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Normalized Quantification of Human Cytomegalovirus DNA by Competitive Real-Time PCR on the LightCycler Instrument

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The development of a novel normalized quantitative competitive real-time PCR on the LightCycler instrument (NQC-LC-PCR) and its application to the quantification of cytomegalovirus (CMV) DNA in clinical samples are described. A heterologous competitor DNA was spiked into test samples and served as an internal amplification control. The internal control (IC) DNA in the test samples was coamplified with the CMV DNA and was tested against a calibrator sample that contained equal amounts of IC DNA and CMV reference standard DNA. An algorithm was developed to normalize possible varying amplification efficiencies between the standard and the samples. After normalization, CMV DNA copy numbers were determined in absolute terms. In a routine clinical setting, normalized quantification by NQC-LC-PCR using a single IC concentration led to results ranging from 500 to 50,000 CMV DNA copies/ml. The results obtained with conventional real-time quantification on the LightCycler instrument were almost identical to those obtained with the NQC-LC-PCR-based quantification. This was the case only for samples in which the PCR was not inhibited. With partially inhibited samples, NQC-LC-PCR was still able to correctly quantify CMV DNA copy numbers even when the PCR was inhibited by about 70%. By analyzing 80 undefined clinical samples, we found that NQC-LC-PCR was suitable for the routine assessment of CMV DNA in clinical plasma samples. Since the ICs were already added to the samples during the DNA purification, almost the entire assay was controlled for sample adequacy. Thus, false negative results were precluded. The NQC-LC-PCR approach developed should be adaptable for additional microbiological applications.

Infection with human cytomegalovirus (CMV) usually leads to lifelong persistence of the viral genome in the host. Those at risk for secondary infection include patients who are immunocompromised due to cancer-related chemotherapy, human immunodeficiency virus infection, or immunosuppressive therapy following solid-organ or bone marrow transplantation.

For the diagnosis of CMV infection, molecular assays based on PCR have been established and have been shown to be superior to virus culture, antigen detection, and serological methods (1, 7, 9, 16, 18, 23). Quantitative molecular assays have been developed and employed to assess CMV DNA loads in patients to identify those at risk for developing CMV disease (1, 6, 10, 11, 15, 20, 22, 23). These quantitative assays include real-time PCR assays using the LightCycler instrument. The LightCycler-based assays are rapid, exhibit great sensitivities, and permit quantification of CMV DNA over a wide range (14, 17, 18; A. M. Kearns, B. Draper, W. Wipat, A. J. Turner, J. Wheeler, R. Freeman, J. Harwood, F. K. Gould, and J. H. Dark, Letter, J. Clin. Microbiol. **39:**2364-2365, 2001). Quantification is usually performed by employing external CMV DNA standards in graded amounts. One prerequisite for this quantification approach is the assumption of equal amplification efficiencies in standards and in samples. However, this may not always be the case, as PCR inhibitors can be present in clinical samples (3). Accordingly, amplification efficiencies may vary from standard to sample and from sample to sample, upsetting quantification results. Internal amplification controls comprised of heterologous or homologous DNA fragments have been shown to be effective for the normalization of varying amplification efficiencies in conventional quantitative competitive PCR (4).

In the present study, we describe the development of a quantitative competitive real-time PCR on the LightCycler instrument, with coamplification of an internal control (IC) for normalization of possible varying amplification efficiencies between the standard and the samples. We apply this approach to the quantification of CMV DNA in clinical plasma samples. For the purpose of this report, we call this approach normalized quantitative competitive LightCycler PCR (NQC-LC-PCR).

MATERIALS AND METHODS

Specimens. Purified genomic DNA of CMV was used as the CMV reference standard (strain AD 169; 10^4 CMV DNA copies/μl; Advanced Biotechnologies, Columbia, Md.). A competitor DNA fragment was generated and used as the IC. A total of 80 undefined clinical plasma samples was used to evaluate the novel PCR assay. As negative clinical control samples, 10 plasma samples derived from patients who showed no clinical symptoms or laboratory findings of infectious diseases were used.

