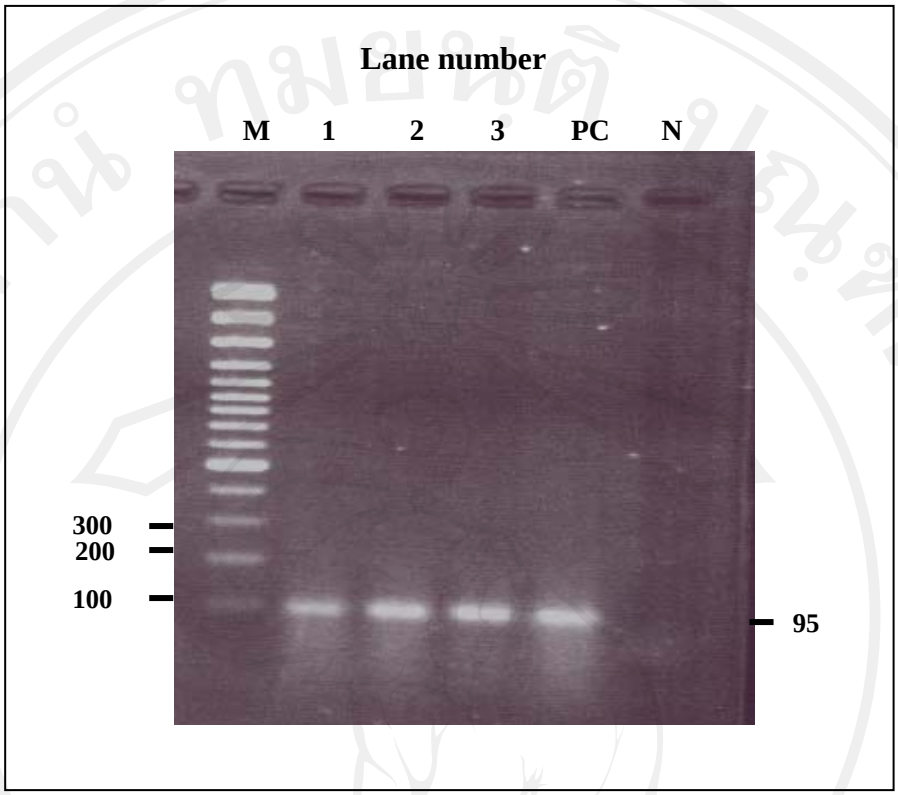

Figure 7 Confirmation of the presence of the HSV-1 DNA fragment (using the specific primers and probe targeting RL2 immediate early protein) in purified plasmid DNA after large scale production. The purified plasmid DNAs were amplified by conventional PCR. Lane M shows the 100 bp DNA ladder, lanes 1-3 show the amplified PCR products of different purified plasmid DNAs, lane PC shows 95 bp of the HSV-1 DNA control, and lane N shows the negative control. All purified plasmid DNAs in lanes 1 to 3 display the 95 bp of the HSV-1 DNA fragment.

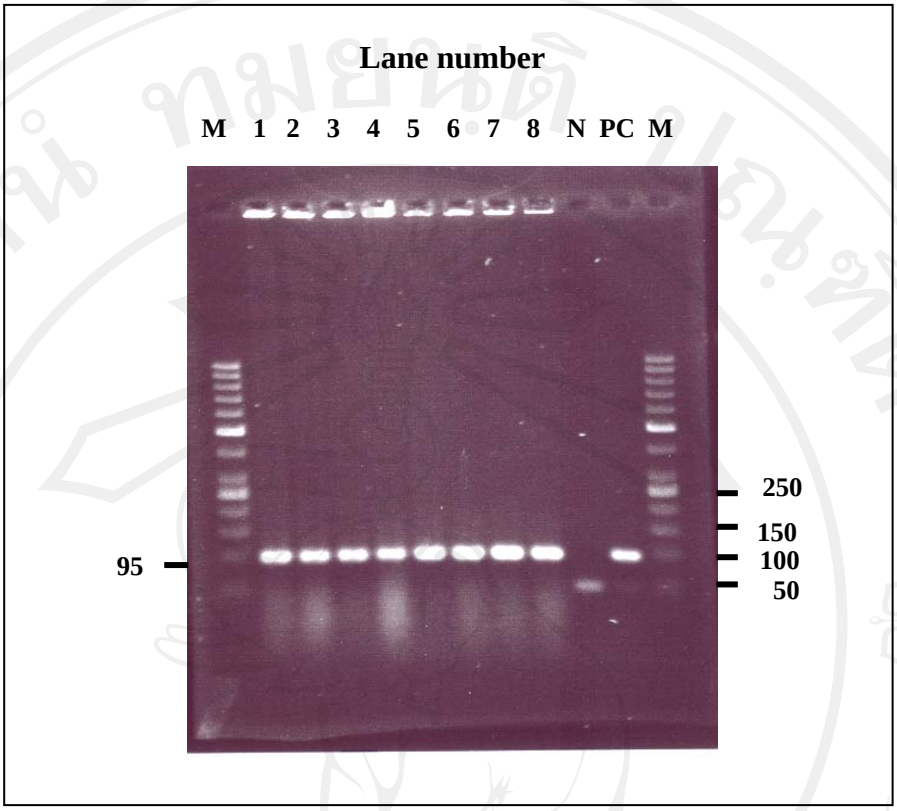

Figure 8 Confirmation of the presence of the HSV-2 DNA fragment (using the specific primers and probe targeting RL2 immediate early protein) in purified plasmid DNA after large scale production. The purified plasmid DNAs were amplified by conventional PCR. Lane M shows the 50 bp DNA ladder, lanes 1-8 show the amplified PCR products of different purified plasmid DNAs, lane N shows the negative control and lane PC shows 95 bp of the HSV-2 DNA control. All purified plasmid DNAs in lanes 1 to 8 display the 95 bp of the HSV-2 DNA fragment.

