Technical Note

Optimization of Human Cytomegalovirus LightCycler Real-Time PCR

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Abstract

Background: Real-time PCR has been widely considered as a powerful tool for the evaluation of Human Cytomegalovirus (CMV) DNA kinetics. Successful PCR relies on optimization, which is an extremely demanding procedure. Nevertheless, certain values could be optimal for most primers in use.

Methodology: Seventeen CMV primer sets recommended in the literature were selected for optimization in terms of $MgCl_2$ and primers concentrations as well as annealing temperature using the LightCycler instrument and SYBR Green I detection format. Optimal values were considered as those showing the lowest crossing point (Cp), the highest fluorescence intensity, the steepest sigmoid curve slope, and the absence of non-specific PCR products.

Results: Optimal values for most studied primers were found to be 3 mM for $MgCl_2$ concentration, 0.5 μM and 0.6 μM for primers concentration, and 55ºC for annealing temperature.

Conclusion: Adopting the resulting values for CMV-specific primers generally used in single-target real-time PCR assays with the same thermal cycler may guarantee their efficient performance minimizing cost and time needed for optimization.

Key Words: CMV primers, real-time PCR, optimization

J Infect Developing Countries 2008; 2(5):406-410.

Received 17 April 2008 - Accepted 18 June 2008

Introduction

Human Cytomegalovirus (CMV) remains a significant cause of morbidity and mortality in immunocompromised patients, putting them at risk of fatal CMV disease upon viral reactivation [1-6]. Real-time PCR has been widely considered as a rapid, efficient, and highly sensitive technique for the evaluation of CMV DNA kinetics [2,7-10], and an effective assay for (i) early diagnosis of CMV disease, (ii) antiviral therapy monitoring, (iii) relapse prediction and (iv) antiviral resistance indication [1,5,11-14].

The key to robust detection of CMV DNA lies in the efficacy and sensitivity of PCR which largely depends on the efficiency of primers as well as on the optimization of PCR conditions [4,15]. However, optimization is a time-consuming, laborious, and costly process. To date, several manufacturers are developing kits that include the PCR components already in their optimal concentrations.

Optimal values for PCR conditions would ensure efficient performance of most of the different oligonucleotide primers in use. To reveal such optimal values, this study aimed at optimizing CMV single-target real-time PCRs in terms of $MgCl_2$ and primers concentrations and annealing temperature using SYBR Green I format.

Materials and Methods

Using "CMV" and "PCR" as keywords, PubMed was searched in the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) for English peer-reviewed articles published between 1993 and 2004. Articles were excluded when (i) they did not scope out CMV PCRs; (ii) they did not describe primers sequences; (iii) they reported on virus genotyping or species-level identification; or (iv) they were listed as editorials, letters to editors, or reviews. The resulting 57 papers with a total of 115 CMV-specific primer pairs were examined. Oligonucleotides with identical sequences or with one additional nucleotide at either the 5' or 3' end of the sequence were identified as synonymous, resulting in 82 primer sets. Primers were included in this study when (i) used in real-time PCRs in the original publications (set 3, set 4, set 7, set 9, set 10, set 12, set 13, set 14, set 16 and set 17); or (ii) evaluated by conventional PCRs and accordingly

recommended (set 1, set 2, set 5, set 6, set 7, set 8, set 11 and set 15) [Table 1]. When nested PCRs or DNA sequencing were described, external primers and sequencing primers were excluded respectively.

Table 1. Optimal empirically-defined PCR conditions for CMV primer sets compared to the original publications.

