

Development and evaluation of a real-time quantitative PCR for the detection of human cytomegalovirus

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Abstract

A novel real-time quantitative PCR (QPCR) assay is described for monitoring CMV DNA load in clinical specimens using the LightCycler™. The assay is rapid (< 40 min), easy to carry out, robust, reliable and is capable of detecting from 10 to over 2×10^5 CMV DNA copies with a wide linear range. Amplification and detection occur simultaneously, avoiding the need for post-PCR analysis and thereby minimising the risk of carryover contamination. The assay proved to be accurate, specific and reproducible when evaluated in three different laboratories. In addition, LightCycler™ results were comparable with those of TaqMan™, an independent real-time QPCR assay. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Both primary and recurrent cytomegalovirus (CMV) infection cause severe disease in individuals with HIV infection, recipients of solid organ and bone marrow transplants, and other patients on immunosuppressive therapy (Fryd et al., 1980; Jacobson and Mills, 1988; Winston et al., 1990; Smyth et al., 1991; Mustafa, 1994; Kanj et al.,

1996). To prevent CMV disease, early identification and treatment of those patients at greatest risk is essential. Pre-emptive therapy reduces the incidence and severity of CMV disease and is dependent on the development of laboratory assays which are sensitive, specific and rapid in order to provide the potential for early diagnosis.

Since CMV persists in the host following primary infection, PCR-based methods which can identify active infection are required. Quantitative PCR (QPCR) techniques have demonstrated a relationship between viral load and risk of CMV disease (Gor et al., 1998; Aitken et al., 1999; Boom et al., 1999; Pellegrin et al., 1999; Emery et

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al., 2000; Sia et al., 2000; Weinberg et al., 2000), providing sensitive and specific methods for predicting the progression to CMV disease. In addition, such assays offer the advantages of assisting in the development of pre-emptive strategies, monitoring the efficacy of antiviral treatment and, indirectly, assessing the emergence of drug resistant strains. Quantitative molecular techniques, including in-house and commercial assays, have been described but are labour-intensive, time-consuming and may require post-PCR handling with the attendant risk of carryover contamination (Gor et al., 1998; Aitken et al., 1999; Boom et al., 1999; Pellegrin et al., 1999; Emery et al., 2000; Sia et al., 2000; Weinberg et al., 2000).

A real-time QPCR assay has been developed for monitoring CMV DNA load in clinical samples on the LightCycler™ (LC; BioGene Ltd, Kimbolton, Cambridgeshire). The instrument performs real-time PCR, combining rapid cycling with fluorescence measurement in glass capillaries (Bernard et al., 1998; Cane et al., 1999). Since amplification, detection and quantification of PCR product occur simultaneously in the same reaction vessel, the need for post-PCR manipulations is obviated and the entire process is complete in under 40 min. Using LC technology, sequence-specific detection of PCR product is achieved using a labelled primer and hybridisation probe, which fluoresce when bound to amplicons in close proximity. The donor (Fluorescein) absorbs light from the blue LED of the LC, the acceptor (Cy5) absorbs the resonance energy and emits light as a result of fluorescence resonance energy transfer (FRET) between the fluorescent moieties. Sequence-specific hybridisation occurs during amplification, allowing real-time identification and quantification of PCR product.

Table 1
Sequences of primers and probe for LightCycler assay

Oligonucleotide	Sequence (5' → 3')	Label	Position ^a
Forward primer	GAG GAC AAC GAA ATC CTG TTG GGC ^a	None	2738–2762
Reverse primer	GTC GAC GGT GGA GAT ACT GCT* GAG G	* 'T' residue labelled with Cy5	2863–2887
Hybridisation probe	GGA CTA CCT CTT CAA ACG CAT GAT TGA C	3' Fluorescein	2836–2863

^a GenBank® Accession No. M22343.

To assess the clinical use of the assay, its performance was evaluated in three different laboratories using a panel of 100 blind-coded whole blood extracts provided by an independent laboratory. The results were compared with those obtained using an in-house TaqMan™-based CMV QPCR assay using the ABI PRISM™ 7700 sequence detection system (Applied Biosystems, Warrington, Cheshire, UK), which has been evaluated in parallel with antigenaemia levels (Guiver et al., in press).

