

# LightCycler Multiplex PCR for the Laboratory Diagnosis of Common Viral Infections of the Central Nervous System

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**A conventional multiplex PCR assay that detects herpes simplex virus type 1 (HSV-1), HSV-2, varicella-zoster virus, and enteroviruses for the diagnosis of central nervous system infections was modified to be performed using the LightCycler system. The sensitivity of detection of each of the viruses using the LightCycler assay was compared to that of the conventional assay using external quality assessment material. The assays had equivalent sensitivities, but the LightCycler assay was more rapid, reduced the risk of contamination, and used an amplicon detection format that demonstrated greater discrimination than a gel electrophoresis method.**

Molecular methods for the diagnosis of viral central nervous system (CNS) infections are now well established in the routine clinical laboratory. Several studies, which have recently been reviewed (14), have demonstrated the high sensitivity, specificity, and utility of the laboratory molecular diagnosis of herpes simplex virus (HSV) and enterovirus CNS infections (9, 15). Molecular methods have replaced conventional virological techniques, such as viral isolation from cerebrospinal fluid (CSF) by cell culturing or detection of a virus-specific antibody response, approaches which, in comparison, are slow or lack sensitivity. The widespread use of the PCR technique, in particular, has improved the laboratory diagnosis and understanding of viral etiologies of clinical syndromes such as aseptic meningitis, for example, when associated with viral reactivation in varicella-zoster virus (VZV) and HSV type 2 (HSV-2) infections in the absence of specific skin lesions (4, 12).

Modifications to the basic PCR technique have been used to increase the sensitivity of detection of viruses, e.g., by using nested primers, or to allow the detection of more than one virus in an assay, e.g., by using multiplex primers. These techniques are particularly relevant to the detection of viruses in CSF because the concentration of viral genomes found in CSF may be low and because several viruses commonly cause similar symptoms. Nested primer PCR has disadvantages, however, in particular, an increased risk of false-positive results caused by contamination.

The LightCycler system (Roche Molecular Biochemicals, Lewes, United Kingdom) is a combined thermal cycler and fluorimeter which allows PCR product detection and identification with a variety of fluorescence chemistries. The LightCycler system can provide the clinical virologist with rapid PCR results because of very fast temperature transition rates and online analysis of data and with reduced risk of contamination because product detection is accomplished in a closed system, advantages that have also been recently described for other applications in clinical virology (2, 5, 6, 11, 13). PCR amplicon detection is possible using the dye SYBR Green 1 (2,

13), which fluoresces only when bound to double-stranded DNA. On completion of PCR, amplicons are identified by measurement of their melting temperatures ( $T_m$ s). It is possible to interpret the results of multiplex PCR using this system if the  $T_m$ s of the specific amplicons are sufficiently different and constant to allow differentiation (2).

The aim of the present study was to adapt an existing conventional thermal cycler nested multiplex PCR assay for the three most common causes of viral CNS infections in the United Kingdom (10) to a final amplification and analysis of results by use of the LightCycler system. The perceived advantages of the new assay would be rapidity and, because of the closed-system detection format, a reduced risk of false-positive results due to contamination.

## MATERIALS AND METHODS

Nucleic acid was extracted and purified from CSF samples and quality control material by using a High Pure viral nucleic acid kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. The sample volume processed was 200  $\mu$ l, and the elution buffer volume applied to the extraction column was 100  $\mu$ l. For some ( $n = 20$ ) of the enterovirus-positive CSF samples, frozen ( $-20^\circ\text{C}$ ) archived extracted nucleic acid was used for the assay. For the herpesvirus and the remaining enterovirus-positive CSF samples and the control material, fresh nucleic acid was extracted from frozen ( $-20^\circ\text{C}$ ) archived CSF samples or virus-infected cell culture fluids.