DNA purification. DNA from normal plasma spiked with CMV reference standard DNA and DNA from clinical samples were purified on the MagNA Pure LC instrument (Roche Applied Science, Vienna, Austria) using a MagNA Pure LC total nucleic acid isolation kit (large volume; Roche Applied Science) according to the manufacturer's instructions. DNA from 1 ml of plasma was purified. Elution was performed with 50 μl of elution buffer. All CMV standard samples including calibrator samples were prepared by combining 400 μl of lysis buffer with 595 μl of CMV-negative (by PCR) normal plasma to which 5 μl of serially diluted CMV reference standard DNA was added. IC DNA was spiked into all samples during the automated purification procedure to yield approximately 5,000 copies/ml of plasma. Each sample was assayed twice in triplicate on 2 different days. A previously tested negative control sample and a calibrator

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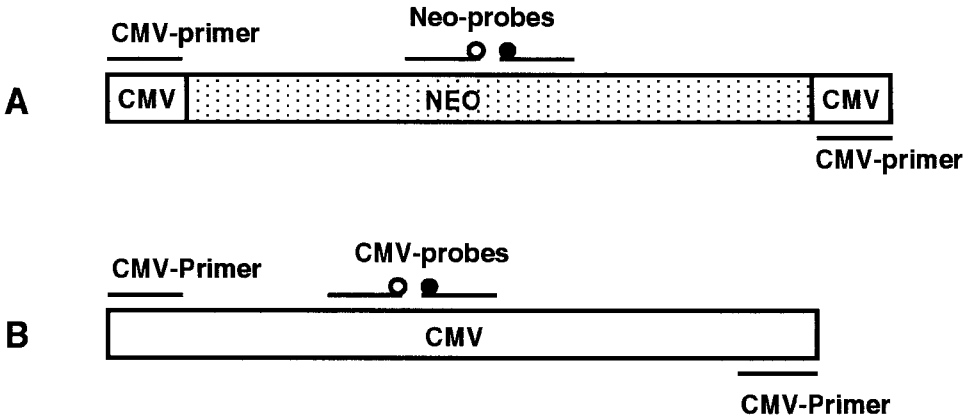

FIG. 1. Schematic sketch of the CMV-specific IC (A) and CMV-specific PCR product (B). The IC DNA fragment consists of the CMV-specific primer sequences flanking the 5′ and 3′ termini of the neo gene sequence, which was used as heterologous DNA. IC DNA and CMV-specific DNA were amplified with the same set of primers. The sizes of the IC-specific and CMV-specific PCR products were 325 and 278 bp, respectively. The 3′ FRET hybridization probe specific for neo was labeled with LC-Red 640 and detected on channel F2 of the LightCycler instrument, whereas the 3′ FRET hybridization probe specific for CMV was labeled with LC-Red 705 and detected on channel F3.

sample were included in all assay runs. In addition, all PCRs included a reagent control comprised of PCR-grade water instead of DNA.

CMV-specific IC. The CMV-specific IC was devised as a PCR competitor by using a stretch of the neomycin phosphotransferase gene (neo) as heterologous DNA (21). Preparative PCR was performed to amplify a 291-bp fragment of the neo gene from a plasmid (bp 312 to 603; GenBank accession no. V00618; neo-specific primer sequences 5′-CGGTGCCCTGAATGAACT-3′ and 5′-ACC GGCTTCCATCCGA-3′). The CMV-specific primer sequences (see below) were used in site-directed mutagenesis to construct the IC DNA fragment (Fig. 1). The fragment was cloned into the plasmid vector pT-Adv by using an AdvanTage PCR cloning kit (Clontech Laboratories Inc., Palo Alto, Calif.) according to the manufacturer's instructions. DNA from the resulting plasmid was purified from transformed bacteria by a standard miniprep procedure and linearized by *Hin*dIII restriction. The DNA concentration was assessed by UV spectrophotometry. The IC fragment was subjected to automated sequence analysis, which confirmed the expected DNA sequence.

Competitive LightCycler CMV PCR. For the LightCycler PCR, the US17 gene of CMV was targeted by using primer sequences similar to those described previously (5′-GACACAACACCGTAAAGC-3′ and 5′-CAGCGTTCGTGTTTC C-3′; GenBank accession no. NC_001347) (8). The CMV-specific PCR products were detected with fluorescence resonance energy transfer (FRET) hybridization probes 5′-TTGCGGGTCATCGTCAGGTCCTC-3′-FL and LC-Red-705-5′-TCC ACGTCAGAGCCCAGCGTGA-p-3′. The IC-specific PCR products were detected with the FRET hybridization probes 5′-GCTGCATACGCTTGATCCG GCT-3′-FL and LC-Red-640-5′-CCTGCCCATTCGACCACCAAGC-p-3′ (bp 516 to 537 and 539 to 560, respectively; GenBank accession no. V00618). The primers were obtained from MWG Biotech, Ebersberg, Germany, and the hybridization probes were obtained from TIB MOLBIOL, Berlin, Germany.