2. Materials and methods

2.1. LightCycler PCR assay design

Primers directed against a 150-bp fragment of the glycoprotein B gene (gp58), the major neutralising epitope within the envelope of CMV, were used as described previously (Darlington et al., 1991; Fox et al., 1995), but with a single nucleotide addition to the reverse primer. In addition, a hybridisation probe was designed to anneal to an internal sequence adjacent to the reverse primer to confirm sequence specificity. The reverse primer was designed with an internal Cy5 label and the hybridisation probe was 3' Fluorescein-labelled (Table 1). During PCR cycling, FRET only occurs when both labelled oligonucleotides hybridise to the amplicon in close proximity, affording sequence-specific detection of PCR product.

2.2. Quantitative standard

A 2-bp mis-match was incorporated into the probe binding region of the 150-bp CMV target

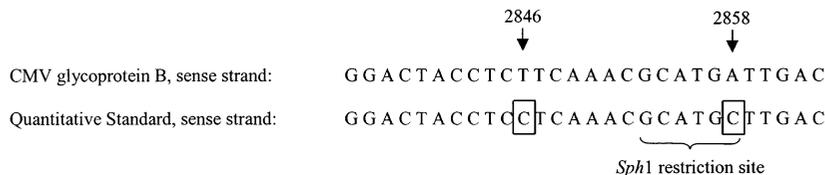


Fig. 1. Partial nucleotide sequence of 150-bp fragment of glycoprotein B gene of CMV, showing probe binding region (GenBank[®] Accession No. M22343, positions 2836–2863). Nucleotide changes are indicated by boxed letters, and the resultant new restriction site in the quantitative standard is shown.

fragment so that it could be readily distinguished from the wild-type sequence using melting curve analysis (vide infra). Mutagenesis was effected by PCR, using an oligonucleotide containing the altered nucleotide sequence, to convert the original T to C at position 2846 and A to C at position 2858 (Fig. 1). The latter base change created a unique restriction site for *Sph*1 in the 150-bp sequence. The modified 150-bp product was purified, cloned into pUC57/T and the mutation confirmed by restriction endonuclease digestion. The plasmid was quantified and then linearised with *Eco*R1, ready for use as quantitative standard (QS) for the assay.

A series of five log₁₀ dilutions corresponding to 2×10^1 to 2×10^5 copies/2 μ l, were prepared and run as external standards in parallel with the test samples. Dilutions of the linearised plasmid were prepared in Tris–EDTA buffer containing 250 pg/ μ l carrier DNA (lambda phage DNA, Sigma, Poole, Dorset, UK) to stabilise the QS's on storage.

2.3. LightCycler PCR assay

An asymmetric PCR approach was used as described by Bernard et al. (1998). To enhance the sensitivity and specificity of the assay, anti-Taq antibody was used. The PCR reaction mixture comprised 3 mM MgCl₂, Taq-based mastermix (BioGene Ltd., Kimbolton, Cambridgeshire) with Platinum anti-Taq antibody (Life Technologies Ltd., Paisley, UK), 0.1 μ M forward primer, 0.45 μ M reverse primer, 0.1 μ M hybridisation probe and 2- μ l DNA template or QS. Reaction mixtures (10 μ l) were spun into glass capillaries which were

hermetically sealed and amplified using the following protocol: an initial denaturation at 96°C for 1 min, followed by 55 cycles of 95°C for 0 s, 55°C for 10 s, 74°C for 10 s, with ramp rates of 20°C/s, 20°C/s and 2°C/s, respectively. Fluorescence was monitored once each cycle at the end of the annealing phase. This was followed by a melting programme of 50°C for 10 s, with slow transition to 85°C (using a ramp rate of 0.1°C/s) and continuous monitoring of fluorescence. The whole cycling process took under 40 min.

Appropriate controls were included in each PCR run: a negative (no template) control, a positive control and five QS's. In addition, on occasion, an independent external quantitative standard (Autogen Bioclear UK Ltd., Wiltshire, UK), diluted to 10⁵ copies/ml in carrier DNA, was included as an independent control to assess the accuracy of quantitation.

2.4. Post-PCR analysis

Computer-assisted interpretation of fluorescence data were carried out using the LC software programs 'Melting curve analysis' and 'Quantification'.

