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Simultaneous Detection and Quantitation of Cytomegalovirus, Epstein-Barr Virus, and Adenovirus by Use of Real-Time PCR and Pooled Standards▽

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Quantitative real-time PCR has become the most widely used preemptive approach for managing cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus infections in immunosuppressed patients. These three assays are normally available as separate tests, each using five quantitation standards that are tested in duplicate. We have developed an adenovirus–CMV–EBV triplex assay that uses one set of five pooled quantitative standards, tested singly rather than in duplicate. This test demonstrated a sensitivity and an accuracy of quantitation equivalent to those of our previous single tests and was shown to be able to detect mixed infections with no loss in sensitivity. This assay is now in routine use in our laboratory and has considerably simplified the work flow of the laboratory, with a resultant improvement in sample turnaround time and significantly reduced costs.

Adenovirus, cytomegalovirus (CMV), and Epstein-Barr virus (EBV) are major causes of morbidity and mortality in immunosuppressed individuals. Detection and quantification of these viruses aid in clinical management, allowing, for example, early treatment in order to prevent disease (1, 8, 19). Accurate monitoring of the level of DNA in blood allows tailoring of treatment.

Real-time PCR has become the standard test for these purposes, because it is fast, sensitive, and quantitative (10, 14, 17). In most cases, singleplex real-time PCR assays are used, although multiplex PCR assays that allow the detection of more than one target have also been described (6, 24). Multiplex PCR assays have many advantages over singleplex assays. For example, with regard to laboratory service, multiplexing reduces test costs, improves turnaround times, and increases test throughput (12, 18, 23, 25). These benefits have a positive effect on clinical service, allowing clinicians to tailor patient management or to initiate antiviral therapy more promptly. However, such assays need careful optimization in order to avoid competition and maintain sensitivity when more than one pathogen is present in the patient sample.

This paper describes a real-time multiplex PCR assay that can simultaneously detect and quantitate CMV, EBV, and adenovirus from plasma specimens. Most published assays quantitate by use of a standard curve formed by at least five standards of known concentrations. Further, each set of viral standards is tested in a different reaction. The standards and samples are often tested in triplicate in order to provide an accurate quantitative result. By multiplexing, the number of standards has been reduced from 15 to 5, each containing known concentrations of all three viruses: adenovirus, CMV, and EBV. We also show that testing both the standards and samples singly rather than in triplicate is acceptable.

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MATERIALS AND METHODS

Sample preparation. Viral DNA was extracted from 200 μl of each plasma sample by using the Qiagen virus kit on the Qiagen MDX extractor extension system according to the manufacturer's instructions (Qiagen, Crawley, West Sussex, United Kingdom).

Real-time PCR conditions. The primers and TaqMan probes for all three viruses have been published elsewhere (14, 20, 21). Each probe was labeled at the 5′ end with a different fluorophore: 6-carboxyfluorescein, VIC, or Cy5 (Table 1). The primers and probes were obtained from Operon and ABI. The primers and probes were optimized prior to use by a previously published optimization technique (11), and each primer and probe was used at a final concentration of 100 μM or 20 μM, respectively.

Singleplex real-time PCR methods. CMV, EBV, and adenovirus DNAs were tested singly using the Invitrogen Platinum Quantitative PCR SuperMix-UDG kit, with the following amplification profile: 2 min at 50°C; 95°C for 2 min; and 40 cycles of 95°C for 8 s and 60°C for 34 s. All real-time assays used a total reaction volume of 15 μl, where 6 μl was the DNA extract. This is an amendment to the recommended protocol. All singleton assays have been in routine use in the West of Scotland Specialist Virology Centre since 2003. Each test has been continually assessed via participation in various external quality assurance schemes, including the National External Quality Assurance Scheme and Quality Control for Molecular Diagnostics (QCMD), and has performed satisfactorily both qualitatively and quantitatively. The triplex test was tested on the 2007 QCMD panels and was shown to give results equivalent to those of the three singleplex assays. Note that the adenovirus panels provided by these schemes included a number of adenovirus serotypes at different concentrations, showing that the assay can accurately quantify numerous adenovirus serotypes (data not shown).