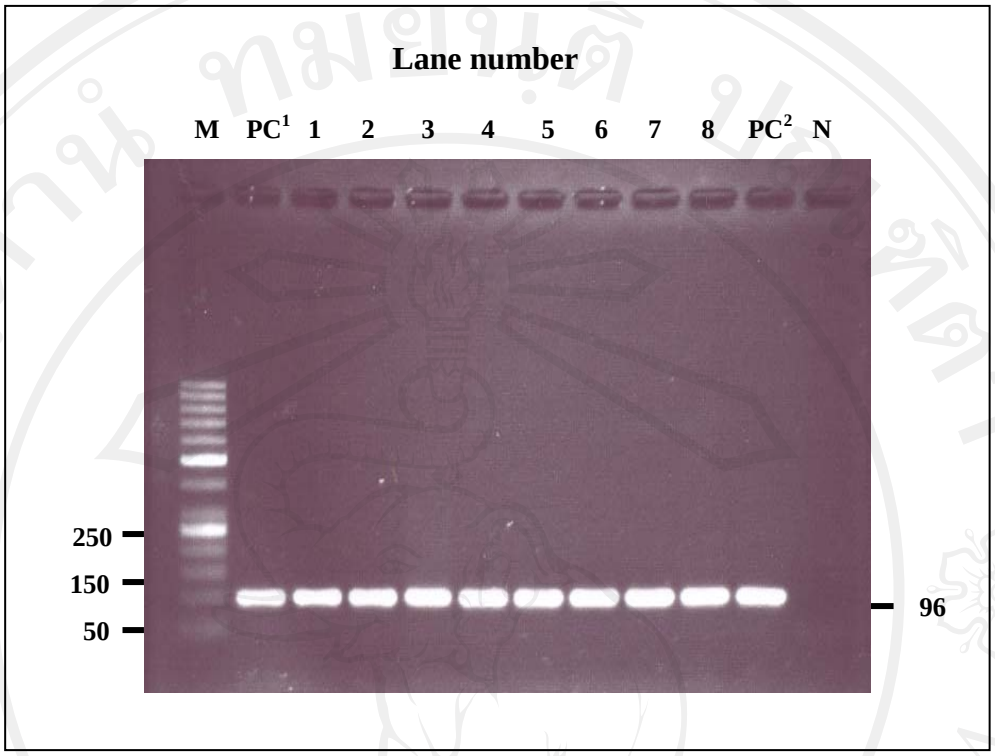

Figure 9 Confirmation of the presence of HSV-1and HSV-2 DNA fragments (using the specific primers and probe targeting UL30: DNA polymerase) in purified plasmid DNA after large scale production. The purified plasmid DNAs were amplified by conventional PCR. Lane M shows the 50 bp DNA ladder, lane PC^1 shows 96 bp of HSV-1 DNA control, lanes 1-4 show the amplified PCR products of different purified plasmid DNAs from the selected HSV-1 transformed colonies, lanes 5-8 show the amplified PCR products of different purified plasmid DNAs from the selected HSV-2 transformed colonies, lane PC^2 shows 96 bp of HSV-2 DNA control and lane N shows the negative control. All purified plasmid DNAs in lanes 1 to 8 show the 96 bp of HSV-1 and HSV-2 DNA fragments.

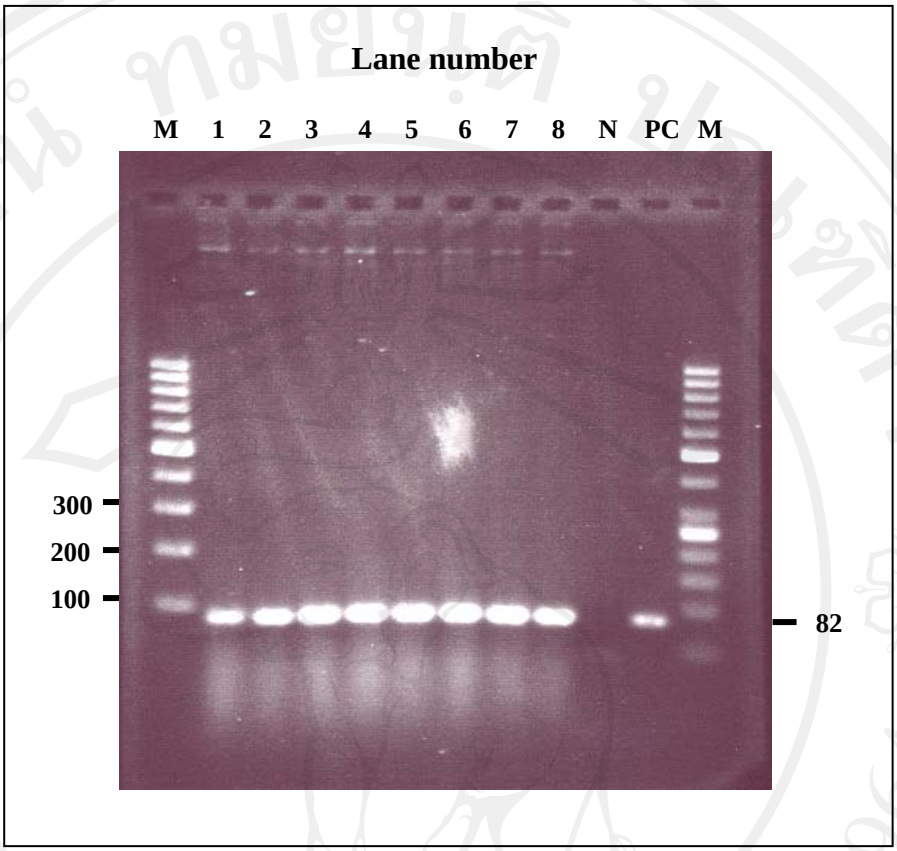

Figure 10 Confirmation of the presence of VZV DNA fragment in purified plasmid DNA after large scale production. The purified plasmid DNAs were amplified by conventional PCR. Lane M shows the 100 bp DNA ladder, lanes 1-8 show the amplified PCR products of different purified plasmid DNAs, lane N shows the negative control and lane PC shows 82 bp of the VZV DNA control. All purified plasmid DNAs in lanes 1 to 8 show the 82 bp of the VZV DNA fragment.

