Comparative evaluation of published cytomegalovirus primers for rapid real-time PCR: which are the most sensitive?

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Standardization of human cytomegalovirus (CMV) PCR is highly recommended. As primer design is essential for PCR sensitivity, this study evaluated all published CMV primer pairs to identify the most sensitive for single-round real-time PCR. PubMed (1993–2004) was searched for original papers aimed at CMV PCR. Fifty-seven papers were identified revealing 82 different primer pairs. Of these, 17 primer sets were selected for empirical study, as they were either used in real-time PCR or were evaluated comparatively by conventional PCR. After optimizing the PCR conditions, these primer sets were evaluated by real-time PCR using a SYBR Green format. Analytical sensitivities were assessed by testing the reference standard CMV strain AD169. A BLAST search was performed to identify mismatches with published sequences. Additionally, 60 clinical samples were tested with the three primer sets showing highest analytical sensitivity and the best match to all CMV strains. Three primer sets located in the glycoprotein B (*UL55*) gene region were found to be the most sensitive using strain AD169. However, two of these showed a considerable number of mismatches with clinical isolates in a BLAST search. Instead, two other pairs from the lower matrix phosphoprotein (*UL83*) gene and DNA polymerase (*UL54*) gene showed reasonable sensitivity and no mismatches with clinical isolates. These three pairs were further tested with clinical samples, which indicated that the two primer sets from *UL55* and *UL54* were the most sensitive. Interestingly, the analytical sensitivity of the PCR was inversely correlated with the size of the PCR product. In conclusion, these two primer pairs are recommended for a standardized, highly sensitive, real-time PCR.

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INTRODUCTION

Real-time PCR is widely considered as an efficient and highly sensitive technique for the evaluation of human cytomegalovirus (CMV) DNA kinetics (Hong *et al.*, 2004; Kearns *et al.*, 2001; Piiparinen *et al.*, 2004; van Doornum *et al.*, 2003), provided that the target sequence and primers are properly selected (Caliendo *et al.*, 2000; Gault *et al.*, 2001). To date, several evaluation studies have demonstrated significant differences in sensitivity of various in-house and commercial PCRs (Allen *et al.*, 1995; Boeckh *et al.*, 2004; Distefano *et al.*, 2004; Weinberg *et al.*, 1998; Zweyberg-Wirgart *et al.*, 1998), which hinder the conduction of comparative studies (Caliendo *et al.*, 2000; Tong *et al.*, 2000) and accurate follow-up of patients (Gault *et al.*, 2001). Moreover, failure of the most frequently used primers to detect some CMV isolates (Allen *et al.*, 1995; Distefano *et al.*, 2004; Zweyberg-Wirgart *et al.*, 1998), and hence

contradictory results between different laboratories (Bustin, 2005; Razonable *et al.*, 2002), has been reported. All of these observations imply that the most appropriate target region for amplification has not yet been defined (Gault *et al.*, 2001) and that standardization is still required for reliable and comparable PCR results (Bustin, 2005; Gault *et al.*, 2001; Tong *et al.*, 2000). As the primers selected dramatically affect the efficacy and sensitivity of PCR, several studies have evaluated the performance of different primer pairs (Allen *et al.*, 1995; Boeckh *et al.*, 2004; Distefano *et al.*, 2004; Mendez *et al.*, 1998; Zweyberg-Wirgart *et al.*, 1998). However, the literature is still replete with many other CMV primers that have never been included in such comparative studies. Our comprehensive study aimed to evaluate comparatively all published CMV primers to identify the most sensitive single-round, real-time PCR primers using a SYBR Green I format. As we aimed only to evaluate primers, all methods employing probes to produce a signal were excluded.

Abbreviations: CMV, human cytomegalovirus; vp, viral particle.