The PCR mixtures were prepared by using LightCycler Fast Start DNA master hybridization probes (Roche Applied Science) supplemented with $MgCl_2$ (4 mM), primers (0.4 μM each), and probes (0.2 μM each). The capillaries were loaded with 15 μl of reaction mixture and with 5 μl of sample DNA by a short centrifugation step (15 s at 735 × *g*). PCR was performed on the LightCycler instrument with the following cycling program: 95°C for 7 min followed by 50 cycles of 2 s at 95°C, 10 s at 60°C, and 15 s at 72°C. The products were analyzed by melting-curve analysis by applying 95°C for 1 min, 60°C for 2 min, and 50°C for 2 s, followed by an increase in temperature from 50 to 85°C (0.2°C/s) and continuous fluorescence recording. IC- and CMV-specific hybridizations were recorded on channels F2 and F3, respectively. The CMV- and IC-specific melting temperatures were 72 and 70°C, respectively. A color compensation file was generated according to the manufacturer's instructions and applied to all PCRs.

Initially, the PCR products were analyzed by agarose gel electrophoresis followed by visualization with ethidium bromide staining and UV illumination to confirm the expected 278- and 325-bp products for CMV and the IC, respectively.

Data acquisition was determined by applying the "fit points" calculation with two points. The noise band was adjusted for the following fluorescence channel settings: F3/F1 for CMV-specific amplification 0.0003 and F2/F1 for IC-specific amplification 0.003. These fluorescence ratios were the mean values plus 2 standard deviations (SDs) from 15 independent measurements with CMV-negative samples without IC DNA. After the noise band was set, the crossing-point (CP) values, as measures of amplification, were used for the automated quantification calculation (LightCycler software, version 3.5.3).

Normalization and quantification. The efficiencies of the CMV-specific and IC-specific amplifications were determined with a standard curve by using CMV reference standard DNA that was serially diluted and spiked with IC DNA and vice versa. After the PCR, logarithmic linear regression analysis was performed with the LightCycler instrument (LightCycler software, version 3.5.3), resulting in the slope values S_{CMV} (CMV-specific amplification) and S_{IC} (IC-specific amplification), according to the following equations:

$$CP_{CMV} = (S_{CMV} \times \log C_{CMV}) + Y_{CMV} \quad (1)$$

$$CP_{IC} = (S_{IC} \times \log C_{IC}) + Y_{IC} \quad (2)$$

CP_{CMV} and CP_{IC} are the CP values of the CMV-specific and IC-specific PCRs, respectively; C_{CMV} and C_{IC} represent the concentrations of CMV DNA and IC DNA, respectively; and Y_{CMV} and Y_{IC} are the respective *y*-axis intercepts.

CMV-specific NQC-LC-PCR was set up with a calibrator that consisted of CMV reference standard DNA and IC DNA, initially with 100 copies of each per capillary. For the clinical application, the calibrator consisted of 5,000 copies of the CMV reference standard/ml and 5,000 copies of IC DNA/ml. IC DNA was spiked into each test sample to yield the same concentration as that in the calibrator. Possible CP value differences between the IC amplification in the calibrator ($CP_{IC\text{-}cal}$) and that in test samples ($CP_{IC\text{-}sample}$) were accounted for by subtracting $CP_{IC\text{-}sample}$ from $CP_{IC\text{-}cal}$, and subsequently, normalization of CP values for the CMV-specific amplification was done by calculating the following: $CP_{CMV} - (CP_{IC\text{-}sample} - CP_{IC\text{-}cal})$. In addition, the differing amplification efficiencies of the CMV-specific PCR (equation 1) and IC-specific PCR (equation 2) were standardized by applying the ratio S_{CMV}/S_{IC} to the term $(CP_{IC\text{-}sample} - CP_{IC\text{-}cal})$. Thus, CMV-specific CP values were standardized and normalized by using the following equation:

$$CP_{CMV\text{-}corr} = CP_{CMV} - [(CP_{IC\text{-}sample} - CP_{IC\text{-}cal}) \times S_{CMV}/S_{IC}] \quad (3)$$

$CP_{CMV\text{-}corr}$ represents the normalized CMV-specific CP value of the test sample, CP_{CMV} represents the CP value of the CMV-specific amplification as retrieved from the LightCycler instrument, $CP_{IC\text{-}sample}$ is the CP value of the IC amplification of the test sample, and $CP_{IC\text{-}cal}$ represents the CP value of the IC amplification of the calibrator.