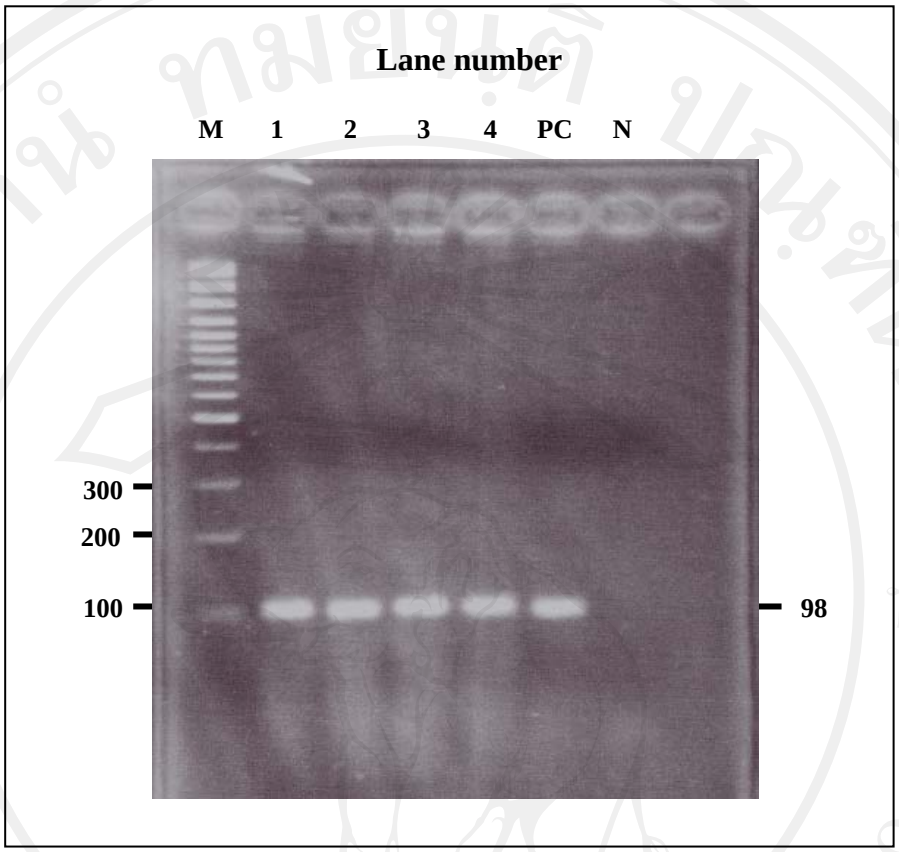

Figure 11 Confirmation of the presence of the *T.gondii* DNA fragment in purified plasmid DNA after large scale production. The purified plasmid DNAs were amplified by conventional PCR. Lane M shows the 100 bp DNA ladder, lanes 1-4 show the amplified PCR products of different purified plasmid DNAs, lane PC shows 98 bp of *T.gondii* DNA control and lane N shows the negative control. All purified plasmid DNAs in lanes 1 to 8 show the 98 bp of the *T.gondii* DNA fragment.

Quantity of DNA controls.

The quantity of each DNA control was measured via the DNA fluorescence assay using the Quant-iT™ dsDNA HS Assay Kits (Invitrogen, USA). Each DNA control was 5-fold serial pre-diluted in distilled water and used as the sample for quantity measurement. Ten µL of standard (10 ng/µL) of the assay kit and 5 µL of each DNA control were diluted in 190 µL and 195 µL of Quant-iT™ dsDNA HS working buffer, respectively. After that, the concentration was read using a Qubit™ fluorometer. The quantity of DNA was calculated by using the following equation (1):

$$\text{Quantity of DNA sample (ng/ µL)} = \frac{\text{O.D.}_{485/530} \text{ of DNA sample} \times \text{Standard conc. (10 ng/ µL)}}{\text{O.D.}_{485/530} \text{ of Standard}}$$

The Quant-iT™ fluorometer gave values of the Quant-iT™ dsDNA HS working assay in ng/mL. This value corresponds to the concentration after each DNA sample was diluted into the assay tube. The concentration of the DNA sample was calculated by using the following equation (2):

$$\text{Concentration of the DNA sample} = \text{QF value} \times \left(\frac{200}{X}\right)$$

Note: QF value = the value (ng/mL) given by the Quant-iT™ fluorometer

X = the number of microliters of the DNA sample that were added to the assay tube.

After DNA controls were diluted using Quant-iT™ dsDNA HS Assay Kits and measured via the Qubit™ fluorometer, the concentration in ng/mL of each DNA control was read and then calculated back to be the concentration of undiluted DNA control corresponding to the pre-diluted factor of each DNA control.

For example, for the HSV-1 DNA control measurement:

Five µL of 1:5 pre-diluted HSV-1 DNA control were diluted in 195 µL of Quant-iT™ dsDNA HS working buffer. The concentration as measured by the Qubit™ fluorometer was 329 ng/mL. Then, the concentration was calculated by using equation 2.

$$\text{Concentration of the sample} = 329 \text{ ng/mL} \times \frac{200}{5}$$

$$= 13{,}160 \text{ ng/mL}$$

$$= 13.160 \text{ µg/mL}$$

Finally, considering the dilution factor, the concentration of HSV-1 DNA was 66 µg/mL. (13.160 µg/mL. x 5 = 66 µg/mL)

The copy number of pGEM®-T Easy vector plasmids with the HSV-1 DNA insert was calculated. The amount of this plasmid DNA, at the concentration of 10 µg/mL was found to be approximately 3 x 10^9 copies/µL. The following correlation formula was used for estimation of amount: (Hammond *et al*., 2005)

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

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

1 mol = 6×10^{23} molecules (copies)

For example, the stock HSV-1 DNA control at the concentration of 10 μg/mL could be converted to number of copies/μL by following calculation:

$$\frac{6 \times 10^{23} \text{ copies/mol} \times 10 \times 10^{-6} \times 10^{-3} \text{ g/μL}}{(3{,}015 \text{ bp} + 95 \text{ bp}) \times 660 \text{ daltons/bp}} = 3 \times 10^{9} \text{ copies/μL}$$

In this study, All DNA controls were further diluted to 10 μg/mL and used as the stock DNA control.

Table 8 The quantity of stock DNA controls.

DNA control	Concentration (μg/mL)	Size of DNA fragment (bp)	Concentration (copies/μL)
1. CMV	10	133	3×10^9
2. HSV-1	10	95	3×10^9
3. HSV-2	10	95	3×10^9
4. HSV	10	96	3×10^9
5. *T.gondii*	10	98	3×10^9
6. VZV	10	82	3×10^9
7. PhHV-1	10	89	3×10^9

2. Reference singleplex real-time PCR.

All specific primers and probes except that for HSV were reproduced according to de Groot-Mijnes *et al*. (de Groot-Mijnes *et al*., 2006). From the optimization step of reference singleplex real-time, we obtained the optimal concentration of each primer and probe (Table 9).

Table 9 The optimal concentrations of primers and probes used in reference singleplex real-time PCR.

Primers/Probes	**Final concentration of primers/probes (nM)**						
	CMV	**HSV-1**	**HSV-2**	**HSV**	***T.gondii***	**VZV**	**PhHV-1**
- FWD primer	300	900	900	300	900	300	50
- RWD primer	900	900	900	300	900	300	50
- Probe	200	200	200	100	200	50	200

2.1 Determination of specificity of reference singleplex real-time PCR.

The specificity of each primer set was investigated by performing a BLAST® search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). From the search, all specific primers sets used were not associated with non-target sequences. Each specific primers set showed only the product unique for its target template sequence.