Multiplex real-time PCR method. The multiplex assay utilized the Qiagen QuantiTect multiplex kit with the following amplification profile: 2 min at 50°C; 95°C for 15 min; and 40 cycles of 95°C for 60 s and 60°C for 60 s. The multiplex assay was also assessed using the Invitrogen Platinum Quantitative PCR SuperMix-UDG kit with the profile outlined above. The real-time assay used a total reaction volume of 15 μl, where 6 μl was the DNA extract. This is an amendment to the recommended protocol.

Development of pooled standards for multiplex quantification. A single set of five standards was developed using plasmid and viral DNAs. The concentrations of each are shown (Table 2). The adenovirus standard was a commercial DNA obtained from Invitrogen. It is an adenovirus serotype 5. The copy number (per milliliter) was provided by the manufacturer. The CMV standard was derived

TABLE 1. Sequences of the primers and probes used in this study

Primer or probe	Sequence
Primers	
AdenovirusF	GCC ACG GTG GGG TTT CTA AAC TT
AdenovirusR	GCC CCA GTG GTC TTA CAT GCA CAT C
CMVs	ACC AAC ATA AGG ACT TTT CAC ACT TT
CMVas	GAA TAC AGA CAC TTA GAG CTC GGG GT
EBV 143F	GGA ACC TGG TCA TCC TTT GC
EBV 143R	ACG TGC ATG GAC CGG TTA AT
Probes	
Adenovirus	Cy5–TGC ACC AGA CCC GGG CTC AGG TAC TCC GA–BHQ-2
CMV	FAM–CTG GCC AGC ACG TAT CCC AAC AGC A–BHQ-1
EBV	VIC–CGC AGG CAC TCG TAC TGC TCG CT–TAMRA

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

from a CMV plasmid and had previously been standardized against reference material provided by QCMD. The EBV standard was generated from the Namwali EBV-infected cell line, and the copy number was determined by the provider (R. Jarrett, University of Glasgow). The same viral DNA controls at the same five concentrations had been used in the singleplex adenovirus, CMV, and EBV assays.

Assessment of the multiplex test. (i) Overview of assessment. The multiplex assay was assessed in various ways in line with established guidelines (3, 4). For example, to ensure that the multiplexing of the three assays did not result in a reduction in the performance of each individual test, we compared the endpoint detection limits of the multiplex assay to those of singleplex real-time quantitative PCR assays by using dilution series derived from positive samples. Various other parameters of the multiplex assay were also examined, including its precision, linearity, efficiency, quantitation limit, and whole-system reproducibility. The ability of the assay to allow sensitive detection of more than one pathogen in a single sample was examined using a series of simulated samples, each containing different concentrations of more than one pathogen. This characteristic was also assessed using clinical samples known to contain more than one pathogen. Finally, the accuracy of the quantification provided by the multiplex assay was assessed by comparing the results to those provided by single assays on routine clinical samples.

(ii) Comparison of the singleplex and multiplex assays using a dilution series of a positive sample. The endpoints of the multiplex assay were compared to those of singleplex assays by using a series of 10-fold dilutions of plasma samples known to contain adenovirus, CMV, and EBV. The nucleic acid was extracted as outlined above, and each dilution series was tested in triplicate and the endpoints compared.

(iii) Precision of the multiplex assay. The precision of the assay was assessed by repeat testing of the standards with low (P1), moderate (P3), and high (P5) concentrations of viruses on 20 different occasions. The standard deviation (SD) was examined for each standard to determine test precision.

(iv) Linearity and efficiency of quantification using standards tested in singleplex assays. The five pooled standards were tested once on 20 separate occasions, and the standard curve was examined. The linearity of the curve and its efficiency were examined for each run. For accurate and reproducible quantification, it is ideal to have a standard curve with a linearity between −3.1 and −3.6 (the linearity of an ideal curve is −3.33) and a reaction efficiency near 100%. Such efficiency values represent a twofold increase in the level of the amplicon after each cycle.