Primers studied		$MgCl_2$ (mM)		Primers (µM)		Annealing temp. (ºC)		
Primer Set[a]	Primer Sequence (5'-3')	Our study[b]	Original Papers	Our study	Original Papers	Our study	Original Papers	Experimental Techniques used in Original Papers
1 [27]	GTA CAC GCA CGC TGG TTA CC GTA GAA AGC CTC GAC ATC GC	3	NA[c]	0.7	0.2 [27]	54	60 [27]	Multiplex conventional PCR [27]
2 [28]	GCA CCG AGA CGC GCA CCG AA CAG CCT CTA CCC TTC CAT CA	3	4 [28]	0.6	NA	55	50 [28]	Conventional PCR [28]
3 [10]	GCC GAT CGT AAA GAG ATG AAG AC CTC GTG CGT GTG CTA CGA GA	3	NA	0.8	NA	55	60 [10]	TaqMan Technology [10]
4 [29]	ACG ATT CAC GGA GCA CCA G GCT GAC GCG TTT GGT CAT C	4	NA	0.8	0.2 [29]	54	60 [29]	TaqMan Technology [29]
5 [30]	GCC TAT CGG TGT CGC TGT ACT C GCG AGG TGT CAT GTT CGA CG	3	7.5 [30]	0.5	NA	55	65 [30]	PCR-ELISA [30]
6 [31, 32]	TCA ATC ATG CGT TTG AAG AGG TA ACC ACC GCA CTG AGG AAT GTC AG	3	1.5 [31]	0.5	0.2 [31]	55	50 [31]	Conventional PCR [31, 32]
7 [33]	TGA GGC TGG GAA GCT GAC AT TGG GCG AGG ACA ACG AA	3	10 [33]	0.7	0.4 [33]	54	60 [33]	TaqMan Technology [33]
8 [28]	GCG GTG GTT GCC CAA CAG GA ACG ACC CGT GGT CAT CTT TA	4	NA	0.7	NA	55	NA	Conventional PCR [28]
9 [34]	GTA GCT GGC ATT GCG ATT GGT TCC AAC ACC CAC AGT ACC CGT	5	5 [34]	0.6	1 [34]	57	66 [34]	Hybridization Probes [34]
10 [13, 17, 18, 24]	ATA GGA GGC GCC ACG TAT TC TAC CCC TAT CGC GTG TGT TC	3	1.5 [17, 18], 4 [13]	0.6	0.4 [18], 0.5 [13, 17]	58	56 [17, 24], 65 [18]. 55 [13]	Conventional PCR [17, 18], Hybridization Probes [13, 24] PCR-ELISA [17]
11 [17]	TGG ACG AGG CTG CCC ATG AGG TGG ACC TGG CCA AAC GAG CCC	3	1.5 [17]	0.4	0.5 [17]	54	56 [17]	
12 [35]	GCA GCC ACG GGA TCG TAC T GGC TTT TAC CTC ACA CGA GCA TT	3	NA	0.6	0.4 [35]	55	60 [35]	TaqMan Technology [35]
13 [20-22]	GGG ACA CAA CAC CGT AAA GC GTC AGC GTT CGT GTT TCC CA	3	4 [22], 5 [21], 6 [20]	0.5	0.5 [20, 22], 0.2 [21]	65	65 [21, 22], 60 [20]	TaqMan Technology [20-22]
14 [36]	GAC ACA ACA CCG TAA AGC CAG CGT TCG TGT TTC C	4	4 [36]	0.4	0.4 [36]	60	60 [36]	Hybridization Probes [36]
15 [19, 23]	GGA TCC GCA TGG CAT TAC CGT ATG T[d] GAA TTC AGT GGA TAA CCT GCG GCG A	3	1.5 [23], 2.5 [19]	0.5	2 [23]	55	55 [19, 23]	Conventional PCR [19, 23]
16 [14]	GGA CGT ATC CAC CTC AGG TAC ACA TAC GTT ACG AAA CTG AGC TCC CAC	3	4 [14]	0.5	0.05 [14]	62	62 [14]	Hybridization Probes [14]
17 [37]	AAA AGT TTG TGC CCC AAC GGT A GCG TGC TTT TTA GCC TCT GCA	3	NA	0.6	0.3 [37]	55	61 [37]	TaqMan Technology [37]

a) Reference(s) of the primer set shown in parentheses. b) Optimal values identified using the LightCycler instrument and SYBR Green I format. c) NA: non-applicable for not being described in the original papers. d) Original primer sequence (GGA TCC GCA TGG CAT TCA CGT ATG T) was corrected to match CMV strain AD169 [38].

Optimal values were empirically identified for all 17 primer sets manufactured by Metabion, Munich, Germany. Different PCR conditions of $MgCl_2$ and primers concentrations and annealing temperature were tested in duplicate over 2-5 mM, 0.3-1 µM and Tm-(Tm-5) ºC ranges at 1 mM, 0.1 µM and 2ºC intervals, respectively, where Tm is the primer-specific melting temperature. Primers concentrations described in the original papers were adopted when only used on the LightCycler instrument. The temperature profile was initiated at 95ºC for 10 minutes, followed by 55 cycles of denaturation at 95ºC for 5 seconds, annealing for 5 seconds, and extension with fluorescence monitoring at 72ºC for a time (in seconds) calculated by the formula recommended by the manufacturer: amplicon length (bp) ÷ 25. PCRs were performed on the LightCycler instrument using the LightCycler-FastStart DNA Master SYBR Green-I Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The amount of CMV strain AD169 DNA template used corresponded to crossing point values ranging between 25 and 35. The product-specific melting point was anticipated using the Tm Utility v1.5 software (Idaho Technology Inc., Utah, USA) to be checked later by the melting-curve analysis. Optimal values were considered when showing (i) the lowest crossing point (Cp), (ii) the highest fluorescence intensity, (iii) the steepest sigmoid curve slope, and (iv) the absence of non-specific products.