The concentration of CMV DNA copies in test samples was calculated according to the following equation:

$$CP_{CMV\text{-}cal} - (S_{CMV} \times \log C_{cal}) = CP_{CMV\text{-}corr} - (S_{CMV} \times \log C_{sample}) \quad (4)$$

$CP_{CMV\text{-}cal}$ represents the CP value of the CMV-specific amplification in the calibrator sample obtained with C_{cal}, which is the concentration of CMV DNA

copies in the calibrator sample. Solving equation 4 in terms of $\log C_{sample}$ yields the following:

$$\log C_{sample} = \log C_{cal} - [(CP_{CMV\text{-}cal} - CP_{CMV\text{-}corr})/S_{CMV}] \quad (5)$$

The CP values and slope values were retrieved from the LightCycler instrument and were imported into Microsoft Excel for spreadsheet calculation of the normalized quantification.

Inhibition experiments. To study the power of normalization, 100 copies of CMV reference standard DNA and 100 copies of IC DNA were coamplified in the presence of graded amounts of hemoglobin (final concentrations, 0.02 to 20 μg/ml) as a PCR inhibitor. Blood from a healthy volunteer was osmotically lysed and spun down. Free hemoglobin from the supernatant was measured by a standard routine laboratory method and applied to the PCR. PCR was carried out, and quantification was performed with and without normalization.

Data analysis. The Spearman rank test was used to calculate coefficients of assumed correlations between different PCR-based quantifications (MedCalc, Mariakerke, Belgium). Quantification results assumed to be different were analyzed by Student's *t* test, and a *P* value of <0.05 was considered significant.

RESULTS

In the first step, the dynamic range of the competitive PCR was examined by using the CMV reference standard DNA, which was serially diluted and combined with 100 copies of IC DNA per capillary. Within a range from 10 to 1,000 CMV DNA copies per capillary, the competitive amplifications appeared to be unaffected by each other. When more than 1,000 CMV DNA copies were used, the amplification of the IC DNA was found to be increasingly inhibited (Fig. 2). When fewer than 10 copies of CMV DNA were used, the CMV DNA was still detectable in the presence of the IC; however, due to increased SDs, normalized quantification could no longer be performed (data not shown).

In the second step, amplification efficiencies for the CMV and IC amplifications were determined using serial dilutions of the CMV reference standard DNA in the presence of 100 IC DNA copies and vice versa. The mean amplification efficiencies (see Materials and Methods) were found to be −3.537 (SD, 0.063; $n = 5$) and −3.864 (SD, 0.068; $n = 5$) for the CMV- and IC-specific PCRs, respectively, and were used for the normalization calculations (equation 3).

The results of normalized quantification of CMV DNA by NQC-LC-PCR were compared with the number of copies of the CMV reference standard DNA applied and with the results of conventional real-time quantification by using a serial dilution of CMV reference standard DNA as the external standard. Figure 3 shows that the results of quantification with NQC-LC-PCR were strongly correlated with the DNA copy numbers used for amplification (Fig. 3A) ($r = 0.972$; $P < 0.001$; 95% confidence interval [CI], 0.946 to 0.985) and with the results of conventional real-time quantification ($r = 0.971$; $P < 0.001$; 95% CI, 0.945 to 0.984) (Fig. 3B).

The power of normalization of NQC-LC-PCR was assessed by performing PCR-inhibitory experiments using hemoglobin in graded amounts. Hemoglobin was found to inhibit the PCR in a dose-dependent manner (Fig. 4). Without normalization, the PCR was markedly and significantly inhibited by hemoglobin concentrations above 0.02 μg/ml. Complete inhibition was obtained at concentrations above 2 μg/ml (Fig. 4). By using the normalization procedure, an almost complete reversion of the inhibition was obtained at a hemoglobin concentration up to 0.2 μg/ml. At this concentration, the PCR was inhibited by 73% (Fig. 4). Hemoglobin concentrations above 0.2 μg/ml resulted in a dose-dependent loss of the normalization capability.