(v) Assessment of the quantitation limits of the multiplex assay. In order to determine the quantitation limit of the assay (i.e., the levels above and below which quantitation becomes inaccurate), the five standards were tested on 20 occasions as unknown samples (i.e., given no quantitation value). The mean quantitation value attributed to the standards by the assay was then compared to the known value. The quantitation limit was the lowest standard for which the value attributed was found to be less than 50% or more than 200% of the known value. This experiment would also determine the viral load above which the assay would become inaccurate.

(vi) Whole-system reproducibility of the multiplex assay. The reproducibility of the assay was assessed by repeat testing (from the point of extraction) and by quantifying single controls known to contain 5,000 copies/ml of adenovirus, CMV, or

TABLE 2. Quantitation standards pooled to generate standard curves for each virus

Pooled standard	Concn[a] of virus		
	CMV	EBV	Adenovirus
P5	1.8×10^6	1.0×10^7	1.0×10^7
P4	1.8×10^5	1.0×10^6	1.0×10^6
P3	1.8×10^4	1.0×10^5	1.0×10^5
P2	1.8×10^3	1.0×10^4	1.0×10^4
P1	1.8×10^2	1.0×10^3	1.0×10^3

[a] Expressed in QCMD copies per milliliter for CMV and in copies per milliliter for EBV and adenovirus.

TABLE 3. Comparison of the endpoint detection limits of the multiplex and singleplex PCR assays

Virus and dilution	Detection limits of assay[a]	
	Singleplex	Multiplex
CMV		
10^{-1}	23.94, 23.83	23.40, 23.73
10^{-2}	27.35, 26.96	26.79, 27.24
10^{-3}	32.20, 32.07	30.21, 30.34
10^{-4}	33.39, 33.28	33.20, 34.09
10^{-5}	36.27, −	37.43, −
EBV		
10^{-1}	23.19, 23.22	23.36, 23.34
10^{-2}	26.37, 26.41	26.22, 26.25
10^{-3}	30.02, 29.58	30.00, 29.52
10^{-4}	32.92, 34.13	33.62, 32.89
10^{-5}	37.13, −	35.47, −
Adenovirus		
10^{-1}	22.20, 22.63	22.40, 22.33
10^{-2}	26.21, 26.60	25.51, 25.60
10^{-3}	30.08, 29.64	29.08, 29.24
10^{-4}	32.99, 33.59	32.58, 32.59
10^{-5}	36.52, −	35.52, 35.09

[a] Expressed as C_T values obtained with samples tested in duplicate. −, no virus detected.

TABLE 4. Precision and reproducibility of the multiplex assay

Target and standard	Mean C_T	SD	Maximum C_T	Minimum C_T
CMV				
P5	23.94	0.60	27.15	22.85
P3	30.70	0.65	34.62	29.51
P1	36.59	1.01	38.27	33.53
EBV				
P5	22.73	0.43	24.16	21.44
P3	29.30	0.53	32.14	28.23
P1	35.92	1.06	39.75	33.29
Adenovirus				
P5	21.21	0.41	22.76	20.46
P3	27.96	0.50	29.55	25.91
P1	34.88	1.01	38.71	32.16

TABLE 5. Linearity and efficiency[a] of the standard curves produced by the pooled standards