In singleplex real-time PCR testing, the specificity of primers and probes were determined by amplifying target and non-target DNA. Any positive

signal was found in non-target DNA amplification. Thus, amplification of target DNA alone confirmed the specificity of each assay.

2.2 Determination of sensitivity of reference singleplex real-time PCR.

The stock DNA controls were 10-fold serial diluted covering 10 μg/mL to 1 x 10^{-2} fg/mL. Ten μL of 10-fold serial diluted DNA control was added in 15 μL of the real-time PCR reaction mixture (DyNAmo^{TM} Probe qPCR kit, New England Biolabs Inc.) that contained an optimal concentration of specific primers and probes. Thus, the concentrations of 10-fold serial diluted DNA controls covering 100 ng/ reaction to 1 x 10^{-4} fg/reaction were tested. After performing the singleplex real-time PCR, the C_T value of each diluted DNA control was recorded. Then, the last 4 dilutions of the DNA control presenting positive signal and negative results were duplicated and retested. A C_T value equal to or greater than 40 was considered as a negative result.

The sensitivities of singleplex real-time PCR of the focal pathogens are presented in Tables 10-16 and summarized in Table 17.

Table 10 The sensitivity test of CMV singleplex real-time PCR.

Reaction	DNA dilution	Average C_T value	DNA conc./reaction
1	1×10^{-7}	29.55	10 fg
2	1×10^{-8}	32.61	1 fg
3	1×10^{-9}	36.29	0.1 fg
4	1×10^{-10}	Neg (N/A)	0.01 fg

N/A: No positive signals were detected in 45 cycles of reference singleplex real-time PCR.

Table 11 The sensitivity test of HSV-1 singleplex real-time PCR.

(targeting RL2 immediate early protein).

Reaction	DNA dilution	Average C_T value	DNA conc./reaction
1	1×10^{-7}	30.84	10 fg
2	1×10^{-8}	33.05	1 fg
3	1×10^{-9}	36.00	0.1 fg
4	1×10^{-10}	39.33	0.01 fg

Table 12 The sensitivity test of HSV-2 singleplex real-time PCR. (targeting RL2 immediate early protein).

Reaction	DNA dilution	Average C_T value	DNA conc./reaction
1	1×10^{-6}	30.32	100 fg
2	1×10^{-7}	33.30	10 fg
3	1×10^{-8}	36.94	1 fg
4	1×10^{-9}	Neg (41.49)	0.1 fg

Table 13 The sensitivity test of HSV-1 singleplex real-time PCR. (targeting UL30: DNA polymerase).

Reaction	DNA dilution	Average C_T value	DNA conc./reaction
1	1×10^{-8}	33.87	1 fg
2	1×10^{-9}	37.89	0.1 fg
3	1×10^{-10}	Neg (N/A)	0.01 fg
4	1×10^{-11}	Neg (N/A)	0.001 fg

Table 14 The sensitivity test of HSV-2 singleplex real-time PCR.

(targeting UL30: DNA polymerase).

Reaction	DNA dilution	Average C_T value	DNA conc./reaction
1	1×10^{-8}	32.44	1 fg
2	1×10^{-9}	36.55	0.1 fg
3	1×10^{-10}	Neg (N/A)	0.01 fg
4	1×10^{-11}	Neg (N/A)	0.001 fg

N/A: No positive signals were detected in 45 cycles of reference singleplex real-time PCR.

Table 15 The sensitivity test of *T.gondii* singleplex real-time PCR.

Reaction	DNA dilution	Average C_T value	DNA conc./reaction
1	1×10^{-8}	33.87	1 fg
2	1×10^{-9}	36.59	0.1 fg
3	1×10^{-10}	38.21	0.01 fg
4	1×10^{-11}	39.44	0.001 fg

Table 16 The sensitivity test of VZV singleplex real-time PCR.

Reaction	DNA dilution	Average C_T value	DNA conc./reaction
1	1×10^{-7}	33.04	10 fg
2	1×10^{-8}	34.41	1 fg
3	1×10^{-9}	38.57	0.1 fg
4	1×10^{-10}	Neg (40.07)	0.01 fg

Table 17 Summary of the sensitivity of singleplex real-time PCR.

Singleplex real-time PCR reaction	Sensitivity	
	DNA conc./reaction	DNA copies/reaction
1. CMV	0.1 fg	30
2. HSV-1[a]	0.1 fg	30
3. HSV-2[a]	1 fg	300
4. HSV-1[b]	0.1 fg	30
5. HSV-2[b]	0.1 fg	30
6. *T.gondii*	0.01 fg	3
7. VZV	0.1 fg	30

[a]: Singleplex real-time PCR using specific primers and probe targeting RL2 immediate early protein.

[b]: Singleplex real-time PCR using specific primers and probe targeting UL30: DNA polymerase.

In addition, the reproducibility of PCR amplification was controlled by observing the threshold cycles (C_T) value of DNA control after each run. Thus, PhHV-1 DNA and DNA controls at the concentration of approximately 0.1-1 fg/reaction were simultaneously performed and used as the positive control in real-time PCR. The C_T values should range from 32 to 35. Only C_T values which within 2 standard deviations of the average from the repeated runs were accepted. These controls were determined for confidence testing for negative results.

2.3 Incidence of the infectious agents in intraocular fluid samples.

In this study, the incidence of each focal infectious agent was investigated in 240 intraocular fluid samples from uveitis patients. The analysis was performed by reference singleplex real-time PCR for each pathogen as described above. In addition, the plasmid DNA controls and PhHV-1 DNA control were performed in parallel with intraocular sample testing. Our series included 240 uveitis patients with an average age of 43 years (range, 4 months-75 years) and the male to female ratio was 1.2:1. None of the samples were inhibited as determined by PhHV-1 analysis. In this population, PanU (80/240, 33%), PU (70/240, 29%) and AU (69/240, 29%) occur in similar percentages. IU was less common (9%) (21/240) (Table 18). Infectious uveitis was found in 96/240 (40.0%) of the tested population. CMV was the most common cause (67/240, 27.9%) in all anatomical types of uveitis. Meanwhile, *T.gondii* (4.2%), VZV (3.3%), HSV-2 (2.5%) and HSV-1(2.1%) were also present (Table 19).