The sensitivity of the LightCycler assay was compared to that of the conventional assay (10) using titrations of HSV-1, HSV-2, and enterovirus isolates that were supplied by the European Union Concerted Action for Quality Control of Nucleic Acid Amplification in Diagnostic Virology (EU CA) program. For VZV, a titration of a previously described isolate was used (10). The specificity of amplicon identification by  $T_m$  measurements was verified by using isolates of HSV, VZV, and enteroviruses from the American Type Culture Collection (ATCC). In order to determine if there were any differences in the  $T_m$ s of amplicons derived from different enterovirus serotypes, control samples containing isolates of coxsackieviruses A9, A16, B2, and B5, echoviruses 1 and 9, and enterovirus 71, supplied either by the EU CA, the United Kingdom National External Quality Assessment Scheme (UK NEQAS), or the ATCC, were assayed by the LightCycler assay. The LightCycler assay was also used to test retrospectively 50 CSF samples found positive by the conventional thermal cycler assay—6 for HSV-1, 9 for HSV-2, 4 for VZV, and 31 for enteroviruses.

PCR primers used for the reverse transcription of enterovirus RNA and the subsequent amplifications of HSV and VZV DNAs and enterovirus cDNA were described previously (10). The reaction conditions and the thermal cycling incubations for the reverse transcription and the first round of PCR amplification using a conventional thermal cycler (Primus 25; MWG Biotech, Milton Keynes, United Kingdom) were described previously (10), except that 25 cycles of amplification were used and the reagent suppliers were those described for the secondary amplifications (see below).

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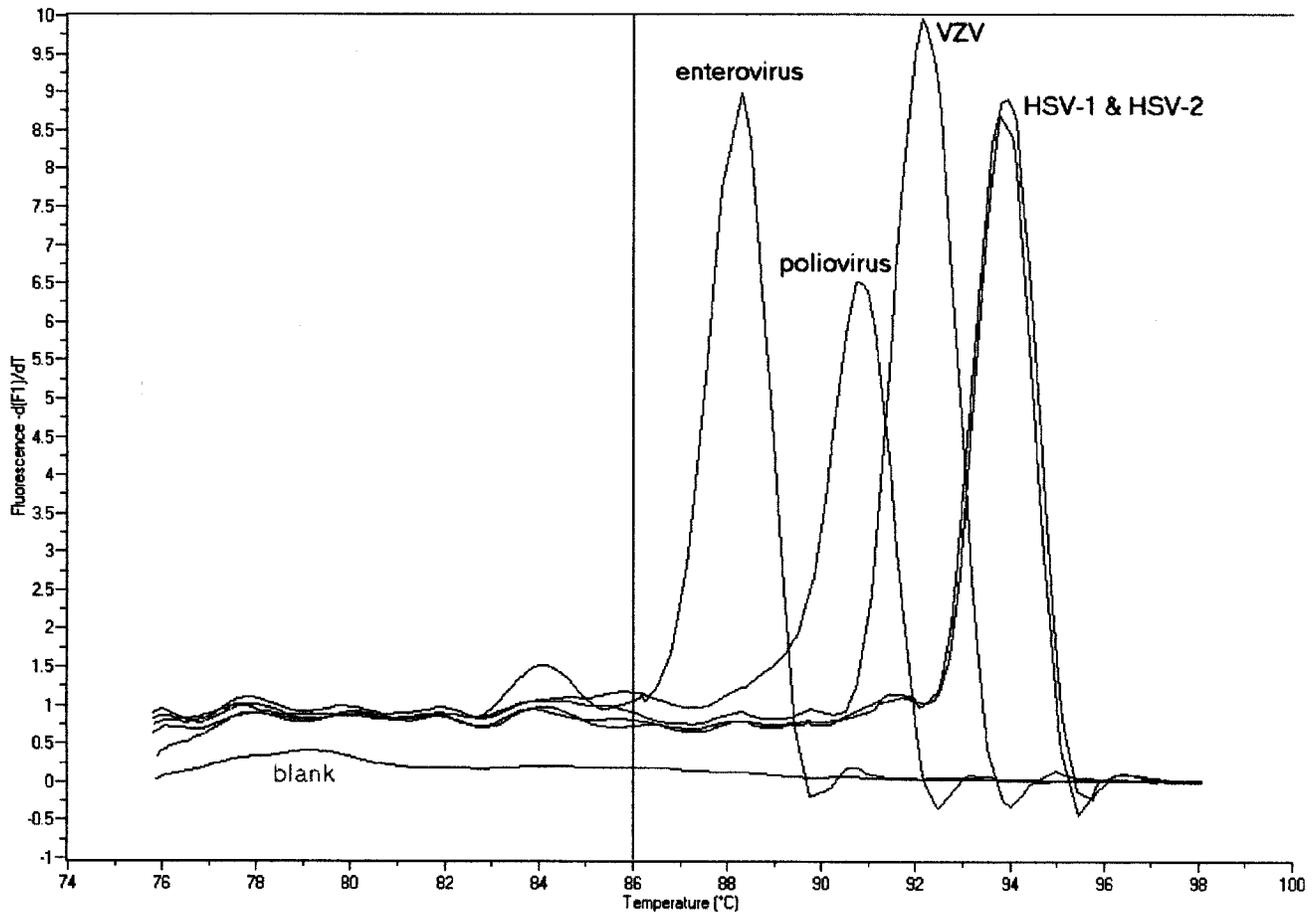