In the clinical approach using the MagNA Pure LC instrument for DNA preparation, 5,000 IC DNA copies per ml of plasma allowed a normalized quantification of CMV DNA from 500 to 50,000 copies/ml in a reliable fashion. When fewer than 500 CMV DNA copies per ml were used, CMV DNA was still detectable down to 250 copies/ml in all replicate experiments. However, due to increased SDs, reproducible normalized quantification was not possible (data not shown). When fewer than 250 copies of CMV DNA were used, inconsistent positive or negative results were obtained. Thus, the detection limit of the assay was found to be 250 CMV DNA copies/ml.

The precision of the CMV-specific NQC-LC-PCR assay was determined by using the results of the CMV and IC amplifications (CP values) derived from 10 replicate calibrator samples. Thus, the intra-assay coefficients of variance were 0.7% for the CMV amplification and 0.2% for the IC amplification. For the calculation of the interassay coefficients of variance, the amplification results of the calibrator samples of 10 assays were selected at random. Thus, the CMV and interassay coefficients of variance were found to be 3.5 and 2.7%, respectively.

In the first evaluation, NQC-LC-PCR was used to detect the presence of and quantify CMV DNA in 80 clinical plasma samples and 10 noninfectious plasma samples. Each assay run included a calibrator sample and a noninfectious negative control sample spiked with IC DNA. The IC DNA was found to be amplified to the expected level in all samples. All 10 noninfectious samples tested negative for CMV DNA. Of the clinical samples tested, 31 samples were found to be positive for CMV DNA. The positive samples exhibited a melting curve similar to that obtained with the CMV reference standard DNA (data not shown). Of the 31 positive samples, 24 were found to have results within the range for normalized quantification (Fig. 5). Three samples were found to contain fewer than 500 CMV DNA copies/ml and were reported as having between 250 and 500 copies/ml (data not shown). The results for four samples exceeded the range for normalized quantification. Since the amplification of the IC DNA was competitively inhibited in these four samples, normalized quantification of the CMV DNA could not be performed. Therefore, these samples were diluted and reanalyzed (Fig. 5).

To compare the normalized quantification of the positive clinical samples with conventional real-time quantification on the LightCycler instrument, the serially diluted CMV reference standard DNA was used as an external standard and samples were analyzed in parallel with NQC-LC-PCR. Figure 5 shows that the results of both quantification methods were in good agreement and significantly correlated with each other within the dynamic range of the NQC-LC-PCR ($r = 0.973$; $P < 0.001$; 95% CI, 0.943 to 0.987). None of the clinical samples appeared to be PCR inhibited as assessed by the amplification of the IC DNA.

The NQC-LC-PCR assay was rapid and convenient when used for assessment of CMV DNA in plasma samples. When 16 samples were analyzed, the entire PCR analysis, including purification of DNA and preparation of PCR mixtures, followed by amplification, detection, and quantification, could be completed within 2[1/2] h.