2.4.1. Melting curve analysis

Following amplification, LC software was used to confirm PCR product identity using melting curve analysis (Bernard et al., 1998; Cane et al., 1999). The temperature at which the probe melts from PCR product is sequence-dependent, such that mutation(s) in the probe binding region result in lower melting temperatures (T_m values).

2.4.2. Quantitative analysis

Using LC software, quantitation was achieved by reference to the five QS's of known copy numbers. Real-time PCR allows monitoring of the exponential phase of amplification and quantitative information is derived from those cycles where DNA increases logarithmically and identification of the cycle number at which fluorescence exceeds a threshold.

Runs were deemed acceptable if: positive and negative controls gave the expected results; the QS's and external quantitative standard were within $0.5 \log_{10}$ of their target value; and the standard curve gave a mean squared error of $> 10^{-2}$.

2.5. TaqMan PCR assay

The assay was carried out as described previously (Guiver et al., in press). Briefly, TaqMan CMV primers and probe target part of the glycoprotein B gene. Amplification and detection was carried out using the Applied Biosystems 7700 Sequence Detection System. The plasmid cloned PCR product was used to generate quantitative standards which showed a linear range of 2×10^1 to 2×10^8 copies/2 μ l input into the PCR reaction. Based on these standards, the TaqMan assay could reproducibly detect down to 20 plasmid copies.

2.6. Pilot study

Following development of the LC assay, its performance was assessed using clinical material. A total of 43 unselected EDTA whole blood samples from immunocompromised patients were extracted using Qiagen DNA Blood mini kit (Qiagen, Crawley, West Sussex, UK). The extracts were assayed in parallel using the in-house qualitative assay performed on a conventional solid-block thermal cycler (as described by Darlington et al., 1991) and on the LC.

2.7. Inter-laboratory study

To evaluate the LC assay more extensively and assess its performance in other laboratories, a

panel of 100 blind-coded extracts was prepared by an independent laboratory (laboratory D). EDTA whole blood specimens from immunocompromised patients were extracted using Genra columns (Flowgen, Leicester) according to the manufacturer's instructions. The extracts were tested in laboratory D using an in-house TaqMan assay (Guiver et al., in press) and then divided into three separate aliquots, ready for distribution.

The sensitivity, accuracy, reproducibility and robustness of the LC assay both within and between laboratories, were assessed using the panel of 100 test samples. All reagents required for the evaluation were distributed to the three sites performing LC CMV QPCR (laboratories A, B and C), where each sample was assayed following a standardised procedure. The results of both LC and TaqMan QPCR assays were returned to laboratory D for analysis.

3. Results

3.1. LightCycler assay performance characteristics

Typical results obtained using melting curve analysis to confirm PCR product identity are shown in Fig. 2. Design of the QS with a 2-bp mismatch in the probe binding region resulted in a 5°C difference in T_m , reliably differentiating wild-type CMV target sequence (typical T_m 68°C , $\pm 1^\circ\text{C}$) from QS (typical T_m 63°C , $\pm 1^\circ\text{C}$). The QS's were stable for at least 2 months at 4°C , and > 12 months when stored at -20°C .

For quantification, the \log_{10} input copy number of each QS was plotted against the cycle number at a threshold crossing to construct a standard curve (Fig. 3). Using LC software, the copy number of CMV DNA-positive test samples was calculated according to their cycle number at threshold crossing by reference to the standard curve. The detection limit of the LC assay was ≤ 10 copies CMV DNA (data not shown). The assay showed a linear relationship from 2×10^1 to 2×10^5 copies/2 μ l input. The quality of the standard curves was excellent, giving mean squared errors between 10^{-2} and 10^{-5} . The QS and exter-

nal quantitative standard results proved reproducible to within 0.5 log₁₀ of the target value both within and between runs.

The pilot study of 43 whole blood extracts showed that LC QPCR and the in-house qualitative assay gave concordant results (24 were PCR-negative and 18 were PCR-positive), except one sample from a patient known to be CMV-positive from previous samples which was detected by the LC assay only (data not shown).

3.2. Comparison of LightCycler and TaqMan

In the inter-laboratory study, 51 of the 100 test samples were CMV DNA-positive by TaqMan. By comparison, laboratories A–C using the LC assay identified 47, 40 and 42 positive samples, giving relative sensitivities of 92.2, 78.4 and 82.4%, respectively. All LC positive samples were also positive by the TaqMan assay, suggesting there were no false positives identified by LC.