Of the 240 patients with uveitis, 74 (31%) were HIV-infected with a mean age of 40 years, and the male to female ratio was 1:1.1. Meanwhile, 166 uveitis

patients (69%) were non-HIV cases. The mean age of non-HIV patients with uveitis was 44 years and the male to female ratio was 1.2:1 (Table 18).

The results of the diagnostic for infectious cause of 74 HIV-infected patients with uveitis are listed in Table 20. Forty-six cases (62.2%) were diagnosed as infectious uveitis, including 2/8 (20.0%) in AU, 1/5 (20.0%) in IU, 18/28 (64.3%) in PU and 25/33 (75.8%) in PanU patients. The most commonly diagnosed infection was CMV (39/74, 52.7% overall; 2/8, 25.0% of AU; 1/5, 20.0% of IU; 15/28, 53.6% of PU; 21/33, 63.6% of panuveitis).

Table 18 General characteristics of 240 uveitis patients in Northern Thailand.

characteristics	**Total**		**HIV-positive uveitis patients**		**Non-HIV uveitis patients**	
	N	**%**	**N**	**%**	**N**	**%**
Number of patients	240	100	74	31	166	69
Male to Female ratio	1.2:1	n.a	1:1.1	n.a	1.2:1	n.a
	Average age (yr)	**Range**	**Average age (yr)**	**Range**	**Average age (yr)**	**Range**
All uveitis	43	0.3-75	40	25-64	44	0.3-75
Anterior uveitis (AU)	47	0.3-75	40	29-64	48	0.3-75
Intermediate uveitis (IU)	42	9-74	42	29-50	41	9-74
Posterior uveitis (PU)	40	6-65	38	25-57	43	6-65
Panuveitis (PanU)	42	14-69	40	25-49	44	14-69

HIV: human immunodeficiency virus n.a.: not applicable

Table 19 Detection of infectious agents in intraocular fluid of uveitis patients by reference singleplex real-time PCR.

Focal infectious agent	Positive results of real-time PCR (%)				
	AU (N=69)	**IU (N=21)**	**PU (N=70)**	**PanU (N=80)**	**Total (N = 240)**
CMV	13 (18.8%)	3 (14.3%)	25 (35.7%)	26 (32.5%)	67 (27.9%)
HSV-1	1 (1.4%)	1 (4.8%)	1 (1.4%)	2 (2.5%)	5 (2.1%)
HSV-2	1 (1.4%)	1 (4.8%)	0 (0.0%)	4 (5.0%)	6 (2.5%)
VZV	3 (4.3%)	0 (0.0%)	2 (2.9%)	3 (3.8%)	8(3.3%)
T. gondii	3 (4.3%)	1 (4.8%)	3 (4.3%)	3 (3.8%)	10 (4.2%)
Total	**21 (30.4%)**	**6 (28.6%)**	**31 (44.3%)**	**38 (47.5%)**	**96 (40.0%)**

AU: anterior uveitis *IU: intermediate uveitis* *PU: posterior uveitis*

Table 20 Detection of interested infectious agents in intraocular fluid of HIV positive patients with uveitis by reference singleplex real-time PCR.

Focal infectious agent	Positive results of real-time PCR (%)				
	AU (N=8)	**IU (N=5)**	**PU (N=28)**	**Panuveitis (N=33)**	**Total (N =74)**
CMV	2 (25.0%)	1 (20.0%)	15 (53.6%)	21 (63.6%)	39 (52.7%)
HSV-1	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (3.0%)	1 (1.4%)
HSV-2	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
VZV	0 (0.0%)	0 (0.0%)	2 (7.1%)	1 (3.0%)	3 (4.1%)
T. gondii	0 (0.0%)	0 (0.0%)	1 (3.6%)	2 (6.1%)	3 (4.1%)
Total	**2 (25.0%)**	**1 (20.0%)**	**18 (64.3%)**	**25 (75.8%)**	**46 (62.2%)**

AU: anterior uveitis *IU: intermediate uveitis* *PU: posterior uveitis*

PanU: Panuveits

Table 21 Detection of interested infectious agents in intraocular fluid of non-HIV patients with uveitis by reference singleplex real-time PCR.

Focal infectious agent	**Positive results of real-time PCR (%)**				
	AU (N=61)	**IU (N=16)**	**PU (N=42)**	**PanU (N=47)**	**Total (N =166)**
CMV	11 (18.0%)	2 (12.5%)	10 (23.8%)	5 (10.6%)	28 (16.9%)
HSV-1	1 (1.6%)	1 (6.3%)	1 (2.4%)	1 (2.1%)	4 (2.4%)
HSV-2	1 (1.6%)	1 (6.3%)	0 (0.0%)	4 (8.5%)	6 (3.6%)
VZV	3 (4.9%)	0 (0.0%)	0 (0.0%)	2 (4.3%)	5 (3.0%)
T. gondii	3 (4.9%)	1 (6.3%)	2 (4.8%)	1 (2.1%)	7 (4.2%)
Total	**19 (31.1%)**	**5 (31.3%)**	**13 (31.0%)**	**13 (27.7%)**	**50 (30.1%)**

AU: anterior uveitis *IU: intermediate uveitis* *PU: posterior uveitis*

PanU: Panuveits

In the non-HIV group, infectious uveitis was found in 50/166 (30.1%) cases and the most frequently diagnosed infection in all anatomical types of uveitis was CMV (28/166, 16.9% of all). VZV and *T.gondii* were documented as secondary diagnosed infections, followed by HSV in AU. This scenario was also found in IU and PU cases, unless VZV was identified. In PanU patients, CMV and HSV-2 were commonly found in comparable percentages (10.6% *vs* 8.5%, respectively). Meanwhile, VZV, *T.gondii* and HSV-1 were found in descending order, respectively (Table 21).

3. Development of multiplex real-time PCR technique to identify organisms involved in uveitis.

3.1 Duplex real-time PCR development

The information obtained from the incidence study showed that CMV was the most common causative agent of infectious uveitis in our study population. However, HSV, VZV and *T.gondii* were also present. Thus, we established duplex real-time PCR to identify herpesvirues mainly for CMV diagnosis, and additionally for HSV, VZV and *T.gondii*. However, *T.gondii* was generally diagnosed by the ophthalmologist, so singleplex real-time PCR should be a satisfactory test.