Run	CMV		EBV		Adenovirus	
	R2	*E*	R2	*E*	R2	*E*
1	0.99	−3.29	0.99	−3.34	0.99	−3.16
2	0.99	−3.49	0.99	−3.49	0.99	−3.15
3	0.99	−3.24	0.99	−3.27	0.99	−3.48
4	0.99	−3.28	0.99	−3.31	0.99	−3.46
5	0.99	−3.31	0.99	−3.42	0.99	−3.22
6	0.99	−3.47	0.99	−3.12	0.99	−3.34
7	0.99	−3.49	0.99	−3.51	0.99	−3.14
8	0.99	−3.31	0.99	−3.10	0.99	−3.24
9	0.99	−3.24	0.99	−3.31	0.99	−3.11
10	0.99	−3.23	0.99	−3.42	0.99	−3.20
11	0.99	−3.11	0.99	−3.39	0.99	−3.30
12	0.99	−3.43	0.99	−3.41	0.99	−3.36
13	0.99	−3.50	0.99	−3.23	0.99	−3.58
14	0.99	−3.37	0.99	−3.49	0.99	−3.27
15	0.99	−3.27	0.99	−3.34	0.99	−3.49
16	0.99	−3.29	0.99	−3.42	0.99	−3.10
17	0.99	−3.52	0.99	−3.28	0.99	−3.18
18	0.99	−3.51	0.99	−3.23	0.99	−3.41
19	0.99	−3.23	0.99	−3.41	0.99	−3.18
20	0.99	−3.30	0.99	−3.58	0.99	−3.39
Mean	**0.99**	**−3.35**	**0.99**	**−3.35**	**0.99**	**−3.28**

[a] R2, reaction efficiency; *E*, linearity.

EBV. These controls are derived from clinical samples containing known levels of adenovirus, CMV, or EBV. These samples were diluted to the appropriate concentration using viral transport medium. Although we report positive results below 5,000 copies/ml, we do not provide quantitative results for samples testing positive but below this cutoff. This cutoff has been routinely used and is based on the theory that the system is likely to become less accurate below this level.

DNAs from the 5,000-copies/ml controls were extracted and tested on 20 different occasions. The mean and standard variation were examined for each control.

(vii) Accuracy of multiplex detection of more than one pathogen. The accuracy of multiplex detection of more than one pathogen was assessed using a chessboard technique, designed to provide a large number of wells, each containing a known concentration of two PCR targets at different ratios. The method used for this assessment is described in detail elsewhere (13). These experiments were designed to determine whether competition is likely to be an issue and can also measure the ratio of each target above which competition can be expected.

In total, three panels of simulated samples were generated using five dilutions manufactured from the strongest quantitation standards: one containing various concentrations of EBV and CMV, one containing adenovirus and CMV, and one containing EBV and adenovirus. The dilution series from which these panels were manufactured were then tested using the singleplex assays. The results are presented as means (unless they were positive/negative).

TABLE 6. Quantitation values attributed to the five pooled standards when tested as unknown samples[a]

Pooled standard	Concn of virus attributed (% difference from known concn)[b]		
	Adenovirus	CMV	EBV
P1	3.12 (+32)	2.5 (+76)	3.06 (+14)
P2	4.02 (+4.7)	3.37 (+30)	4.07 (+17.4)
P3	5 (0)	4.24 (−3.5)	5.04 (+9.6)
P4	6.81 (+45)	5.35 (+24)	6.03 (+7.2)
P5	7.24 (+74)	6.24 (−3.5)	7.12 (+32)

[a] Values >50% less than or >200% greater than the expected value are deemed unacceptable.

[b] Virus concentrations are expressed in log copies per milliliter. + and −, the concentration of virus determined was greater or less, respectively, than the known concentration by the indicated percentage.

TABLE 7. Whole-system reproducibility of the multiplex assay

Virus	Concn of virus (log copies per ml)				
	Expected	Mean	SD	Minimum	Maximum
EBV	3.7	3.88	0.21	3.50	4.21
Adenovirus	3.7	3.75	0.24	3.34	4.15
CMV	3.7	3.84	0.20	3.42	4.24

Three clinical samples known to contain three pathogens (as determined by singleplex testing) were also tested. To assess the benefit of the Qiagen QuantiTect multiplex kit, both the panels of simulated mixed samples and the clinical samples were tested using both the Qiagen QuantiTect multiplex kit and the Invitrogen Platinum Quantitative PCR SuperMix-UDG kit.

(viii) Accuracy of multiplex quantification of clinical samples in comparison to singleplex assays. A total of 30 citrated blood samples, previously shown to be positive and quantified for either adenovirus, EBV, or CMV by singleplex real-time PCR, were used to assess whether the quantitative values provided by the multiplex assay were accurate. These samples were tested by the multiplex assay, and the results were compared to those obtained by the singleplex assays.