FIG. 1. LightCycler system melting curve analysis of ATCC isolates amplified by multiplex PCR. Enterovirus is echovirus type 1, ATCC VR-31,  $T_m$  of 88.20°C; poliovirus is poliovirus type 2 (attenuated), ATCC VR-301,  $T_m$  of 90.79°C; VZV is ATCC VR-586,  $T_m$  of 92.17°C; HSV-1 is ATCC VR-260,  $T_m$  of 93.83°C; and HSV-2 is ATCC VR-734,  $T_m$  of 93.91°C. The line designated "blank" represents the analysis of a PCR mixture without the addition of a target. The vertical line at 86°C represents the incubation temperature at which the levels of fluorescent signals were measured during each cycle of PCR. The y axis is the negative differential of fluorescence over temperature ( $-dF/dT$ ).

Secondary amplifications with nested primers and online analysis of PCR products were performed with the LightCycler system. Each reaction had a total volume of 20  $\mu$ l containing 1  $\mu$ l of the primary reaction solution, 16 mM  $(\text{NH}_4)_2\text{SO}_4$ , 67 mM Tris-HCl (pH 8.8 at 25°C), 0.01% (wt/vol) Tween 20, 5.0 mM  $\text{MgCl}_2$ , 0.25 mM each deoxynucleotide triphosphate (Sigma Ltd., Poole, United Kingdom), 0.5  $\mu$ M each oligonucleotide primer (MWG Biotech), 1.25 U of *Taq* polymerase (Qiagen Ltd.), 1  $\mu$ l of  $10\times$  SYBR Green 1 (Sigma), and 0.01 mg of bovine serum albumin (Sigma).

The thermal cycling incubations used for the nested primer amplifications on the LightCycler instrument were done at 96°C for 1 s, 55°C for 5 s, 72°C for 5 s, and 86°C for 5 s. At the end of each 86°C incubation, the fluorescent signal of each reaction was measured at a wavelength of 530 nm using the LightCycler fluorimeter. PCR amplification with the nested primers was done by 25 cycles, following which the LightCycler system melting curve analysis program was performed. In this program, the incubation temperature was increased from 75°C to 98°C at a transition rate of 0.2°C/s, with measurement of the emitted fluorescent signals at each step increase. The  $T_m$  of the dominant PCR product from each reaction was measured from the downloaded data using LightCycler version 3 software, and these temperatures were compared to the  $T_m$ s for positive control reactions containing HSV-1, VZV, and nonpoliovirus enterovirus genomes, which were included in each batch of tests.

## RESULTS

The molecular sensitivity of detection by the LightCycler assay was compared to that of the conventional thermal cycler assay by use of the EU CA virus titrations for HSV-1, HSV-2,

and enteroviruses and by use of the titration of a well-characterized isolate for VZV. Similar sensitivities for the detection of HSV-1, HSV-2, and VZV were measured, but a slightly lower sensitivity was observed with the LightCycler assay than with the conventional assay for the enterovirus titrations tested (Table 1).