3.1.1 Optimization of duplex real-time PCR and determination of sensitivity and specificity of duplex real-time PCR

The sensitivity and specificity of duplex real-time PCR were simultaneously investigated during the optimization step, which determined the C_T value of each infectious agent. Briefly, mixed plasmid DNA controls with CMV and other plasmid DNA (HSV-1, HSV-2, VZV or *T.gondii*) at various concentrations were analyzed. At first, the CMV plasmid DNA control at a fixed concentration of 1 pg/reaction (100 ng/mL) was mixed with various concentrations of other plasmid DNA controls ranging from 1 fg/reaction to 1 pg/reaction (100 ng/mL to 100 fg/mL). After that the concentrations of other plasmid DNA controls were fixed and mixed together with various concentration of CMV plasmid DNA control. All mixed plasmid DNA controls were used as the samples for duplex real-time PCR determination. In addition, the same concentration of each plasmid DNA control was investigated in parallel by singleplex real-time PCR. In each run of amplification

plasmid DNA control was also added for reproducibility determination. The concentrations of each primer and probe that were used were as previously described in singleplex real-time PCR. (Tables 5 and 9) The results were given in Table 22-25. The HEX positive signal was determined for CMV detection. The FAM positive signal was determined for HSV-1, HSV-2, *T.gondii* or VZV detection.

Table 22 Results of CMV/HSV-1 duplex real-time PCR reaction. Mixing of fixed concentration of CMV DNA control and various concentrations of HSV-1 DNA control determination using CMV/HSV-1 duplex real-time PCR.

Mixed plasmid DNA control	**Plasmid DNA control conc./reaction**		**Average C_T value**		
			duplex real-time PCR		**singleplex real-time PCR (FAM)**
			FAM	**HEX**	
C/H1-1	CMV	1 pg	Neg (N/A)	25.83	ND
	HSV-1	1 pg	24.62	Neg (N/A)	24.67
C/H1-2	CMV	1 pg	Neg (N/A)	25.11	ND
	HSV-1	100 fg	28.02	Neg (N/A)	27.87
C/H1-3	CMV	1 pg	Neg (N/A)	24.73	ND
	HSV-1	10 fg	31.24	Neg (N/A)	30.63
C/H1-4	CMV	1 pg	Neg (N/A)	25.37	ND
	HSV-1	1 fg	Neg (N/A)	Neg (N/A)	39.44

Table 23 Results of CMV/HSV-1 duplex real-time PCR reaction. Mixing of fixed concentration of HSV-1 DNA control and various concentration of CMV DNA control determination using CMV/HSV-1 duplex real-time PCR.

Mixed plasmid DNA control	**Plasmid DNA control conc./reaction**		**Average C_T value**		
			duplex real-time PCR		**singleplex real-time PCR (HEX)**
			FAM	**HEX**	
H1/C-1	CMV	1 pg	Neg (N/A)	25.48	26.96
	HSV-1	1 pg	29.43	Neg (N/A)	ND
H1/C -2	CMV	100 fg	Neg (N/A)	27.03	29.26
	HSV-1	1 pg	28.11	Neg (N/A)	ND
H1/C -3	CMV	10 fg	Neg (N/A)	34.21	36.23
	HSV-1	1 pg	28.37	Neg (N/A)	ND
H1/C -4	CMV	1 fg	Neg (N/A)	Neg (N/A)	Neg (N/A)
	HSV-1	1 pg	28.53	Neg (N/A)	ND

N/A: No positive signals were detected in 45 cycles of reference singleplex real-time PCR.

ND: Not determined

The sensitivity of CMV/HSV-1 duplex real-time PCR for CMV DNA and HSV-1 DNA detection were found at the same value which presented in the reaction that contained 10 fg/reaction of DNA. In CMV singleplex real-time PCR, the lowest concentration of CMV DNA that could be detected was 10 fg/reaction. The sensitivity of HSV-1 singleplex real-time PCR was also 10 fg/reaction. From the results both sets of real-time PCR resulted in comparable sensitivity in CMV and HSV-1 DNA detection. In addition, the CMV DNA and HSV-1 DNA at the concentration of 1 pg/reaction in all duplex real-time PCR amplification had similar C_T values at approximately of 25 and 28, respectively. (Table 22, Table 23) Thus, CMV/HSV-1 duplex real-time PCR could be used for CMV and HSV-1 determination without the effects of real-time PCR mixture and both DNA concentrations.

Table 24 Results of CMV/HSV-2 duplex real-time PCR reaction. Mixing of fixed concentration of CMV DNA control and various concentrations of HSV-2 DNA control determination using CMV/HSV-2 duplex real-time PCR.

Mixed plasmid DNA control	**Plasmid DNA control conc./reaction**		**Average C_T value**		
			duplex real-time PCR		**singleplex real-time PCR (FAM)**
			FAM	**HEX**	
C/H2-1	CMV	1 pg	Neg (N/A)	Neg (N/A)	ND
	HSV-2	1 pg	20.29	Neg (N/A)	20.30
C/H2-2	CMV	1 pg	Neg (N/A)	Neg (N/A)	ND
	HSV-2	100 fg	25.71	Neg (N/A)	25.49
C/H2-3	CMV	1 pg	Neg (N/A)	Neg (N/A)	ND
	HSV-2	10 fg	30.25	Neg (N/A)	30.06
C/H2-4	CMV	1 pg	Neg (N/A)	Neg (N/A)	ND
	HSV-2	1 fg	Neg (N/A)	Neg (N/A)	36.84

N/A: No positive signals were detected in 45 cycles of reference singleplex real-time PCR.

ND: Not determined

The sensitivity of HSV-2 DNA detection of both duplex and singleplex real-time PCR were 10 fg/reaction. (Table 24) The samples which contained 1 pg/reaction of CMV DNA and the various concentrations of HSV-2 DNA were determined using CMV/HSV-2 duplex real-time PCR and CMV singleplex real-time PCR. A positive signal of CMV detection was found in all amplification reactions. (Table 24) However, CMV DNA alone used as the reaction control had C_T values of 32.82 and 31.25 in CMV/HSV-2 duplex real-time PCR and CMV singleplex real-time PCR, respectively (data not shown) Thus, the CMV/HSV-2 duplex real-time PCR reaction may not suitable for CMV DNA detection in the presence of HSV-2 DNA.