RESULTS

Comparison of the singleplex with the multiplex assay using a dilution series of a positive sample. The multiplex and singleplex assays were shown to have similar endpoints when the dilution series was tested (Table 3). For adenovirus, the endpoint detection limit was shown to be the fifth dilution, whereas for CMV and EBV, the limits were shown to be between the fourth and fifth dilutions. This shows that the use of the additional primers has no effect on the sensitivity of each individual test and also that the Qiagen kit is as sensitive as the Invitrogen kit.

Precision of the multiplex assay. Examination of the results of repeat testing of the P1, P3, and P5 standards shows that the assay is precise: all standards were detected at highly similar threshold cycles (C_Ts) over the 20 runs (Table 4). This precision is reflected in the SDs, which are low for each standard.

Linearity and efficiency. Examination of the slopes of the 20 runs shows that all runs had acceptable slopes (all between −3.10 and −3.60) and were found to be highly efficient (all 0.99) (Table 5). These results show that the multiplex assay using pooled standards singly will provide accurate and robust quantification over a wide dynamic range (5 log units).

Assessment of the quantitation limit of the multiplex assay. The quantitation value attributed to each standard (when tested as an unknown) was found to be very similar to the expected value (Table 6). For adenovirus, CMV, and EBV, the mean quantitation values for the lowest standards were very similar to the expected values. Consequently, the quantitation limits of the adenovirus and EBV assays are 1,000 copies/ml, whereas CMV has a quantitation limit of 180 copies/ml. All assays were found to be accurate at the quantitation maximum. This was 10^6 copies/ml for CMV and 10^7 copies/ml for adenovirus and EBV. Samples with levels of virus above these values may not be accurately quantified.

Whole-system reproducibility (including extraction). Examination of the C_Ts and the quantified mean log copies per milliliter and SDs of the 20 extracted controls shows that the whole system is reproducible at 5,000 copies/ml (our quantification cutoff), with little difference in the C_T or quantification value observed over the 20 runs (Table 7). In each case the SD

TABLE 8. C_Ts (in duplicate) obtained by testing of simulated samples containing both CMV and EBV with the Invitrogen Platinum Quantitative PCR SuperMix-UDG kit[a]

CMV component[a]	C_T for CMV or EBV with the indicated CMV component and the following EBV component[a]:									
	EBV 1 (20.58)		EBV 2 (23.25)		EBV 3 (26.08)		EBV 4 (29.84)		EBV 5 (>40)	
	CMV	EBV	CMV	EBV	CMV	EBV	CMV	EBV	CMV	EBV
CMV 1 (21.56)	20.96	20.33	21.71	22.81	21.52	25.58	21.38	29.18/>40	21.62	>40
CMV 2 (24.00)	23.60	20.42	23.75	23.20	24.06	25.83	23.98	28.51	23.20	>40
CMV 3 (27.62)	26.74	20.36	26.96	23.18	27.03	26.24	27.30	28.68	26.73	>40
CMV 4 (31.06)	>40	20.32	29.54/>40	23.24	31.19	26.22	30.85	28.97	30.30	>40
CMV 5 (>40)	>40	20.30	>40	23.22	>40	26.32	>40	29.98	>40	>40

[a] The number(s) in parentheses after each CMV or EBV component of the simulated sample is the C_T obtained for that component by the singleplex CMV or EBV assay, respectively.

was <0.25 log copies/ml. As a result, samples detected at or above this level will be quantified accurately.

Accuracy of multiplex quantification of more than one pathogen. Simulated mixed samples were tested so as to assess the ability of the multiplex test to detect mixed infections by using either the Qiagen QuantiTect multiplex kit or the Invitrogen Platinum Quantitative PCR SuperMix-UDG kit. The Qiagen QuantiTect multiplex kit allowed the detection of both pathogens irrespective of the concentration difference between the targets. Differences greater than 5 log units were reliably detected by the assay. The Invitrogen Platinum Quantitative PCR SuperMix-UDG kit was less useful at detecting mixed infections, particularly when the difference in the concentration of the two targets was large. Examples showing the superiority of the Qiagen QuantiTect multiplex kit over the Invitrogen Platinum Quantitative PCR SuperMix-UDG are shown in Tables 8 and 9. These examples show that a high concentration of EBV can result in false-negative reactions in the CMV component of the assay when the Invitrogen Platinum Quantitative PCR SuperMix-UDG kit is used (Table 8). However, the Qiagen QuantiTect multiplex kit allows the detection of all targets irrespective of the concentration.

