

# Sequencing and Resolution of Amplified Herpes Simplex Virus DNA with Intermediate Melting Curves as Genotype 1 or 2 by LightCycler PCR Assay

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**DNA from 101 specimens containing herpes simplex virus (HSV) produced atypical intermediate melting curves compared with those expected for HSV type 1 or HSV type 2 subsequent to real-time PCR. Nucleic acid sequence analysis of amplified target DNA revealed 1- or 3-bp polymorphisms in the probe region which allowed designation of these viruses as HSV genotype 1 or HSV genotype 2. These two subpopulations of HSV were also identified as HSV genotype 1 or HSV genotype 2 using another commercially available PCR method. Amplified HSV target DNA producing intermediate melting curves could be designated as HSV genotype 1 or HSV genotype 2 without performing sequencing or another PCR method with 96/101 (95%) specimens by adding known intermediate HSV DNA characteristic for the two subpopulations as controls.**

Herpes simplex virus (HSV) causes superficial and systemic infections within every major organ system of the body in both normal and immunocompromised patients. Over a recent 5-year period, HSV was the most common virus recovered in the diagnostic laboratory using conventional and shell vial cell culture methods (5). Nevertheless, HSV is rarely recovered from cerebrospinal fluid (CSF) specimens; PCR was demonstrated to have at least equal sensitivity to cell culture recovery of the virus from brain biopsy tissue and is now recognized as the “gold standard” for the laboratory diagnosis of central nervous system infections due to this virus (8).

We converted culture-based assays and conventional PCR (gel electrophoresis and Southern blotting or enzyme-linked immunosorbent assay) used for CSF specimens to real-time molecular amplifications of this virus target nucleic acid for dermal and genital specimens beginning in May 2000 and later for CSF specimens. Implementation of LightCycler real-time PCR (Roche Molecular Biochemicals, Indianapolis, Ind.) in the routine laboratory became possible, since amplification and detection of target HSV DNA occurs in a closed system, thereby virtually eliminating the threat of carry over amplicon contamination events. Trend analysis in our laboratory has provided an increased sensitivity for the routine detection of HSV infections from genital (34.3% to 38.4%; 12% increase) and dermal (23.6% to 27.7%; 17% increase) specimens compared to shell vial cell cultures, in agreement with the experience of others (2, 4, 6, 9). The assay also provides simultaneous genotyping of HSV using melting curve analysis of the fluorescence resonance energy transfer (FRET) hybridization probes. However, some amplified DNA of HSV exhibits atypical melting temperatures ( $T_m$ ) that occur between the predicted  $T_m$ s obtained with HSV genotype 1 and genotype 2. We sequenced the intermediate HSV PCR products to determine

the polymorphism(s) responsible for the atypical melting curves and to designate these as HSV genotype 1 or 2.

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## MATERIALS AND METHODS

This study was approved by the Institutional Review Board of the Mayo Foundation. Nucleic acid from specimens previously yielding intermediate melting curves with amplified target DNA of HSV ( $n = 101$ ; genital, 56; dermal, 41; cerebrospinal fluid, 4) were extracted using the MagNA Pure LC (Roche Molecular Biochemicals, Indianapolis, Ind.) automated extraction system according to the manufacturer's instructions. HSV DNA was amplified by LightCycler PCR using herpes simplex virus 1/2 Primer/Hyb Probes ASR, which included HSV type 1 and type 2 reference DNA as controls (GenBank accession number M16321, catalog no. 3 315 177; Roche Applied Science, Indianapolis, IN). After amplification, the PCR product was processed for DNA sequencing with shrimp alkaline phosphatase and exonuclease I. DNA sequence was determined bidirectionally using dye terminator chemistry on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA) with HSV primers (3). A second assay was used to resolve the specimens with target HSV DNA producing intermediate melting curves (RealArt HSV 1/2 LC PCR Reagent; Artus GmbH, Hamburg, Germany) as HSV genotype 1 or HSV genotype 2.