Table 25 shows the results of CMV and *T.gondii* DNA detection by CMV/ *T.gondii* duplex real-time PCR and *T.gondii* singleplex real-time PCR. The sample that contained 1 fg/reaction of *T.gondii* DNA had C_T values of 37.42 and 36.84 in CMV/ *T.gondii* duplex real-time PCR and *T.gondii* singleplex real-time PCR, respectively. These C_T values could be interpreted as the sensitivity of the reactions. However, further dilutions of *T.gondii* DNA should be investigated to confirm this assumption. Meanwhile, 1 pg/reaction of CMV DNA mixed together with the higher concentration of *T.gondii* DNA had a weaker positive signal compared to the sample which contained a lower concentration of *T.gondii* DNA. (Table 25)

Table 25 Results of CMV/ *T.gondii* duplex real-time PCR reaction. Mixing of a fixed concentration of CMV DNA control and various concentrations of *T.gondii* DNA control determination using CMV/ *T.gondii* duplex real-time PCR.

Mixed plasmid DNA control	**Plasmid DNA control conc./reaction**		**Average C_T value**		
			duplex real-time PCR		**singleplex real-time PCR (FAM)**
			FAM	**HEX**	
C/T-1	CMV	1 pg	Neg (N/A)	37.59	ND
	T.gondii	1 pg	25.41	Neg (N/A)	24.04
C/T-2	CMV	1 pg	Neg (N/A)	33.93	ND
	T.gondii	100 fg	28.24	Neg (N/A)	28.98
C/T-3	CMV	1 pg	Neg (N/A)	30.67	ND
	T.gondii	10 fg	32.83	Neg (N/A)	32.53
C/T-4	CMV	1 pg	Neg (N/A)	29.37	ND
	T.gondii	1 fg	37.42	Neg (N/A)	36.84

N/A: No positive signals were detected in 45 cycles of reference singleplex real-time PCR.

ND: Not determined

Table 26 Results of CMV/VZV duplex real-time PCR reaction. Mixing of a fixed concentration of CMV DNA control and various concentrations of VZV DNA control determination using CMV/ VZV duplex real-time PCR.

Mixed plasmid DNA control	**Plasmid DNA control conc./reaction**		**Average C_T value**		
			duplex real-time PCR		**singleplex real-time PCR (FAM)**
			FAM	**HEX**	
C/V-1	CMV	1 pg	Neg (N/A)	30.84	ND
	VZV	1 pg	26.06	Neg (N/A)	26.63
C/V-2	CMV	1 pg	Neg (N/A)	29.03	ND
	VZV	100 fg	31.09	Neg (N/A)	31.89
C/V-3	CMV	1 pg	Neg (N/A)	30.21	ND
	VZV	10 fg	35.12	Neg (N/A)	351.9
C/V-4	CMV	1 pg	Neg (N/A)	29.87	ND
	VZV	1 fg	Neg (N/A)	Neg (N/A)	38.43

N/A: No positive signals were detected in 45 cycles of reference singleplex real-time PCR.

ND: Not determined

The sensitivities of CMV/VZV duplex real-time PCR and VZV singleplex real-time PCR for VZV DNA detection were found to be similar in the reaction that contained 10 fg/reaction of VZV DNA. In addition, the CMV DNA at the concentration of 1 pg/reaction had a C_T value of approximately 30 in all duplex real-time PCR amplifications (Table 26). Thus, CMV/VZV duplex real-time PCR could be used for CMV and VZV determination regardless of the real-time PCR mixture and the concentrations of both DNAs.

3.1.2 Diagnostic efficiency determination of duplex real-time PCR

The previous duplex real-time PCR determination using plasmid DNA controls showed that CMV/HSV-1 duplex real-time PCR and CMV/VZV duplex real-time PCR could be used for detection of each type of DNA. Thus, we used duplex real-time PCR to investigate positive samples for CMV, HSV-1 or VZV. Unfortunately, HSV-1 positive samples were not available at the time, so only CMV/VZV duplex real-time PCR was tested.

Seventy-six CMV positive samples and 9 VZV positive samples were re-analyzed using CMV/VZV duplex real-time PCR and compared to the singleplex real-time PCR for each pathogen. In addition, 15 samples that were negative for the focal pathogens were included. Table 27 presents the diagnostic efficiency of the duplex real-time PCR of CMV and VZV determination. The CMV/VZV duplex real-time PCR had comparable diagnostic efficiency compared to CMV singleplex real-time PCR and VZV singleplex real-time PCR, with efficiencies of approximately 85% and 67%, respectively. However, false negative results were found in CMV detection by singleplex and duplex real-time PCR at 10% and 16%, respectively. For VZV

Table 27 Diagnostic efficiency determination of CMV/VZV via duplex real-time PCR.

Sample	Results of CMV singleplex real-time PCR		Results of VZV singleplex real-time PCR		Results of CMV/VZV duplex real-time PCR	
	Positive	Negative	Positive	Negative	Positive	Negative
CMV positive sample (N=76)	66 (87%)	10 (13%)	ND	ND	64 (84%)	12 (16%)
VZV positive sample (N=9)	0 (0%)	9 (100%)	6 (67%)	3 (33%)	6 (67%)	3 (33%)
Negative sample (N=15)	0 (0%)	15 (100%)	0 (0%)	15 (100%)	0 (0%)	15 (100%)

ND: Not determined

determination, false negative results were found in 33% by both singleplex and duplex real-time PCR amplification. In addition, the known negative samples were all negative.

3.2 Multiplex real-time PCR development

Based on the incidence data of infectious uveitis of our study population and the advantages of multiplex real-time PCR (QIAGEN, Inc. QIAGEN® Multiplex PCR Handbook, 2010), the diagnostic benefits of this technique included:

- reduced reagent cost for each intraocular fluid sample investigation.

- elimination of amplification variation that may occur in each intraocular fluid sample among separately singleplex real-time PCR testing for each infectious agent. All infectious agents were simultaneously co-amplified by multiplex real-time PCR in the same test tube.

- reduction of the volume of extracted DNA from intraocular fluid samples used for l investigating infectious agents as compared to separate identification by singleplex real-time PCR. This allows for the remaining extracted DNA from intraocular fluid samples to be retested.

- increased throughput in each run since more targets can be analyzed per run on a real-time cycle.

3.2.1 Optimization of multiplex real-time PCR

The duplex real-time PCR investigation suggested that CMV/HSV-1 duplex real-time PCR and CMV/VZV duplex real-time PCR could be used for detection of each DNA. However, CMV/HSV-2 duplex real-time PCR was not as efficient.

Thus, we designed new primers and probes that could detect both HSV-1 and HSV-2. Using the Primer premier 5 software, with primers and probes specific to UL30 gene, the conserved regions among HSV-1 and HSV-2 were obtained. (Table 5)

For the amplification optimization, the concentrations of primers and probes for HSV were determined. Finally we obtained the optimal forward primer, reverse primer and probe at the final concentrations of 300 nM, 300 nM and 100 nM, respectively. (Tables 9 and 28)

Table 28 The results of HSV singleplex real-time PCR optimization.