Of the CMV-positive test samples, the viral load ranges obtained by both QPCR assay techniques were very similar (Fig. 4). The quantitative results using TaqMan ranged from 1 × 10² to

3 × 10⁶ copies/ml (median 3.3 × 10⁴); LC results ranged from 1.1 × 10² to 1.7 × 10⁶ copies/ml (median 3.2 × 10⁴). Of the 50 samples positive by both assays, comparison of the TaqMan and mean LC viral loads showed that the majority of results (36; 72%) were within 0.5 log₁₀; 7 (14%) differed by 0.5–1 log₁₀ and 7 (14%) by > 1 log₁₀. Assessing the inter-laboratory reproducibility of the three centres performing LC QPCR, of the 42 samples positive at more than one laboratory, the majority of viral loads (33; 78.6%) were within 0.5 log₁₀; 4 (9.5%) differed by 0.5–1 log₁₀ and 5 (11.9%) by > 1 log₁₀.

Linear regression analysis was performed to investigate the relationship between quantitative data obtained by both real-time PCR assays (Fig. 5). A significant relationship was shown both between the TaqMan and LC results (Fig. 5, i–iii; $R = 0.82–0.89$, $P < 0.001$) and when the data from each of the three laboratories using LC technology were compared (Fig. 5, iv–vi; $R = 0.76–0.86$, $P < 0.001$).

4. Discussion

QPCR assays have been shown to be useful clinically and are becoming increasingly important tools in diagnostic laboratories for monitoring viral infections. Standard QPCR techniques such as competitive PCR and hybridisation assays are laborious, time-consuming and many require post-PCR handling steps with the attendant risk of carryover contamination. Real-time PCR offers a significant advance over such cumbersome QPCR techniques, providing rapid results and precise quantification in an easy to use platform, without the requirement for post-PCR manipulations (since amplification and detection take place in closed tubes).

QPCR techniques for human CMV have demonstrated a relationship between viral load and the risk of CMV disease, and have proved useful in monitoring response to therapy (Gor et al., 1998; Aitken et al., 1999; Boom et al., 1999; Pellegrin et al., 1999; Emery et al., 2000; Sia et al., 2000; Weinberg et al., 2000). To capitalise on the advantages of real-time PCR, recent efforts have

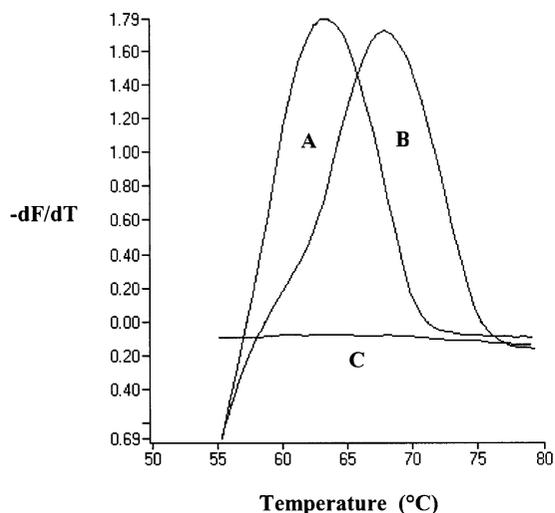


Fig. 2. Melting curve graph showing (A) quantitative standard (T_m 63°C), (B) CMV target (T_m 68°C) and (C) negative control trace. Melting peaks were derived by plotting the negative derivative of fluorescence with respect to temperature ($-dF/dT$ vs. temperature).