The measurement of amplicon  $T_m$ s for the ATCC isolates of HSV-1, HSV-2, VZV, and enteroviruses allowed for unequivocal interpretation of the multiplex PCR results by analysis with the LightCycler system melting curve program (Fig. 1). For the nonpoliovirus enteroviruses supplied by EU CA, UK NEQAS, and ATCC, the range of amplicon  $T_m$ s across the serotyped isolates was only 1.37°C (Table 2). Amplicons from the three poliovirus isolates tested, representing serotypes 1, 2, and 3, had  $T_m$ s (average  $T_m$ , 90.46°C) that were consistently approximately 2°C higher than those of the amplicons derived from the nonpoliovirus enteroviruses tested (average  $T_m$ , 88.09°C). No difference in amplicon  $T_m$ s was observed between HSV-1 and HSV-2.

In the retrospective testing of 50 CSF samples which had been found positive by the conventional thermal cycler assay, 49 samples were also found positive by the LightCycler assay,

TABLE 1. Comparison of the sensitivity of detection of HSV-1, HSV-2, and enteroviruses by the conventional thermal cycler assay and the LightCycler system assay

Virus	Virus concn <sup>a</sup>	Results of the following assay <sup>b</sup> :	
		Conventional thermal cycler	LightCycler system
HSV-1	$2 \times 10^2$ – $6 \times 10^2$	Pos	Pos
	$2 \times 10^1$ – $6 \times 10^1$	Pos	Pos
	$2 \times 10^0$ – $6 \times 10^0$	Neg	Neg
HSV-2	$3 \times 10^2$ – $7 \times 10^2$	Pos	Pos
	$3 \times 10^1$ – $7 \times 10^1$	Pos	Pos
	$3 \times 10^0$ – $7 \times 10^0$	Neg	Neg
VZV	0.1	Pos	Pos
	0.01	Pos	Pos
	0.001	Neg	Neg
Coxsackievirus A9	4.0	Pos	Pos
	0.4	Pos	Pos
	0.04	Equiv <sup>c</sup>	Neg
Coxsackievirus B5	3.0	Pos	Pos
	0.3	Pos	Pos
	0.03	Equiv <sup>c</sup>	Neg

<sup>a</sup> For HSV-1, HSV-2, coxsackievirus A9, and coxsackievirus B5, this information was supplied by EU CA. The VZV isolate used has been described previously (10). For HSV-1 and HSV-2, virus concentration is the estimated number of viral genomes per milliliter, and for VZV and the enteroviruses, concentration is expressed as virus infectivity (50% tissue culture infective doses per milliliter).

<sup>b</sup> Pos, positive; Neg, negative.

<sup>c</sup> All samples were tested as replicates of three. These samples were inconsistently positive and so were classified as equivocal (Equiv).

the exception being an enterovirus-positive sample which failed to be reamplified from archived extracted RNA.

## DISCUSSION

The advantages for the clinical virology laboratory of performing PCR with the LightCycler system are several. The speed of laboratory diagnosis of a CNS infection is important because a definitive laboratory finding of a viral etiology in suspected meningitis may resolve further patient investigation, associated health expenditure, and anxiety. For an unusual or unexpected result, where confirmation is required, a rapid assay may also be repeated during the same work day. Despite the disadvantages of the technique, nested PCR is often used for the diagnosis of viral CNS infection because of the requirement for high sensitivity. Nested PCR was used for this assay because the sensitivity of assays performed with the LightCycler system may be compromised by the small volume of extracted nucleic acid that can be added to the glass capillary reaction vessels used in the instrument (7). An existing, well-optimized multiplex PCR assay was therefore adapted so that the secondary amplification, product detection, and identification steps were performed with the LightCycler system using a template from the conventional thermal cycler reverse transcription-multiplex primary PCR.