Testing of clinical samples using both kits confirmed the superiority of the Qiagen QuantiTect multiplex kit (Table 10). Two of the three samples contained adenovirus at a very high level with lower levels of CMV and EBV. Testing using the Qiagen QuantiTect multiplex kit allowed the detection of all three pathogens at C_Ts similar to those with individual tests. Multiplexing using the Invitrogen Platinum Quantitative PCR SuperMix-UDG kit failed to detect the CMV and EBV in two of the three samples. This failure was probably a result of the strong adenovirus outcompeting the other target. The third sample contained adenovirus and CMV at similar concentrations, whereas the concentration of EBV was almost 2 log units higher. However, no significant difference in results was obtained with either kit.

Accuracy of quantification on clinical samples. The data show that the multiplex PCR provided quantification values similar to those provided by the singleplex assays (Table 11). These data show that testing the pooled standards and samples singly rather than in duplicate can provide accurate quantification data.

DISCUSSION

From the results presented, it can be seen that the multiplex PCR assay for adenovirus, CMV, and EBV is a highly suitable alternative to singleplex assays. Not only is the assay as sensitive as the single assays; it also offers accurate and reproducible quantitation to a level of at least 5,000 copies/ml.

The quantitation is achieved using a single set of five quantitation standards, each containing a different concentration of adenovirus, CMV, and EBV. The presence of more than one target within each individual standard had no effect on the performance of the individual test components. Consequently, the standard curves produced were consistently linear and efficient.

The ability to detect and accurately quantitate >1 target is directly related to the use of the Qiagen QuantiTect kit. As mentioned above, this kit is especially designed for multiplexing and thus reduces test interaction and competition. Conse-

TABLE 9. C_Ts (in duplicate) obtained by testing of simulated samples containing both CMV and EBV by the Qiagen QuantiTect multiplex kit[a]

CMV component[a]	C_T for CMV or EBV with the indicated CMV component and the following EBV component[a]:									
	EBV 1 (22.75)		EBV 2 (26.24)		EBV 3 (29.68)		EBV 4 (32.93)		EBV 5 (36.44/>40)	
	CMV	EBV	CMV	EBV	CMV	EBV	CMV	EBV	CMV	EBV
CMV 1 (25.56)	24.15	22.58	24.68	25.97	24.80	29.15	24.47	31.56	24.33	35.19/>40
CMV 2 (27.84)	28.05	22.62	28.10	25.96	27.58	29.20	27.38	32.62	27.88	35.55/>40
CMV 3 (32.51)	32.43	22.64	31.75	26.01	31.58	29.63	31.32	32.34	31.53	36.02/>40
CMV 4 (35.34)	35.85	22.88	34.16	25.93	37.36	29.54	35.16	32.44	34.44	>40
CMV 5 (36.15/>40)	>40	22.92	>40	26.04	>40	29.53	35.65/>40	32.70	37.26/>40	36.02/>40

[a] The number(s) in parentheses after each CMV or EBV component of the simulated sample is the C_T obtained for that component by the singleplex CMV or EBV assay, respectively.

TABLE 10. Comparison of the Qiagen QuantiTect multiplex kit and the Invitrogen Platinum Quantitative PCR SuperMix-UDG kit on clinical samples containing >1 target

Sample	C_T for the indicated virus by:								
	Single testing			Invitrogen multiplex kit			Qiagen multiplex kit		
	Adenovirus	CMV	EBV	Adenovirus	CMV	EBV	Adenovirus	CMV	EBV
A	31.04	31.34	25.54	31.02	31.19	24.38	32.82	32.65	24.65
B	11.46	28.86	31.30	11	Neg[a]	Neg	10.19	29.62	29.62
C	12.12	31.50	30.42	11.02	Neg	Neg	10.59	29.28	29.27

[a] Neg, negative result.

quently, real-time PCR assays can be simply multiplexed with little optimization required (5, 7, 15, 16, 22).