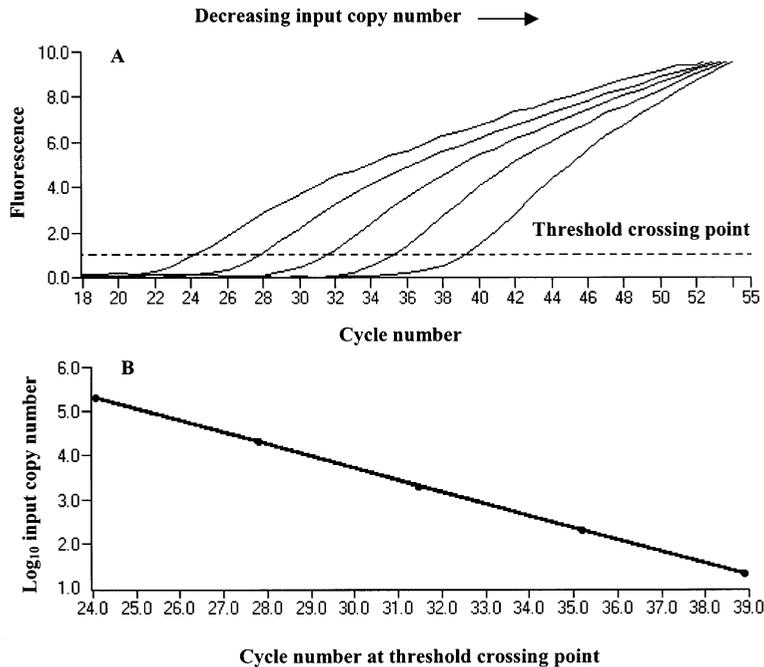


Fig. 3. Amplification traces (A) and standard curve (B) of serial 10-fold dilutions of quantitative standard from 2×10^1 to 2×10^5 copies/reaction. The threshold crossing point is calculated using LightCycler software and the data used to generate a standard curve.

focussed on the development of real-time QPCR assays for CMV, primarily based on TaqMan chemistry and the ABI PRISM™ 7700 sequence detection system (Guiver et al., in press, Nitsche et al., 1999; Machida et al., 2000; Nitsche et al., 2000; Tanaka et al., 2000; Yun et al., 2000). In this paper we describe the development and evaluation of a real-time PCR assay using LC technology for the quantitative detection of CMV DNA in the routine diagnostic laboratory.

The LC assay described above was rapid, the entire process being completed within 2 h (45 min for DNA extraction; 40 min for cycling and detection combined). The assay proved to be at least as sensitive as the standard in-house solid block-based qualitative test (with a significant reduction in assay time: 2 h vs. 7 h), and was specific, showing no evidence of false positives. Design of the assay also resulted in the reliable differentiation of QS from CMV target sequence.

The detection limit of the LC real-time PCR assay was ≤ 10 copies. Applying acceptance lim-

its of $\pm 0.5 \log_{10}$ for inter- and intra-run variation in viral load measurement, the assay provided reproducible results and quantification over a broad dynamic range of 2×10^3 to 5×10^8 copies/ml whole blood. Independent monitoring of assay performance using an external quantitative standard confirmed the accuracy of the quantitative results.

The LC and TaqMan in-house assays compared in this study are independent real-time QPCR platforms and use different chemistries and instrumentation. Despite this, the results of the inter-laboratory study showed good correlation between the two assays. One sample was positive by TaqMan only (9×10^3 copies/ml) but could not be confirmed using the LC assay. Of the LC apparent false-negatives, TaqMan gave viral load measurements close to the detection limit of the LC assay (1×10^2 to 9×10^3 copies/ml). These low copy number discrepant results may be genuine, or they may be attributed to sampling variation. Alternatively, they may be accounted for by

the fact that following assay of the samples by TaqMan, the extracts were frozen and thawed prior to aliquotting and distribution for LC QPCR-testing which may have resulted in the loss or degradation of DNA template. Limited sample volume prevented repeat testing of these extracts.

The overall viral load ranges obtained using both assay techniques were very similar. Analysis of CMV DNA-positive results showed good correlation between both QPCR assays, the majority of viral loads (72%) being within $0.5 \log_{10}$. Similarly, the majority of quantitative results (78.6%) obtained by the three LC centres were within $0.5 \log_{10}$, demonstrating the inter-laboratory reproducibility of the LC assay. Regression analysis of the data from all four laboratories showed a significant relationship not only in viral load measurements

between the TaqMan and LC assays ($P < 0.001$), but also in the quantitative data obtained between each of the three LC sites ($P < 0.001$).

A recent study examined the suitability of both TaqMan and LC for the detection of CMV DNA (Nitsche et al., 1999). Both systems gave similar results and proved equally suitable for quantitative analysis of CMV, and the data presented here support these observations. The ABI PRISM™ 7700 sequence detection system offers the advantage of the simultaneous analysis of 96 samples for facilitating high throughput of a single assay. In contrast, the LC instrument accommodates 32 samples per run, but has faster run times, offering the flexibility of being able to process a smaller number of samples and perform a wide range of diagnostic assays.

