Short Communication

PCR optimization: Improving of human cytomegalovirus (HCMV) PCR to achieve a highly sensitive detection method†

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Abstract
Polymerase chain reaction (PCR) is a rapid and simple technique with high sensitivity and specificity. In the recent years, PCR has been used for rapid detection of viral nucleic acids, such as Human cytomegalovirus (HCMV), whereas, PCR optimization is an important task to be done, especially before it's diagnostic application. Annealing temperature, ion concentration (especially Mg^{2+} ion) and the cycling program and enhancer compounds are important optimization parameters. Peripheral blood leukocytes (PBLs) were isolated from samples collected from renal transplant recipients suffering from severe and symptomatic CMV disease. PBLs DNA was extracted and used for PCR. Annealing temperature and $MgCl_2$ concentration and cycling condition were optimized. Dimethyl sulfoxide (DMSO) and gelatin were checked as enhancer components. The optimized condition obtained through this study was: 1x PCR buffer (20 mM Tris-HCl pH 8.6, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.25 μM of each primers, 0.25 unit/25 μl Taq DNA polymerase, 5% DMSO, 500 μg/ml gelatin and 50-150 ng template DNA in 25 μl final volume. PCR was performed as: 95°C 5 min (pre-denaturation), 94°C 50 sec, 58°C 1 min, 72°C 1 min for 35 cycles and 72°C 5 min (final extension). Using these conditions, it was shown that optimized PCR was five fold more sensitive than initial PCR; which can be used for diagnostic application of HCMV in renal transplant patients.
Keywords: *Polymerase chain reaction (PCR), Optimization, Human Cytomegalovirus (HCMV), Renal transplant recipients.*

The polymerase chain reaction (PCR), which uses two oligonucleotides (primers), complementary to sense and antisense strands of a defined DNA fragment, a (heat-stable) DNA polymerase and several cycles of denaturation, primer annealing and extension, is a technique for amplifying specific nucleic acids *in vitro*. This technique has been used with success for amplification of human genomic sequences such as HLA genes (Scharf *et al*. 1986) and now for rapid detection of various viral nucleic acid sequences (Brice *et al*. 1992; Brytting *et al*. 1992; Teleni *et al*. 1990; Zaaijer *et al*. 1993; Kaneko *et al*. 1989 and Jackson *et al*. 1993). Recently, PCR techniques have become a powerful and sensitive tool in a broad field of research that is, molecular biology, medical and food diagnostics, and population genetics. Since it's inception in 1988 (Saiki *et al*. 1988), PCR has become one of the main techniques in modern molecular biology and is arguably the most important biotechnological innovation to date, and is rapidly becoming a standard technique in molecular diagnostic. A major principle concerning the use of PCR, especially in diagnostic application is optimization of PCR. When setting up PCRs for first time with new template DNA, new primers or a new

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preparation of thermostable DNA polymerase enzyme, amplification will generally be less than optimal. Fine-tuning of the reaction is usually required to suppress nonspecific amplification and/or to enhance the sensitivity of reaction. Therefore, depending on how successful is the amplification, it may be necessary to optimize the conditions. In this study, the effect of PCR optimization was shown with step-by-step optimization by using CMV DNA, which was obtained from renal transplant patients to achieve a rapid, simple and sensitive HCMV detection method.

Study population was of renal transplant patients who had severe and symptomatic CMV disease, which is defined with fever, leucopenia, thrombocytopenia, elevated serum creatinine, abnormal liver function tests, pneumonia, gastrointestinal involvement, positive pp65 Antigenemia assay [Monoclonal mouse anti-cytomegalovirus, FITC-conjugated F $(ab')_2$ fragment of rabbit anti-mouse immunoglobuline, DAKO A/S, Denmark] and positive cell culture in human fetal foreskin fibroblast–derived (HFFF) cell line (Sia and Patel, 2000 and Gerna *et al*. 1994). EDTA-blood samples (10 ml) were collected. Peripheral blood leukocytes (PBLs) were separated from whole EDTA-blood by sedimentation of erythrocytes for 30 min at 37°C in equal volume of 5% dextran T-500 (Fluka Chemica AG, Switzerland) solution in phosphate buffered saline (PBS). A quick alkaline lysis technique was used for DNA extraction. Briefly, portion of 5×10^5 PBLs were boiled in 100 µl of 50 mM NaOH for 20 min. Then, 20 µl of 1M Tris-HCl pH 7.6 was added. After centrifuging at 3500 Xg for 2 min, the supernatant was used for PCR. A 406 bp fragment from the 4^{th} exon (*Hind III-X* fragment) of the CMV immediate early (*IE*) gene was amplified by using two primers which, primer A, 5′-GAA TTC AGT GGA TAA CCT GCG GCG A–3′ (nucleotides 197424-197448) was complementary to the sense DNA strand and primer B, 5′-GGA TCC GCA TGG CAT TCA CGT ATG T–3′ (nucleotides 197042-197066) was complementary to the antisense DNA strand (Mendez *et al*. 1988 and Drouet *et al*. 1993).