METHODS

Literature search. PubMed (from 1993 to June 2004) was searched for English articles using the terms 'CMV' and 'PCR', resulting in 1081 papers. Articles were excluded when they (i) did not describe the CMV PCR in detail or did not describe the primer sequences; (ii) reported on virus genotyping or species-level identification; (iii) were listed as editorials, letters to editors or reviews. This resulted in 57 papers with a total of 115 CMV-specific primer pairs. 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 they were (i) used in real-time PCRs in the original

publication (sets 3, 4, 7, 10, 12, 13, 14, 16 and 17); or (ii) evaluated comparatively by conventional PCR and recommended accordingly (sets 2, 6, 7, 8, 11 and 15). When nested PCRs were described, only internal primers were considered. Three additional primer sets were included; these had been used in our in-house PCR (set 9), used in the AMPLICOR CMV Test (Roche Molecular Systems) (set 5) or were for two annealing sites (set 1; Table 1).

Optimization. Different PCR conditions were tested in duplicate, and optimal values of MgCl₂, primer concentration and annealing temperature were identified for all 17 primer sets, which were manufactured by Metabion. Accordingly, the temperature profile adopted was 95 °C for 10 min, followed by 55 cycles of denaturation

Table 1. Optimal PCR conditions for CMV primer sets assessed experimentally

Primer set 6 was also used by Distefano *et al.* (2004); primer set 10 was also used by Bai *et al.* (2000), Pang *et al.* (2003) and Sekhon *et al.* (2004); primer set 13 was also used by Exner & Lewinski (2002) and Gouarin *et al.* (2002); and primer set 15 was also used by Mendez *et al.* (1998).

Primer set	Reference	Sequence (5'→3')	Target region	Optimal PCR conditions			
				MgCl ₂ (mM)	Primers (µM)	Annealing temp. (°C)	Elongation time (s)
1	Markoulatos <i>et al.</i> (2001)	GTACACGCACGCTGGTTACC GTAGAAAGCCTCGACATCGC	<i>TRL11, IRL11</i>	3	0.7	54	11
2	Zweyberg-Wirgart <i>et al.</i> (1998)	GCACCGAGACGCGCACCAGAA CAGCCTCTACCCCTCCATCA	<i>UL54</i>	3	0.6	55	7
3	van Doornum <i>et al.</i> (2003)	GCCGATCGTAAAGAGATGAAGAC CTCGTGCGTGTGCTACGAGA	<i>UL54</i>	3	0.8	55	6
4	Sanchez & Storch (2002)	ACGATTCACGGAGCACCAG GCTGACGCGTTTGGTCATC	<i>UL54</i>	4	0.8	54	3
5	Weinberg <i>et al.</i> (1998)	GCCTATCGGTGTCGCTGTACTC GCGAGGTGTCATGTTCCGACG	<i>UL54</i>	3	0.5	55	15
6	Jones <i>et al.</i> (2000)	TCAATCATGCGTTTGAAGAGGTA ACCACCGCACTGAGGAATGTCAG	<i>UL55</i>	3	0.5	55	4
7	Boeckh <i>et al.</i> (2004)	TGAGGCTGGGAAGCTGACAT TGGGCGAGGACAACGAA	<i>UL55</i>	3	0.7	54	3
8	Zweyberg-Wirgart <i>et al.</i> (1998)	GCGGTGGTTGCCCAACAGGA ACGACCCGTGGTCATCTTTA	<i>UL55</i>	4	0.7	55	4
9	Ibrahim <i>et al.</i> (2005)	GTAGCTGGCATTGCGATTGGT TCCAACACCCACAGTACCCGT	<i>UL55</i>	5	0.6	57	9
10	Allen <i>et al.</i> (1995)	ATAGGAGGCGCCACGTATTC TACCCCTATCGCGTGTGTTC	<i>UL55</i>	3	0.6	58	11
11	Allen <i>et al.</i> (1995)	TGGACGAGGCTGCCCATGAGG TGGACCTGGCCAAACGAGCCC	<i>UL75</i>	3	0.4	54	10
12	Griscelli <i>et al.</i> (2001)	GCAGCCACGGGATCGTACT GGCTTTTACCTCACACGAGCATT	<i>UL83</i>	3	0.6	55	7
13	Gault <i>et al.</i> (2001)	GGGACACAACACCGTAAAGC GTCAGCGTTCGTGTTTCCCA	<i>UL83</i>	3	0.5	65	12
14	Stöcher & Berg (2002)	GACACAACACCGTAAAGC CAGCGTTCGTGTTTCC	<i>UL83</i>	4	0.4	60	12
15	Drouet <i>et al.</i> (1995)	GGATCCGCATGGCATTACCGTATGT* GAATTCACTGGATAACCTGCGGCGA	<i>US7, 8</i>	3	0.5	55	17
16	Razonable <i>et al.</i> (2001)	GGACGTATCCACCTCAGGTACACA TACGTTACGAAACTGAGCTCCAC	<i>US9</i>	3	0.5	62	12
17	Machida <i>et al.</i> (2000)	AAAAGTTTGTGCCCCAACGGTA GCGTGCTTTTTAGCCTCTGCA	<i>US17</i>	3	0.6	55	7