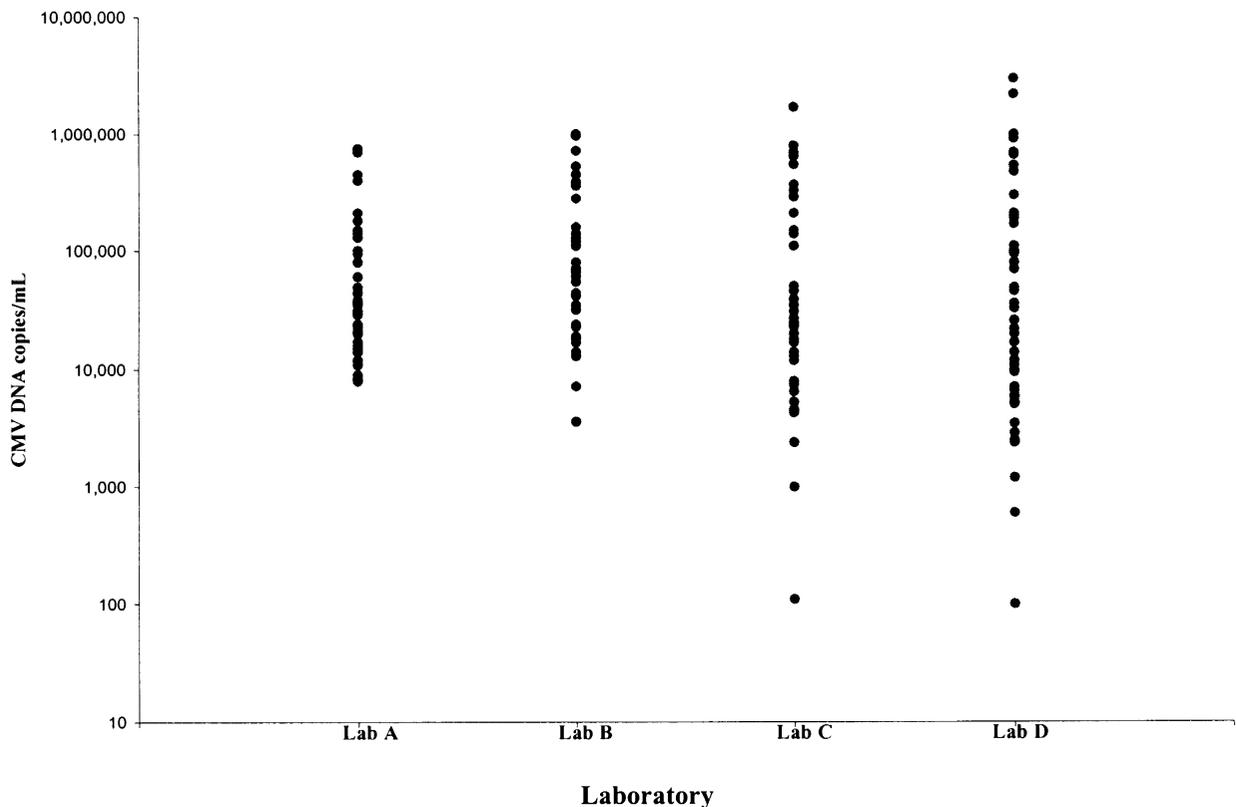


Fig. 4. Distribution of viral loads in CMV DNA-positive samples using LightCycler (laboratories A–C) and TaqMan (laboratory D).