The initial PCR condition was consisted of 1X PCR buffer (20 mM Tris-HCl pH 8.6, 50 mM KCl) (Cinnagen Inc, Iran), 1.5 mM $MgCl_2$ (Cinnagen Inc, Iran), 0.2 mM of each dNTPs (Cinnagen Inc, Iran), 0.25 µM primer A and B, 0.25 unit/25 µl Taq DNA polymerase (Cinnagen Inc, Iran) and 50-150 ng template DNA in 25 µl final volume. Thirty PCR cycles consisting of (first denaturation: 95°C for 10 min), 95°C for 1 min, 55°C for 1 min and 72°C for 1 min (ended by extension at 72°C for 7 min) performed by using an Mastercycler® Personal thermal cycler (Eppendorff Germany). The amplification products were electrophoresed on 1.3% agarose gel (Roche molecular biochemicals, Germany) and photographed by a gel documentation system (ETS Vilber-Lourmat, France). Leucocytes DNA extracted from healthy and normal embryo-cord blood samples were used as negative control. Initial PCR detected HCMV DNA and no other band aside from product was visualized. This product was confirmed by using different restriction enzymes such as *Cfo-I* (Boehringer Mannhein, Germany), *SauA3-I* (GibcoBRL, Germany), *Dde-I* (GibcoBRL, Germany), *Sst-I* (GibcoBRL, Germany), *Mbo-I* (GibcoBRL, Germany) and *BstX-I* (Fermentas AB, Lithuania) (Figure 1).

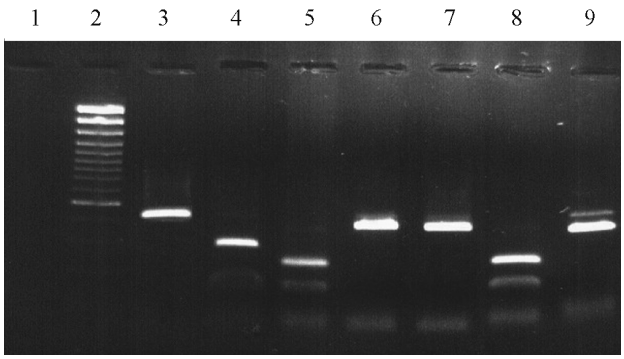

Figure 1: Pattern of restriction enzymes digestion on HCMV PCR-product in 2.5% agarose gel. Lane1: Negative control of HCMV PCR, Lane2: 100 bp DNA ladder plus (Fermentas AB, Lithuania), Lane3: 406 bp undigested HCMV PCR-product, Lane4: *Cfo-I* digestion pattern (271 and 135 bp fragments), Lane5: *Sau3A-I* digestion pattern (208, 131 and 67 bp fragments), Lane6: *Dde-I* digestion pattern (358 and 48 bp fragments), Lane7: *Sst-I* digestion pattern (356 and 50 bp fragments), Lane8: *Mbo-I* digestion pattern (208, 131 and 67 bp fragments), Lane9: *BstX-I* digestion pattern (335 and 71 bp fragments).

Table 1. Work plan matrix and results of annealing temperatures and $MgCl_2$ concentrations optimization.

$MgCl_2$ concentrations (mM)	Annealing temperatures (°C)					
	53	55	58	60	62	64
0.5	-	-	-	-	-	-
1.0	+	+	++	+	+	-
1.5	+	+	+++	+	+	-
2.0	++	++	+++	+	+	+
2.5	++	+++	++++	++	++	+

Description of signs: Minus: No band, +: Weakly band, ++: Semi-strong band, +++: strong band, ++++: High-strong band

Table 2. Work plan matrix and results for DMSO and gelatin optimization.