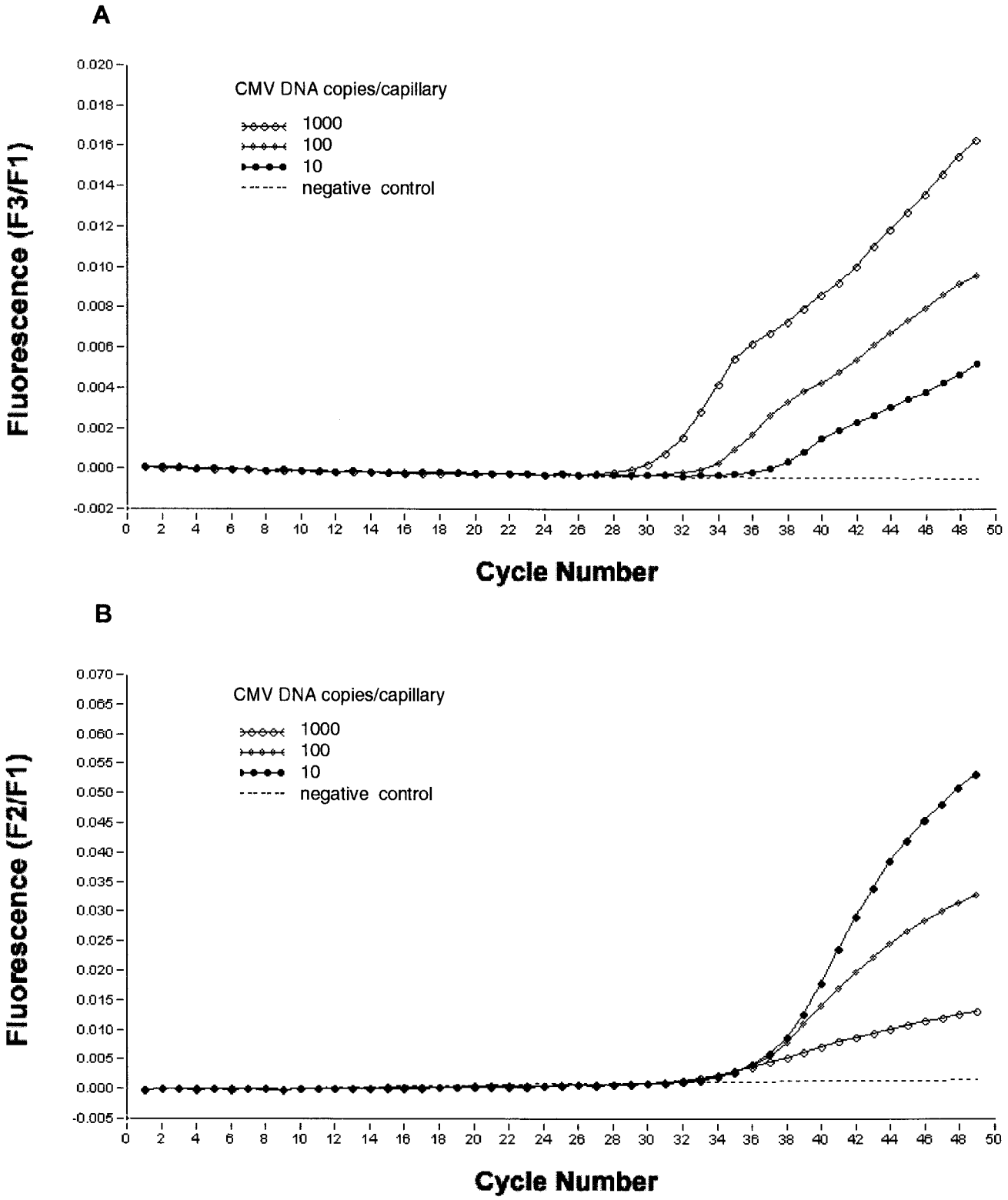

FIG. 2. Real-time fluorescence plot of the CMV-specific amplification (A) and the IC-specific amplification (B). One hundred copies of the IC and the indicated number of copies of CMV DNA were applied to each capillary. Note in panel B that with increasing amounts of CMV DNA, the IC amplification was competitively inhibited.