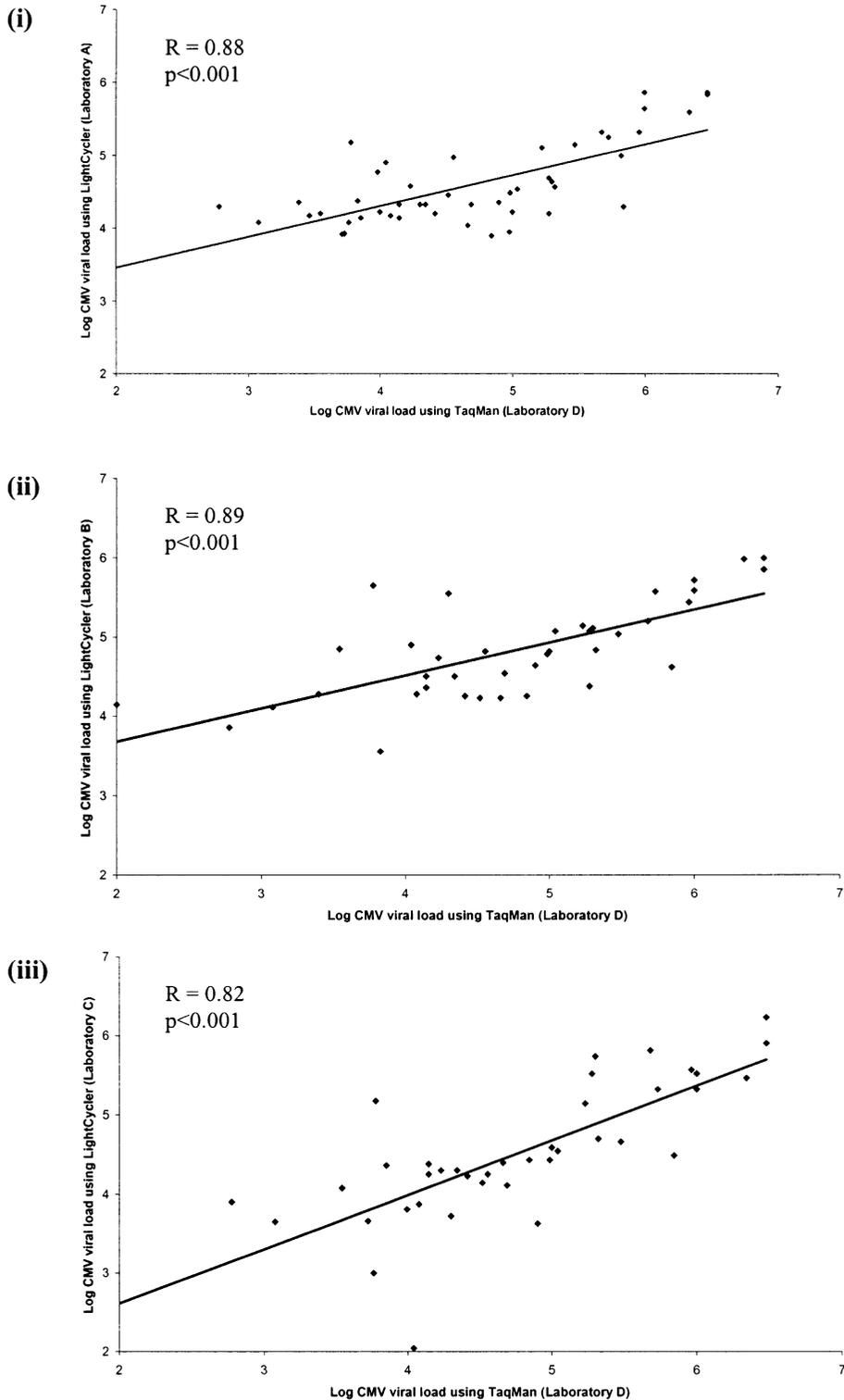


Fig. 5. Linear regression analysis of \log_{10} quantitative data to compare LightCycler (laboratories A, B and C) and TaqMan (laboratory D) results.