Greater discrimination based on differences in amplicon GC content in addition to length is a potential advantage of the LightCycler SYBR Green 1 detection format compared to gel

electrophoresis (2). Differences in amplicon  $T_m$ s could potentially be exploited to allow some enterovirus serotyping and the differentiation of HSV-1 and HSV-2. A requirement of this assay, however, is the use of primers for highly conserved targets in the viral genome so that false-negative results, caused by sequence heterogeneity, are avoided. The primers used in this assay for the amplification of conserved regions of the enterovirus and HSV genomes therefore precluded any potential for greater discrimination. Usefully, however, it was possible to use  $T_m$  measurements to discriminate polioviruses from the other enteroviruses tested. Although there is little clinical benefit in enterovirus serotyping, in the United Kingdom it is a requirement of good laboratory practice that poliovirus be excluded when an enterovirus infection is diagnosed, in order to comply with the surveillance requirement of the World Health Organization Eradication of Poliovirus Program. The apparent differences in  $T_m$ s between amplicons from poliovirus and nonpoliovirus enteroviruses in this assay need to be confirmed using further serotyped viral isolates.

It is possible to derive quantitative data from the LightCycler assay, as amplicon concentration is monitored in real time and emitted fluorescence will increase more rapidly in samples with a higher initial concentration of target viral nucleic acid. The relative concentrations of viral nucleic acids in different samples of CSF may therefore be compared by recording the cycle at which the fluorescence signal increases above the background level. Furthermore, if external quantitation standards with known copy numbers of viral genomes are included in batches of tests, then an accurate measurement of the actual viral genome concentration is possible. For CNS infection, quantitation may be useful for monitoring antiviral treatment and establishing prognosis in HSV encephalitis (3), monitoring chronic enterovirus infections in patients with hypogammaglobulinemia, or monitoring VZV infection, where the clinical significance of qualitative VZV detection is uncertain (1).

It is interesting that the noise band crossing point for enterovirus-positive CSF samples assayed in this study ranged

TABLE 2.  $T_m$  data for virus amplicons from CSF samples and control material<sup>a</sup>

Virus amplicon and source (no. of samples assayed)	$T_m$	$T_m$ mean	$T_m$ range
HSV-1 from CSF (6)		93.39	93.22–93.48
HSV-2 from CSF (9)		93.44	93.18–93.51
VZV from CSF (4)		92.09	91.60–92.75
Enterovirus from CSF (31)		89.01	88.38–89.81
HSV-1 isolate (3)		93.55	93.20–93.83
HSV-2 isolate (3)		93.60	93.29–93.91
VZV isolate (3)		92.27	92.17–92.38
Coxsackievirus A9 isolate (1)	88.08		
Coxsackievirus A16 isolate (1)	88.95		
Coxsackievirus B2 isolate (1)	87.81		
Coxsackievirus B5 isolate (1)	88.15		
Echovirus 1 isolate (1)	88.20		
Echovirus 9 isolate (1)	87.58		
Enterovirus 71 isolate (1)	87.87		
Poliovirus 1 isolate (1)	90.25		
Poliovirus 2 isolate (1)	90.79		
Poliovirus 3 isolate (1)	90.35		

<sup>a</sup>  $T_m$  data are reported in degrees Celsius.

from cycles 12 to 22; for the HSV-1 samples, the crossing points ranged from cycles 5 to 12, and for the HSV-2 samples, all of which were from adult females with meningitis, the range was cycles 12 to 19. If there were equivalent PCR efficiencies for different enterovirus serotypes and for both HSV types, then these data would indicate a broad range of enterovirus RNA concentrations in CSF during illness and would confirm what has been postulated previously—that there is a higher CSF viral load in HSV-1 disease than in HSV-2 disease (8, 10).

This report demonstrates that, by exploiting differences in amplicon  $T_m$ s, multiplex PCR assays may be adapted to the LightCycler system in order to provide a more rapid laboratory diagnosis, a greater safeguard against contamination, and additional diagnostic information compared to the results obtained with an ethidium bromide-stained agarose gel electrophoresis detection format.

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