*The original primer sequence (5'-GGATCCGCATGGCATTACCGTATGT-3') was corrected to match CMV strain AD169 (Habbal *et al.*, 2005).

at 95 °C for 5 s, annealing at the experimentally determined optimal temperature for 5 s and extension with fluorescence monitoring at 72 °C for a time (in seconds) calculated by the formula recommended by the manufacturer: amplicon length in bp/25 (Table 1). The product-specific melting point (T_m) was anticipated using the T_m Utility version 1.5 software (Idaho Technology) and was checked later by melting-curve analysis. PCRs were performed on a LightCycler instrument using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics), following the manufacturer's recommendations, and using 5 µl CMV strain AD169 as DNA template.

Sensitivity assessment. DNA was extracted from 200 µl aliquots of CMV strain AD169 reference standard containing 3.22×10^{10} virus particles (vp) ml⁻¹ (Advanced Biotechnologies) by using a High Pure Viral Nucleic Acid kit (Roche Diagnostics) according to the manufacturer's instructions. Three 50 µl extracts were pooled, and serial 10-fold dilutions of the pool were tested, so that all PCR runs were undertaken with DNA from the same extraction. In an initial evaluation, the 10-fold dilutions were tested in duplicate using all 17 primer sets. Only 12 primer sets with a positive result up to a dilution of at least 10^{-8} were further tested in a second evaluation using 10 replicates of three dilutions (the last two dilutions with positive reactions and the next dilution with a negative reaction in the initial evaluation).

Statistical analysis. Sensitivities were assessed by probit analysis using SPSS 10.0 for Windows software. Regression analysis was carried out by using Prism 4 for Windows and Macintosh software (GraphPad Software).

BLAST search. All 17 primer sets were used for a BLAST search against the reference genomic sequences and nucleotide collection (nr/nt) databases of CMV. Mismatches with the published sequences of CMV strains and isolates were analysed.

Evaluation of clinical samples. Serum samples from 60 kidney transplant recipients with proven CMV infection were extracted as described above. None of the patients was under antiviral therapy when the blood sample was taken.

RESULTS AND DISCUSSION

It has been firmly established that standardization is essential to overcome the observed pitfalls of real-time CMV PCR in both diagnostics and research, despite its well-known benefits (Bustin, 2005; Gault *et al.*, 2001). Therefore, several previous studies have compared CMV oligonucleotides, and examined their reliability and sensitivity (Allen *et al.*, 1995; Boeckh *et al.*, 2004; Distefano *et al.*, 2004; Mendez *et al.*, 1998; Zwegberg-Wirgart *et al.*, 1998). Nevertheless, there remain many CMV-specific primer sets available in the literature that have not undergone such evaluation studies, especially for real-time PCR. Thus this study aimed to take all available published CMV primers as well as literature recommendations into consideration. We aimed only to evaluate primer sensitivities as an essential step, and to evaluate primer and probe combinations in future studies. Therefore, we adopted a SYBR Green I detection format on a LightCycler instrument, and excluded specific hybridization and hydrolysis probe detection formats.