Gelatin (µg/ml)	DMSO (%)		
	0	5	10
0	–	++	–
50	–	+	–
100	+	++	–
150	+	++	–
300	+	+	–
500	–	++++	–

Description of signs: Minus: No band, +: Weak band, ++: Semi-strong band, +++: strong band, ++++: High-strong band

Sensitivity of initial PCR was determined by performing a PCR on serial dilutions of extracted DNA for first step of optimization. Results showedthat dilution of 10^{-2} of DNA was amplified. $MgCl_2$ concentrations and annealing temperatures optimization were done with different $MgCl_2$ concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mM) at different annealing temperatures (53, 55, 58, 60, 62 and 64°C) by using three dilutions (five µl of 10^{-1}, one µl of $10^{-1}$and five μl of 10^{-2}) of template DNA in a matrix format. The best concentration of $MgCl_2$ was found to be 2.5 mM and the best annealing temperature was 58°C (Table 1). For enhancer components optimization, two enhancer compounds were selected. DMSO (Sigma-Aldrich Co, England) with concentrations of 0, 5 and 10% and gelatin (Sigma-Aldrich Co, England) with concentrations of 0, 50, 100, 150, 300 and 500 μg/ml were examined in a matrix format. Results demonstrated that 5% DMSO and 500 µg/ml gelatin were the most optimal concentrations for this PCR (Table 2). Cycling condition consisted of 25 to 45 cycles in steps of 5 cycles were examined after enhancer's optimization. Optimization program showed that 35 cycles is the best protocol for HCMV PCR. Increasing the cycles resulted in non-specific products. Primers concentrations were optimized as well, by checking different concentrations of primers (0.125, 0.25, 0.5, 0.75 and 1 µM) in the PCR reaction. Results showed that primer concentrations more than 0.25 µM produced primer-dimers and concentrations less than 0.25 µM resulted in less amount of PCR products. Therefore, primers with 0.25 µM concentration were shown to be optimal for this PCR assay.

For comparison the sensitivity of initial (non-optimized) and optimized PCR, two sets of experiments with similar dilutions of template DNAwere prepared and PCR was performed under two conditions, one with optimized PCR and one with initial PCR. Sensitivity of these PCRs was determined by amplification's ability of target gene on serial dilutions of DNA. Comparison of initial and optimized HCMV PCR showed that the sensitivity of optimized PCR was five fold more than initial PCR (Figure 2). Therefore, optimized PCR reaction and program consisting of: 1X PCR buffer (20 mM Tris-HCl pH

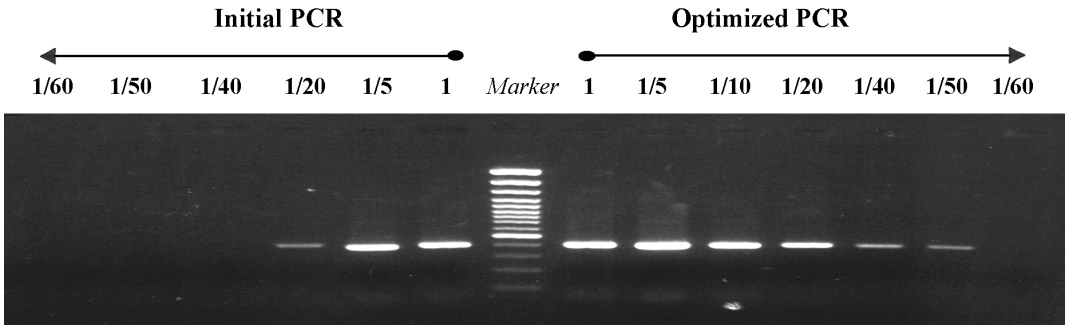

Figure 2. **Result of sensitivity of initial (non-optimized) and optimized PCR by using identical serial dilutions of DNA (which, marked above of each lanes) in 1.3% agarose gel.** The amplified DNA shows that sensitivity of the optimized PCR is five folds more than initial PCR. Marker: 100 bp DNA ladder plus (Fermentas AB, Lithuania).