## RESULTS

Two subpopulations within the 101 specimens containing HSV DNA with intermediate  $T_m$ s were identified based on the DNA sequences obtained and were designated intermediate HSV type A (55%) and intermediate HSV type B (45%). Of these specimens (genital, 56; dermal, 41; CSF, 4), 5 (dermal, 3; genital, 2) contained HSV intermediate DNA that could not be resolved as genotype 1 or 2. Intermediate HSV type A had one polymorphism, and type B had three polymorphisms (Table 1) in the probe region. The polymorphisms resulted in an altered FRET probe melting curve with a peak  $T_m$  of 61.8°C for type A and 62.7°C for type B, which fall between the  $T_m$  of HSV genotype 1 (55.3°C) and the  $T_m$  of genotype 2 (69.7°C) (Fig. 1). PCR results using another LightCycler assay (RealArt HSV 1/2 LC PCR Reagent) found all intermediate HSV type A

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TABLE 1. Polymorphisms in the probe region observed in HSV intermediate strains compared to reference strains

Virus	Nucleotide <sup>a</sup> at position:				
	3086	3093	3105	3114	3117
Reference HSV genotype 1	A	T	C	G	T
Reference HSV genotype 2	G	C	C	G	T
Intermediate HSV type A ( <i>n</i> = 56)	A	C <sup>a</sup>	C	G	T
Intermediate HSV type B ( <i>n</i> = 45)	G	C	T <sup>a</sup>	C <sup>a</sup>	C <sup>a</sup>

<sup>a</sup> Represents polymorphism in the nucleic acid sequence with respect to the sequence with GenBank accession no. M16321.

strains to be HSV genotype 1 and all 101 of the intermediate HSV type B strains to be HSV genotype 2.

## DISCUSSION

An advantage of the LightCycler hybridization FRET probes is that they allow PCR product detection even in the presence of polymorphisms. This is especially important for dependable detection of viruses due to the large number of polymorphisms often present in viral specimens. Nevertheless, some specimens containing amplified DNA of HSV had polymorphisms under the FRET probes that produced atypical melting curves which precluded designation as HSV genotype 1 or HSV genotype 2. We emphasize that all of the  $T_m$  patterns were diagnostic for HSV DNA. Using primer and probe sequences within the DNA polymerase gene, Anderson et al. found that 15 of 745 (2.0%) genital and cutaneous specimens

contained HSV DNA that produced melting curve peaks that were not specific for either genotype of HSV (1). In their practice, these melting curves from 15 samples conformed to six different  $T_m$  patterns. We found a 2.5-fold higher incidence of these intermediate strains at the Mayo Clinic using the Roche HSV1/2 primer/Hyb Probes ASR comprising two patterns. For example, during a recent 9-month period of time, 164 of 3,269 (5.0%) specimens containing HSV demonstrated intermediate melting curves.

For diagnostic service to clinicians, specific resolution of intermediate specimens of HSV into specific genotypes of the virus may have importance in patient management. For example, the genotype of HSV (1 or 2) may influence the frequency of reactivation of infection and is important for the management of recrudescence since it confirms that the recurrent infection is due to the same HSV genotype (10). In addition, infection with HSV type 1 can influence the clinical manifestations that may develop after subsequent infection with HSV type 2. In one study, for example, four patients who developed HSV type 1 genital lesions first had a more prolonged episode and more frequent recurrences following the acquisition of HSV type 2 (7).

In the absence of commercially available reference DNA of intermediate HSV-1 and HSV-2, several general approaches can be used to characterize these specimens detected by the Roche ASR. The most accurate method is to sequence the PCR amplicon from intermediate specimens, but this is not always feasible. A second approach is to retest the intermedi-