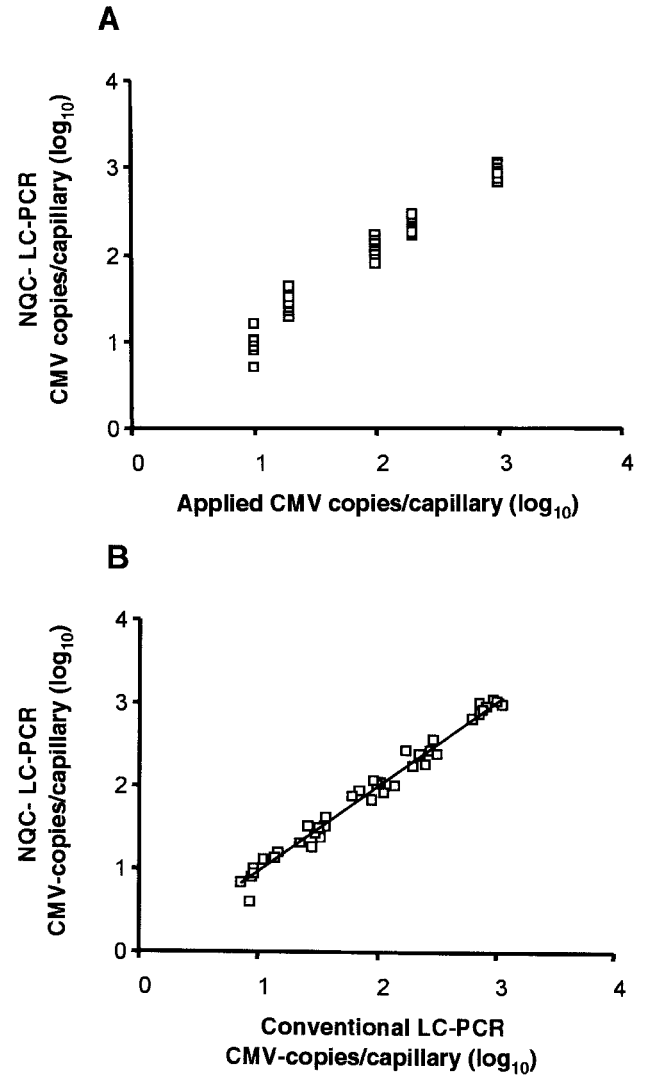

FIG. 3. Normalized quantification of CMV reference standard DNA. Ten, 20, 100, 200, and 1,000 copies of CMV reference standard DNA per capillary ($n = 8$) were assessed by NQC-LC-PCR and conventional LightCycler PCR. (A) CMV copies measured by NQC-LC-PCR were plotted against applied CMV copies ($r = 0.972$; $P < 0.001$; 95% CI, 0.946 to 0.985). (B) CMV copies measured by NQC-LC-PCR were plotted against CMV copies measured by conventional LightCycler PCR ($r = 0.971$; $P < 0.001$; 95% CI, 0.945 to 0.984; linear regression analysis, $y = 1.02x - 0.05$). Note that none of the samples was inhibited.

DISCUSSION

An important requirement for accurate PCR-based nucleic acid quantification is the evaluation of the quality of samples. Therefore, in previous studies, IC DNA that has been coamplified with the sample DNA within the same reaction vessel has been introduced into the PCR (2, 4, 6, 13). Previously reported DNA quantifications on the LightCycler instrument were mostly performed using external reference standards (7, 8, 19). Therefore, amplification efficiencies have not been monitored for variations from standard to sample or from sample to sample.

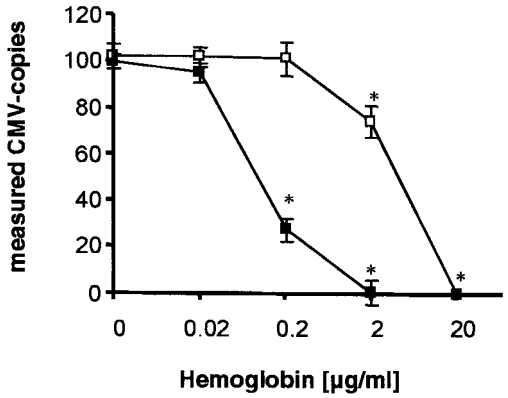

FIG. 4. Normalized quantification of CMV DNA in PCR-inhibited samples by NQC-LC-PCR. Approximately 100 copies of CMV DNA and 100 copies of IC DNA were amplified in the presence of hemoglobin added in graded amounts. Quantification was performed in normalized fashion (open squares) and by the conventional real-time LightCycler method (closed squares). Quantified CMV copies in samples with hemoglobin were compared with quantified CMV copies in samples without hemoglobin by Student's t test. Symbols marked with an asterisk indicate significantly different CMV copy numbers ($P < 0.005$) between samples with and without hemoglobin; symbols without an asterisk indicate that results were not significantly different ($P > 0.05$).

In the present report, we addressed this issue and described a novel LightCycler-based approach for the accurate normalized quantification of CMV DNA, NQC-LC-PCR. The NQC-LC-PCR includes an IC introduced into the CMV-specific real-time PCR for coamplification in competitive fashion. The

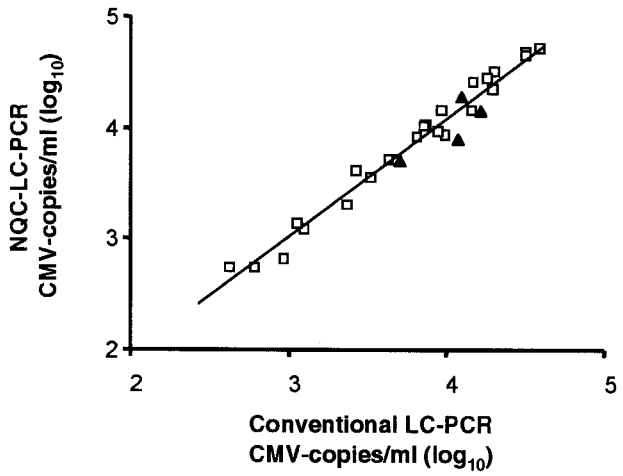

FIG. 5. Correlation between quantitative CMV DNA values from clinical samples analyzed by NQC-LC-PCR assay and by conventional LightCycler assay. The scatter diagram and the regression line show the relation of mean numbers of CMV DNA copies (squares) for 28 CMV-positive clinical samples assessed by both methods ($r = 0.973$; $P < 0.001$; 95% CI, 0.943 to 0.987; linear regression analysis, $y = 1.07x - 0.18$). The NQC-LC-PCR assay used a single calibrator sample for normalized quantification. For the conventional LightCycler assay, a serial dilution of CMV reference standard DNA was used as the external standard. In the NQC-LC-PCR, the results for four samples exceeded the range of quantification. These samples were diluted 1:10 and retested (triangles). Note that none of the samples appeared to be inhibited.