Results

The overall optimal $MgCl_2$ concentrations varied between 3 and 5 mM for all studied primers. However, 3 mM was an optimal $MgCl_2$ concentration for 13 primer sets out of 17 (76%). Moreover, this concentration showed the highest mean value of fluorescence intensities and the least mean value of crossing points [Figure 1]. When $MgCl_2$ concentrations were increased above 3 mM, the mean value of fluorescence intensities and the sigmoid curve slope steepness decreased though the mean value of crossing points remained constant. When $MgCl_2$ concentrations were decreased to 2 mM, a higher mean value of crossing points was shown [Figure 1]. Amplification always failed with $MgCl_2$ concentration at 1 mM.

Optimal primer concentrations ranged from 0.4 to 0.8 µM for all 17 primers. However, 0.5 µM and 0.6 µM were the optimal concentrations for 10 primer sets out of 17 (59%) [Figure 2]. The highest mean value of fluorescence intensities and the least mean value of crossing points were shown at primer concentrations between 0.5 and 1 µM. But non-specific products were encountered when primer concentrations were raised above 0.6 µM for set 5, set 6 and set 11.

Figure 1. Variation of crossing points mean value and fluorescence intensities mean value according to magnesium chloride concentration for all 17 primer sets studied. $MgCl_2$ concentration of 3 mM showed to be the optimum.

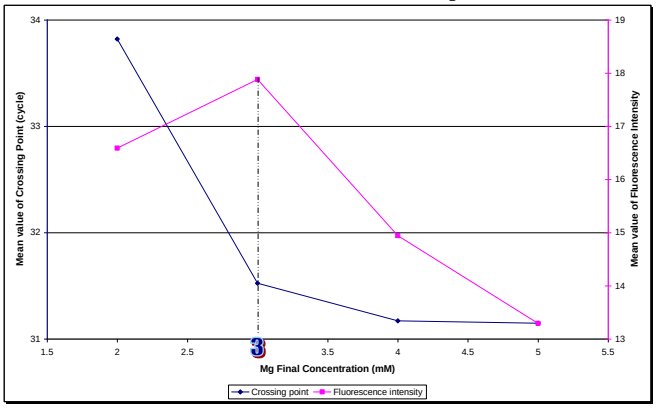
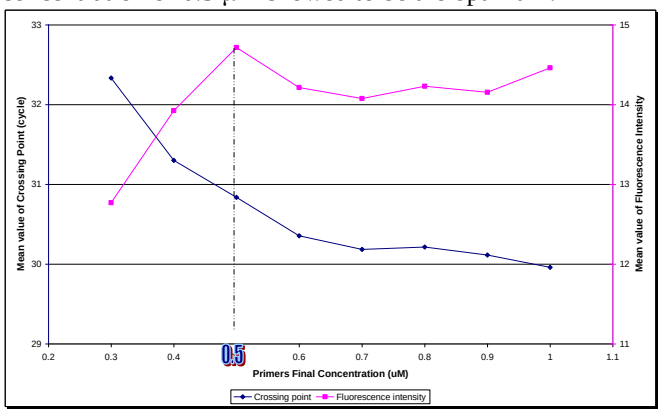
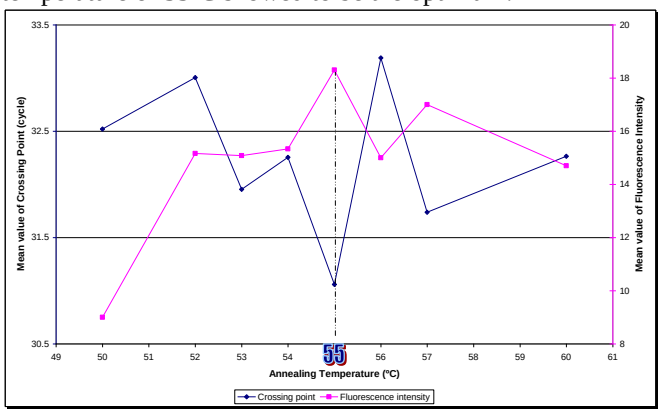


Figure 2. Variation of crossing points mean value and fluorescence intensities mean value according to concentration of all 17 primer sets studied. Primer concentration of 0.5 µM showed to be the optimum.