HSV singleplex real-time PCR condition	**DNA control conc./reaction**	**Average C_T value of detection for**	
		HSV-1 DNA	**HSV-2 DNA**
Condition I : **- FWD primer : 300 nM** **- RWD primer : 300 nM** ***- Probe : 200 nM***	1 pg	28.16	29.22
	0.01 pg	34.11	33.59
Condition II : **- FWD primer : 300 nM** **- RWD primer : 300 nM** ***- Probe : 100 nM***	1 pg	28.34	31.04
	0.01 pg	36.72	37.89
Condition III : **- FWD primer : 300 nM** **- RWD primer : 300 nM** ***- Probe : 50 nM***	1 pg	31.73	31.78
	0.01 pg	35.75	35.18

The sensitivity of the optimal HSV singleplex real-time PCR was measured. Serially diluted HSV-1 and HSV-2 DNA controls that contained 1fg/reaction to 0.001 fg/reaction were tested. The sensitivity of HSV-1 and HSV-2 detection was the same at 0.1 fg/reaction (Table 29).

Table 29 The sensitivity of HSV singleplex real-time PCR.

Reaction	HSV DNA control conc./reaction	Average C_T value of detection for	
		HSV-1 DNA	HSV-2 DNA
1	1 fg	30.79	31.39
2	0.1 fg	32.57	33.31
3	0.01 fg	38.12	39.63
4	0.001 fg	40.24	39.70

The optimal concentrations of primers and probes for CMV, HSV and VZV (Table 5) were mixed and investigated for CMV, HSV-1, HSV-2 and VZV DNA detection. Using serially diluted plasmid DNA controls for each pathogen, satisfactory detection signals were obtained (Table 30).

3.2.2 Determination of sensitivity and specificity of multiplex real-time PCR

In parallel with the optimization step, the sensitivity and specificity of multiplex real-time PCR for CMV, HSV and VZV were determined and compared to

singleplex real-time PCR for each pathogen (Table 31, 32, 33). The sensitivity of multiplex real-time PCR are listed in Table 30.

Table 30 The sensitivity test of multiplex real-time PCR.

Multiplex real-time PCR reaction for detection of	**Sensitivity**	
	DNA conc./reaction	**DNA copies/reaction**
1. CMV	0.1 fg	30
2. HSV-1	1 fg	300
3. HSV-2	0.1 fg	30
4. VZV	0.1 fg	30

Table 31 The average C_T value of multiplex real-time PCR compared to CMV singleplex real-time PCR.

CMV DNA control conc./reaction	**Average C_T value**	
	CMV singleplex real-time PCR	**Multiplex real-time PCR**
1 fg	34.21	33.54
0.1 fg	38.23	36.24

Table 32 The average C_T value of multiplex real-time PCR compared to HSV singleplex real-time PCR.

DNA control		**Average C_T value**	
Type	**conc./reaction**	**HSV singleplex real-time PCR**	**Multiplex real-time PCR**
HSV-1	1 fg	34.86	34.40
HSV-1	0.1 fg	38.49	39.48
HSV-2	1 fg	34.20	33.81
HSV-2	0.1 fg	38.56	38.42

Table 33 The average C_T value of multiplex real-time PCR compared to VZV singleplex real-time PCR.

CMV DNA control conc./reaction	**Average C_T value**	
	VZV singleplex real-time PCR	**Multiplex real-time PCR**
1 fg	34.94	33.46
0.1 fg	38.33	38.13

3.2.3 Diagnostic efficiency determination of multiplex real-time PCR

The known positive samples for CMV, HSV and VZV were retested by multiplex real-time PCR and compared to singleplex real-time PCR of each pathogen. Mulitplex real-time PCR showed comparable efficiency for CMV and VZV diagnosis to singleplex real-time PCR with 100% of true positives. However, in HSV detection, only 4/11 (36%) were diagnosed by multiplex real-time PCR. As well as the HSV singleplex real-time PCR, the same 4 samples presented a positive signal of amplification. Meanwhile, 7/11 (67%) gave negative results in both singleplex and multiplex real-time PCR (Table 34).

Table 34 Diagnostic efficiency determination of multiplex real-time PCR.

Sample	Results of CMV singleplex real-time PCR		Results of HSV singleplex real-time PCR		Results of VZV singleplex real-time PCR		Results of Multiplex real-time PCR	
	Positive	Negative	Positive	Positive	Negative	Negative	Positive	Negative
CMV positive sample (N=40)	40 (100%)	0 (0%)	ND	ND	ND	ND	40 (100%)	0 (0%)
HSV positive sample (N=11)	ND	ND	4 (36%)	7 (64%)	ND	ND	4 (36%)	7 (64%)
VZV positive sample (N=8)	ND	ND	ND	ND	8 (100%)	0 (0%)	8 (100%)	0 (0%)

ND: Not determined

4. Evaluation of the diagnostic efficiency of Goldmann-Witmer coefficient analysis and Real-time PCR technique in identifying the causes of uveitis.

Sixty-six available paired plasma and intraocular fluid samples of 166 non-HIV patients with uveitis (40%) were further investigated by GWC analysis. When GWC was performed as the additional diagnostic tool, it provided a 22% (5/23) increase in infectious uveitis identification of AU patients and an approximately 8% (5/66) increase overall. In contrast to 13/23 (57%) who were positive solely by real-time PCR. For identification of infectious uveitis using GWC together with real-time PCR, 23 positive were found and 10 of these positive results (10/23, 43%) were diagnosed by GWC. However, most of these uveitis patients (43/66, 65%) showed negative results in both tests (Table 35).

Table 35 Diagnostic results of non-HIV patients with uveitis analyzed by real-time PCR and GWC analysis.

Anatomical uveitis type (N)	**Diagnostic results (%)**			
	PCR+/GWC+	**PCR+/GWC-**	**PCR-/GWC+**	**PCR-/GWC-**
AU (23)	2 (9%)	6 (26%)	5 (22%)	10 (43%)
IU (4)	0 (0%)	0 (0%)	0 (0%)	4 (100%)
PU (20)	0 (0%)	4 (20%)	0 (0%)	16 (80%)
PanU (19)	3 (16%)	3 (16%)	0 (0%)	13 (68%)
Total (66)	**5 (8%)**	**13 (20%)**	**5 (8%)**	**43 (65%)**