obtained data are introduced into an algorithm for normalization of possible differences in amplification efficiencies, followed by quantification of CMV DNA copies in absolute terms. Thus, the above-mentioned quality of samples is controlled for and also normalized for by testing of the sample's IC against the IC of a calibrator sample, which is not inhibited. The power of the normalization procedure was investigated by PCR-inhibiting experiments. These experiments showed that the NQC-LC-PCR was able to correctly quantify CMV DNA even when the PCR was inhibited by up to approximately 70% when hemoglobin was used as the PCR inhibitor (Fig. 4). Similar data were obtained when EDTA was used as the PCR inhibitor (data not shown).

After normalization, quantification by NQC-LC-PCR was performed with the CMV DNA present in the calibrator and used as the sole standard. Comparison of the results with those from conventional real-time quantification using a serial dilution of CMV reference standard DNA (Fig. 3A) suggested that the use of a single CMV DNA concentration is sufficient to accurately quantify CMV DNA copies by NQC-LC-PCR.

Using a single concentration of IC DNA, the range of quantification with NQC-LC-PCR was found to cover about 3 orders of magnitude (10 to 1,000 copies per capillary). Due to the nature of competitive PCR, the IC amplification was gradually inhibited when more than 1,000 DNA copies per capillary were used. Quantification was still possible over several orders of magnitude at concentrations greater than 1,000 copies of DNA per capillary, albeit only without normalization. However, by increasing the amount of IC DNA, the range of normalized quantification could be adjusted to cover higher CMV DNA concentrations at the expense of the lower normalized quantification limits (data not shown). So, depending on the amount of IC DNA used for NQC-LC-PCR, various normalized quantification ranges can be defined to cover various analytical needs.

The possible utility of NQC-LC-PCR in routine laboratory testing of CMV DNA in clinical samples was investigated using automated DNA purification on the MagNA Pure LC instrument. This instrument has recently been shown to purify herpesvirus DNA from clinical samples in a highly reproducible fashion (12). These previous findings are supported by our own observations in this study of low interassay coefficients of variance for the CMV and IC amplifications (3.5 and 2.7%, respectively). NQC-LC-PCR can also be performed when the DNA is purified using a manual procedure.

The quantification range of the CMV-specific NQC-LC-PCR was chosen to meet the clinical need for sensitive detection and clinically relevant quantification (1, 5, 9, 11, 14). Thus, the dynamic range was adjusted to extend from 500 to 50,000 CMV DNA copies/ml of plasma. This covers most of the reported clinical cutoff values for CMV loads in plasma samples. Samples that exceeded the upper limit of quantification were diluted, and testing was repeated. But, as mentioned above, the assay can be adjusted to enable normalized quantification of CMV DNA copies at higher concentrations. In the clinical evaluation, PCR-inhibited samples were not found, which was reflected in the good agreement between the results of NQC-LC-PCR and conventional real-time quantification. This might be due to the good quality of the DNA purification procedure, which may have removed possible PCR inhibitors, the sole use of plasma samples in this study, or the limited number of CMV-positive samples among the clinical samples. Nevertheless, the PCR-inhibiting experiments showed that NQC-LC-PCR can normalize for inhibited amplifications in a wide range. Also, the necessity of using ICs in clinical PCRs has been stressed, and ICs are especially important in the evaluation of samples that had tested negative to preclude false negative results.

In conclusion, we described a novel normalized quantitative competitive PCR approach using the LightCycler instrument. In its clinical application, i.e., quantification of CMV DNA in plasma samples, this assay proved to be sensitive, accurate, and practical for clinical routine laboratory testing. In addition, the clinical assay controlled for sample adequacy from the DNA purification procedure through the amplification process, and false negative test results were precluded. Our novel approach should also prove useful for the accurate enumeration of other clinical microbial targets.

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