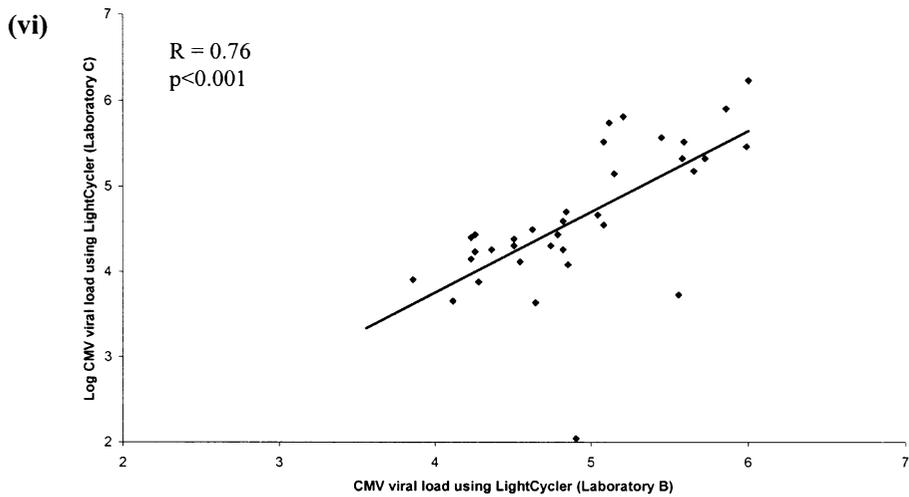
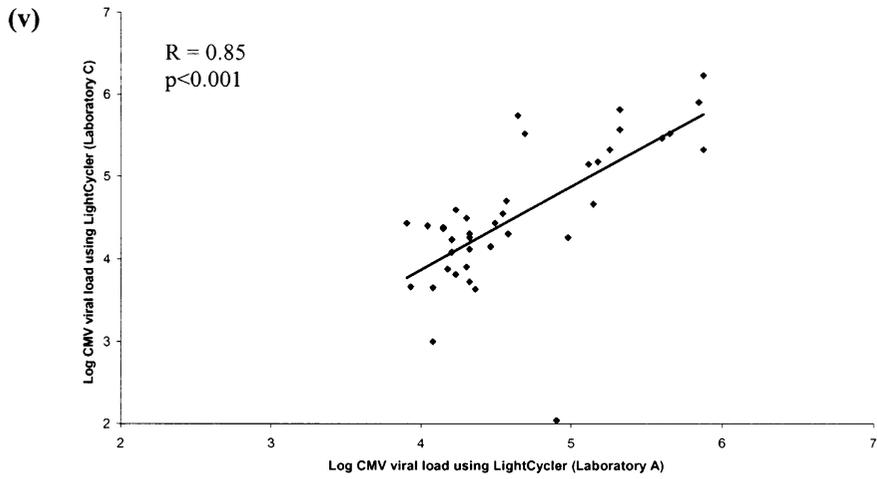
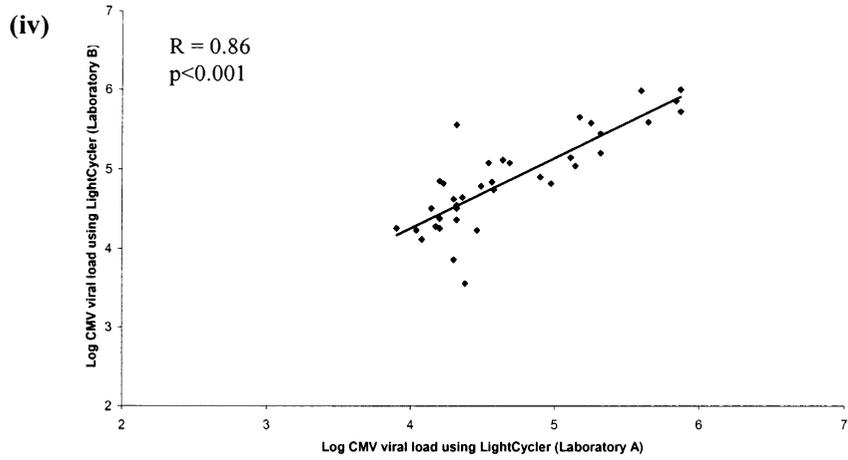


Fig. 5. (Continued)

In conclusion, the real-time PCR assay was found to be labour-saving, and an easy to use platform suitable for routine diagnostic use. The LC-based QPCR assay for the quantitative analysis of CMV in clinical material provided rapid, reliable, accurate and reproducible quantification in a closed tube system, thereby significantly reducing the risk of PCR product carryover contamination. Performance of the assay following a standardised procedure showed the results were reproducible between laboratories, facilitating the comparison of data between independent sites.

Capitalising on the rapid reaction time of the LC assay (within 2 h), multiple runs per day can be carried out, allowing the rapid exclusion of CMV as a cause of disease in patients in whom CMV DNA is not detectable. Such a rapid turnaround also facilitates the timely confirmation of new PCR-positive samples from 'at risk' patients and determination of the rate of increase of viral load which may be important in identifying patients at greatest risk of CMV disease (Emery et al., 2000). Studies are underway to assess further the clinical utility of the LC QPCR assay in monitoring patients prospectively, and to establish PCR-breakpoints for CMV disease in order to determine appropriate parameters for therapeutic intervention.

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