The use of this kit also ensured that the presence of as many as three targets in the simulated and clinical samples had no effect on the performance of any aspect of the assay. Although the clinical significance of mixed infections in immunocompromised patients is unclear, dual and triple infections do occur. A recent audit of patients from the Bone Marrow Transplant Unit at the Glasgow Royal Infirmary showed that 20% had CMV, EBV, or adenovirus present in the samples tested. Of these patients, 13.2% had dual infections and 1.3% had triple infections. Although such infections would have been detected through the use of single assays, multiplexing using alternative PCR kits may have resulted in false-negative results or inaccurate quantitation, and this may, in turn, have had implications for clinical management.

TABLE 11. Comparison of the quantitation values provided by the singleplex and the multiplex assay on 30 clinical samples

Patient sample	Virus detected	Concn of virus (log copies per milliliter) detected by:	
		Routine quantitation	Multiplex quantitation
1	EBV	4.58	4.72
2	EBV	5.61	5.46
3	EBV	ND[a]	3.66
4	EBV	3.95	4.13
5	EBV	5.40	5.44
6	EBV	3.63	4.46
7	EBV	5.45	5.29
8	EBV	ND	3.25
9	EBV	3.93	4.10
10	EBV	4.02	4.39
11	EBV	4.13	4.35
12	Adenovirus	7.26	7.24
13	Adenovirus	6.93	7.27
14	Adenovirus	6.49	6.54
15	Adenovirus	3.99	3.82
16	Adenovirus	6.57	6.65
17	Adenovirus	4.01	3.84
18	Adenovirus	5.59	5.54
19	Adenovirus	5.79	5.76
20	Adenovirus	8.61	8.90
21	CMV	3.25	2.70
22	CMV	3.40	3.29
23	CMV	3.33	3.16
24	CMV	3.88	3.88
25	CMV	3.79	3.74
26	CMV	3.39	3.24
27	CMV	2.91	3.17
28	CMV	3.99	3.76
29	CMV	4.92	4.98
30	CMV	3.58	3.23

[a] ND, not determined.

The multiplex assay will have several positive outcomes for routine service (2, 12). For example, each sample is now tested by only one multiplex PCR assay rather than by three separate PCR tests. This will reduce the cost of the service significantly (because smaller amounts of PCR master mix, controls, and standards are needed) and will also improve turnaround times. Since implementing this assay in our laboratory, we have seen a reduction of at least a day in turnaround times, despite receiving an increased number of samples and despite the fact that all samples are now quantified (previously our laboratory quantified only CMV- and EBV-positive samples). The use of only 5 standards per PCR run (instead of 15) and the fact that all samples are now tested singly (instead of triplicate) will allow more clinical samples to be tested per PCR run. This will reduce test costs and turnaround times further. Both these examples highlight how the developments described here have simplified service, which in turn should reduce the number of technical errors occurring. Clinical service will also be improved, since the assay ensures that all relevant results are available immediately rather than sequentially. Consequently, patient management can be tailored more rapidly. The use of the multiplex assay will also free up laboratory equipment for other assays, which may, in turn, reduce their turnaround time.

The benefits of the Qiagen QuantiTect kit could also be applied to the use of other real-time PCR tests, particularly in situations where mixed infections are common. For example, the Qiagen QuantiTect kit would prove useful for respiratory and gastroenteritis multiplex assays. The use of the kit could also aid the development of internal-control systems, since internal-control PCR assays can now be more easily added to existing assays. We have now added an internal control to the triplex assay described here (9). The internal-control assay detects murine CMV, which is added to the samples prior to extraction. The addition of this assay has had no effect on the performance of the multiplex kit and now allows detection in samples containing inhibitors that may have led to false-negative results or inaccurate quantification (data not shown).

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