While overall primer-specific melting temperatures ranged from 52ºC to 66ºC, optimal empirically-determined annealing temperatures ranged between 54ºC and 66ºC. However, 12 primer sets out of 17 (71%) showed optimal performance at annealing temperatures of 54ºC or 55ºC. Furthermore, Figure 3 illustrates that the highest mean value of fluorescence intensities and the least mean value of crossing points were shown at annealing temperature of 55ºC.

Figure 3. Variation of crossing points mean value and fluorescence intensities mean value according to annealing temperature for all 17 primer sets studied. Annealing temperature of 55ºC showed to be the optimum.



Discussion

Optimal primer design as well as optimal conditions are essential for maximal efficiency, sensitivity, and specificity of real-time PCR and, hence, for robust CMV diagnosis and monitoring [4,15,16]. Thus this study aimed at taking all published CMV primers as well as literature recommendations into consideration. Out of 82 published CMV-specific primer pairs, 17 primer sets were selected carefully in terms of specificity and sensitivity so that undesired reaction results should not be attributed to poor design of primers.

Although adopted PCR conditions for most of the CMV primers included in this study were already described in the original papers, optimal conditions were to be redefined empirically in accordance to the thermal cycler, detection format, target multiplicity, and commercial kit used in our study. It has been well-known that such factors play an essential role in optimizing PCR reaction [16]. This is underlined by the fact that different authors [13,17-24] using different experimental techniques employed different optimal conditions for the very same primer pair [Table 1]. In addition, optimal values determined in our study were different from those described in the original publications, which indicates the necessity for optimizing PCR conditions empirically when using different experimental techniques.

On the other hand, when the thermal cycler, detection format, target multiplicity, and commercial kit used are unified, it seems there are certain conditions that deliver supreme PCR efficiency and primer performance for most primers in use, the fact reflected here by the highest fluorescence intensity, the lowest crossing point, the steepest sigmoid curve slope, and the absence of non-specific products. Even though several manufacturer companies are increasingly providing kits with most PCR components optimized, PCR optimization remains a considerably time-consuming, laborious, and costly procedure. Therefore, identifying and adopting such optimal values introduces an exemption from optimizing reactions performed in defined circumstances.

PCR conditions were optimized in terms of $MgCl_2$ and primers concentrations and annealing temperature according to the manufacturer's instructions. Other PCR components concentrations were not tested because they were already optimized by the manufacturer. Our results indicated that $MgCl_2$ concentration of 3 mM, primers concentration of 0.5 µM and 0.6 µM, and annealing temperature of 54ºC and 55ºC were the optimal conditions for most primers studied. However, the crossing point values increased at $MgCl_2$ concentrations lower than 3 mM for less DNA polymerase and primers annealing efficiencies [25,26]; whereas the reaction more quickly reached the plateau phase with decreasing fluorescence intensity and sigmoid curve slope steepness at $MgCl_2$ concentrations more than 3 mM. This could be ascribed to template

reannealing effect which inhibits the formation of template-primer hybrids. Moreover, decreasing primer concentration below 0.5 μM resulted in a reduced PCR efficiency, but increasing primer concentration above 0.6 μM gave rise to non-specific products for 3 primer sets out of 17 sets. Otherwise, non-specific products were not encountered at all. This could be due to hot start PCR utilization as well as careful selection of all 17 studied primer sets among all published sets according to literature recommendations. Furthermore, albeit governed by the widely variable primer-specific melting temperature, annealing temperature of 55ºC could be acceptable for most primers, ensuring the balance between generating detectable yield and amplification of undesired products.

In conclusion, we empirically determined the optimal values of CMV single-target real-time PCRs to be adopted for most CMV-specific primers using SYBR Green I as well as sequence-specific probe detection formats on the LightCycler instrument. However, the latter format may require elevated $MgCl_2$ concentrations by 0.5-2 mM over the SYBR Green I optimum. Starting with such values guarantees efficient PCR performance, minimizing cost and time needed for optimization.

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Conflict of interest: No conflict of interest is declared.