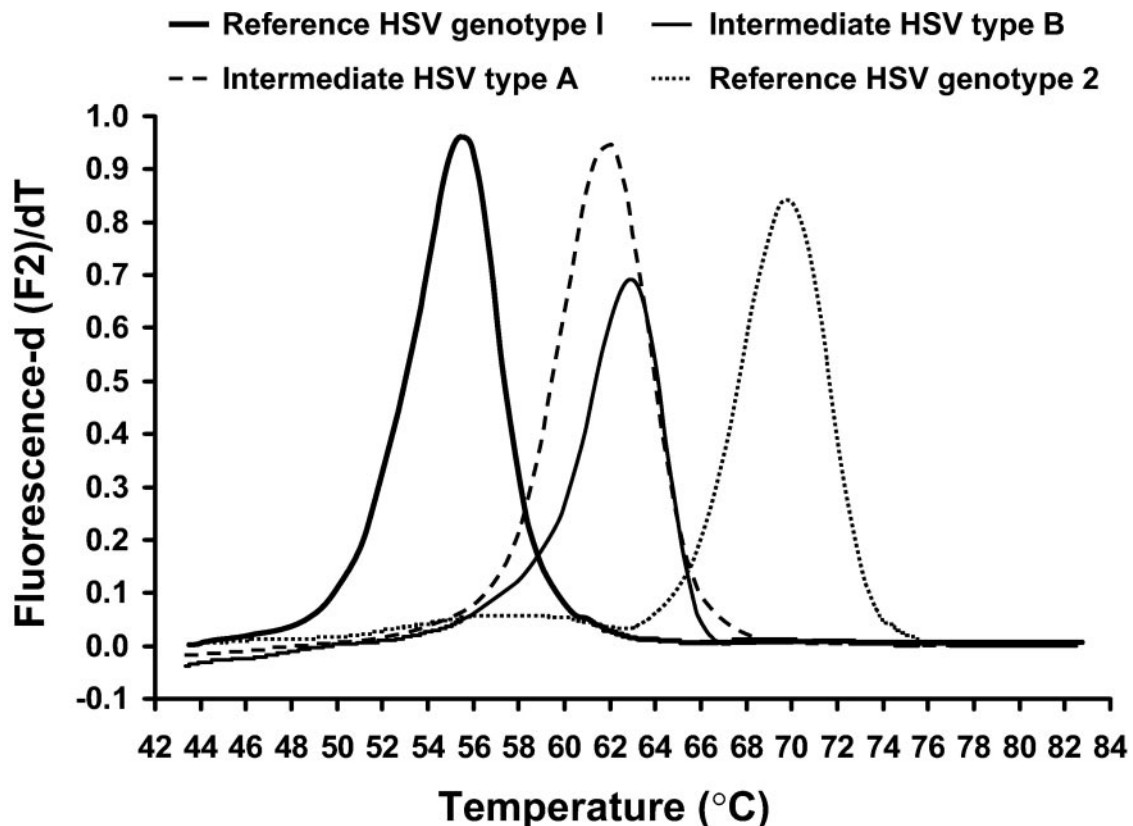


FIG. 1. Melting curves of HSV genotype 1, HSV genotype 2, and the intermediate HSV.

ate specimens with another reference assay. A third approach would be to retest intermediate specimens in the same test run with specific target controls representing polymorphic HSV, genotypes characteristic for HSV-1 and HSV-2. The two intermediate genotypes can be specifically identified by small ( $0.9^{\circ}\text{C}$ ) but characteristic and reproducible melting curve peak  $T_m$  differences.

For routine laboratory practice we have optimized the third option. For example, we attempted to differentiate the two intermediate HSV strains by determining a melting temperature and were successful in achieving 100% specificity and more than 95% sensitivity on one LightCycler instrument; however, the variation in  $T_m$  was too great to achieve consistent differentiation when different reagent lots and instruments were compared. We were able to resolve and designate the two subpopulations of amplified HSV DNA that produced intermediate melting curves by using intermediate strains with known sequence and genotype as controls within the run and designate the unknown intermediate HSV specimens as HSV genotype 1 and HSV genotype 2 by comparing the melting temperatures of these unknown specimens to the melting temperatures of known intermediate strains. Using known intermediate strains as controls with each run enabled us to correctly type 96 specimens (95%) out of the 101 intermediate specimens.

**Conclusion.** HSV specimens that cannot be initially placed into genotype 1 or 2 by melting curve analysis using LightCycler PCR were found to contain polymorphisms. These intermediate specimens can be designated as HSV genotype 1 or HSV genotype 2 by adding known intermediate strains as controls or by testing with another PCR method.

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