Optimal conditions

Table 1 shows the optimal empirically defined PCR conditions ranging from 3 to 5 mM for MgCl₂, from 0.4 to 0.8 µM for primer concentrations and from 54 to 65 °C for annealing temperatures. The extension times calculated according to the length of the PCR product determined by each primer pair are also listed in Table 1. Although PCR conditions for most of the CMV primers evaluated in our study had already been described in the original papers, optimal conditions were redefined empirically in accordance with the thermal cycler, detection format and commercial kit used in our study. It is commonly accepted that these factors play an essential role in optimizing PCRs. This is underlined by the fact that different authors often employed different optimal conditions for the same CMV primer pair (Allen *et al.*, 1995; Bai *et al.*, 2000; Drouet *et al.*, 1995; Exner & Lewinski, 2002; Gault *et al.*, 2001; Gouarin *et al.*, 2002; Mendez *et al.*, 1998; Pang *et al.*, 2003; Sekhon *et al.*, 2004). Our optimal conditions were different for most primer sets from the values adopted in the original papers.

Sensitivity assessment

Five primer sets were excluded after the initial evaluation because they showed positive results only up to dilutions of 10^{-6} (set 15) or 10^{-7} (sets 5, 6, 11 and 16). The remaining 12 primer sets showed positive results up to dilutions of 10^{-8} (set 4), 10^{-9} (sets 1, 7, 8, 9, 10, 13 and 17) or 10^{-10} (sets 2, 3, 12 and 14) in the initial evaluation. In the second evaluation using 10 replicates per dilution, the predicted detection limit corresponding to probit of 0.90 was fewer than 10 vp ml⁻¹ for sets 7, 8 and 10, and ranged from 20 to 50 vp ml⁻¹ for sets 1, 2, 3, 9 and 12 (Table 2).

Annealing at more than one target site on the CMV strain AD169 genome may have resulted in the high sensitivity (30 vp ml⁻¹) of primer set 1. However, the sensitivity of this primer set may be less favourable in a diagnostic setting, as the internal repeat long sequences included in the genome of laboratory CMV strains are not usually found in the genome of clinical CMV isolates (Murphy *et al.*, 2003). Moreover, a large number of primer sets evaluated in this study proved to be more sensitive than primers adopted in frequently used commercial kits (set 5; >322 vp ml⁻¹) (Weinberg *et al.*, 1998).

BLAST search

As we used a laboratory-adapted strain for evaluation of sensitivity, we added a BLAST search to identify mismatches with other CMV strains and isolates. Three primer sets (sets 6, 11 and 12) did not show any mismatches with published sequences of CMV strains and isolates. However, sets 6 and 11 were initially excluded for showing poor sensitivity. The remaining 14 primer sets showed mismatches with some published CMV sequences, mostly related to laboratory CMV strains such as Toledo and Towne (Table 2).

Table 2. CMV primer sets assessed experimentally in terms of sensitivity and computationally by BLAST search

NA, Not applicable: primers not tested because their last positive dilution was higher than 10^{-8} in the initial evaluation; hence sets 5, 6, 11, 15 and 16 were excluded.