8.6, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.25 µM of each primers A and B, 0.25 unit/25 µl (1 unit) Taq DNA polymerase, 5% DMSO and 500 µg/ml gelatin and 50-150 ng/25 µl template DNA in 25 µl final volume. The program used consisted of a pre-denaturation step of 95°C for 5 min, 35 cycling of denaturation 94°C for 50 sec, annealing temperature 58°C for 1 min, extension 72°C for 1 min, and a final extension step of 72°C for 5 min. In this study, by examination of these variables, HCMV PCR was optimized which, can with confidence be used for HCMV DNA detection on PBLs of renal transplant recipients.

For PCR optimization, the most important parameters that influence reaction specificity are the annealing temperature, buffer concentration and the cycling protocol. This work showed the effects of PCR optimization on HCMV DNA detection which obtained from renal transplant recipients PBLs. A common source of human DNA is blood, which should be collected into tubes containing 1 mg/ml EDTA to avoid coagulation. Heparin a common anti-coagulant should be avoided, as it is a potent PCR inhibitor (Taylor, 1997). Other substances in blood, perhaps prophyrin compounds, also inhibit PCR but can be removed by lysing red blood cells (RBC) and collecting the white cells by centrifugation for DNA preparation (McPherson and Moller 2000). In spite of
washing process, a wide range of compounds derived from the biological specimens used to extract DNA may inhibit PCR. If inhibition is encountered , it is often useful to dilute the DNA sample. In this study, three times washing protocol for removing RBC inhibitors after lysis step was followed. The concentration of Mg^{++} is critical. It can exist as dNTPs-Mg^{++} complexes that interacts with the sugar-phosphate backbone of nucleic acids and influence the activity of Taq DNA polymerase (McPherson and Moller 2000). The common strategy for assaying the effect of Mg^{++} ion concentration is to adjust the standard buffer so that the $MgCl_2$ varies between 0.5 and 5.0 mM, usually in steps of 0.5 or 1.0 mM. In this work, 0.5 to 2.5 mM $MgCl_2$ concentrations, in steps of 0.5 mM were tested from which, 2.5 mM $MgCl_2$ gave the best result.

The success of a PCR relies heavily on the specificity with which a primer anneals only to its target (and not non-target) sequence. So it is important to optimize this molecular interaction. Whether a primer can anneal only to its perfect complement or also to sequences that have one or more mismatches to the primers depends critically upon the annealing temperature. In general, the higher the annealing temperature, the more specific annealing of the primer to its perfect matched template occurs, leading to decrease amplification of non-target sequences. If there is poor recovery of product and a high background of nonspecific products, then empirical determination of an optimal annealing temperature may be necessary, coupled with optimization of the $MgCl_2$ concentration (McPherson and

Moller 2000). In this study, annealing temperature and $MgCl_2$ were optimized in a matrix. Annealing temperature at 58°C with 2.5 mM $MgCl_2$ concentration showed the best result.

Various enhancer compounds have been reported to improve the specificity and efficiency of PCR. These include chemicals that increase the effective annealing temperature of the reaction, DNA binding proteins and commercially available reagents. Such additives can be added to PCR mixture to enhance primer-annealing specificity, reduce mismatch primer annealing and improve product yield and length. There is no magic additive that will ensure success in every PCR and it may be necessary to test different additives under different conditions, such as annealing temperature, etc. The following compounds have been used as enhanced for PCR system: DMSO 1-10% (v/v), bovine serum albumin (BSA) 10-100 µg/ml [0.01-0.1% (w/v)], glycerol 5-20% (v/v), spermidine, formamide 1.25-10% (v/v), polyethylene glycol 6000 (PEG 6000) 5-15% (w/v), gelatin 0.01-0.1% (w/v), tween-20 0.05% (v/v), nonidet P40 (NP40) 0.5% and triton-X100 0.01% (Lu and Negre 1993; Sarkar *et al*. 1990; Ahokas and Erkkila *et al*. 1993; Varadaraj and Skinner 1994 and Weyant *et al*. 1990). In this study, DMSO and gelatin additives under different conditions in a matrix were tested. Results shown that 5% DMSO and 500 µg/ml of gelatin were the best to be used. No band was observed at 10% DMSO which shows the negative effect of DMSO at high concentration.

In general, the number of cycles should be kept to the minimum required to generate sufficient product for future analysis or manipulation. At this point, the yield of specific amplification products should be maximal, whereas nonspecific amplification products should be barely detectable, if at all. Therefore, after cycling optimization in this study, 35 cycles were shown to give optimal result.

In conclusion, optimization is strongly recommended prior to application of PCR for diagnosis of infectious agents in clinical specimens.

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