Primer set	Amplicon (bp)	Sensitivity assessment		CMV strains not matching
		End point sensitivity in vp ml ⁻¹ *	90/95 % detection limit in vp ml ⁻¹ †	
1	257		30/36	Merlin, Toledo and Towne and other isolates
2	161		35/104	TB40/E clone TB40-BAC4, W2 and S3 and other isolates
3	133		20/42	Merlin and other isolates
4	61		67/81	Towne
5	362	>322	NA	Merlin and Towne; Toledo-BAC and other isolates
6	94	>322	NA	None
7	66		8/10	FIX-BAC, TR-BAC, Toledo-BAC and other isolates
8	94		8/10	Towne and isolate 1342/93
9	213		50/104	Towne; isolates Toledo-BAC, TR-BAC and FIX-BAC
10	254		9/11	F19/BRAIN and F14/BM; isolates N28, N22, N23, N24, N25, N26, N27, N42 and N44
11	226	>322	NA	None
12	159		49/95	None
13	283		67/109	Toledo and PH-BAC isolate
14	279		908/2805	Toledo
15	407	>3220	NA	Merlin and Toledo and other isolates
16	286	>322	NA	Sw2 and v7868
17	151		74/137	Toledo and Towne

*Last dilution with at least one positive replicate in the initial evaluation using two replicates per dilution.

†Calculated by probit analysis in the final evaluation using 10 replicates per dilution. Probit analysis is given for 90 and 95 %.

Thus our results indicated that three primer sets (sets 7, 8 and 10) selected from the glycoprotein B (*UL55*) gene region were the most sensitive, with detection limits of 8 vp ml⁻¹ (sets 7 and 8) and 9 vp ml⁻¹ (set 10). However, with regard to matching published CMV sequences by BLAST search, set 8 was superior to sets 7 and 10 because it showed only two mismatches with laboratory strain Towne and the 1342/93 isolate. Therefore, it was potentially considered as a recommended candidate for a standardized highly sensitive CMV real-time PCR. Two other primer sets (set 12, *UL83*, and set 4, *UL54*) with reasonable sensitivity (49 and 67 vp ml⁻¹, respectively) also did not show any mismatches except for one mismatch with the laboratory strain Towne for set 4. Hence, they were also considered for further evaluation using CMV clinical isolates.

Evaluation of clinical samples

Three primer sets (sets 8, 4 and 12) were further tested using 60 clinical samples from patients with proven CMV infection. Positive reactions were obtained in 59/60 (98 %), 58/60 (97 %) and 50/60 (83 %) samples using sets 8, 4 and 12, respectively. Unlike set 8, all negative reactions using sets 4 and 12 were observed in the samples with low viral load. These results favour the use of set 8 or 4. As a next

step, different probes will need to be designed for further evaluations (e.g. quantitative analysis) employing the various probe formats such as hydrolysis or hybridization probes. For sets 4 and 12, probes have already been described by the respective authors (Griscelli *et al.*, 2001; Sanchez & Storch, 2002), whilst set 8 has so far only been used in a conventional PCR.

PCR product size

The correlation between PCR sensitivity and PCR product size was examined. Interestingly, as shown in Fig. 1, the analytical sensitivity of the PCR was inversely correlated with the length of the PCR product ($r=0.601$, $P=0.011$). Although there was no clear cut-off for the length of the PCR product, PCRs with product sizes of less than 100 bp generally revealed higher sensitivities (Table 2), although one primer set (set 6) proved to be an exception. The influence of product size on sensitivity is likely to be due to viral DNA fragmentation or a DNA degradation process in the sample, resulting in DNA fragments with a higher chance of annealing to both primers when the PCR covers a shorter fragment (Boom *et al.*, 2002). This has been shown for an Epstein–Barr virus PCR (Stevens *et al.*, 2005). Accordingly, we recommend shorter PCR products to render higher sensitivities.

Fig. 1. Correlation of the PCR sensitivity with the PCR product size. Closed symbols indicate the detection limit as the last dilution with at least one positive replicate (out of two per dilution) in the five primer sets not passing the initial evaluation. Open symbols show the 90 % detection limit in probit analysis based on the final evaluation using 10 replicates per dilution.

In conclusion, we found that the primers investigated differed significantly in sensitivity, and we suggest a primer pair specific for the *UL55* gene as a candidate for a standardized PCR. Moreover, two additional pairs specific for the *UL83* and *UL54* genes combined reasonable sensitivity with excellent matching with published sequences of CMV strains, favouring the primer set from *UL54* when testing clinical samples.

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