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Unlabeled Probes for the Detection and Typing of Herpes Simplex Virus

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Background: **Unlabeled probe detection with a double-stranded DNA (dsDNA) binding dye is one method to detect and confirm target amplification after PCR. Unlabeled probes and amplicon melting have been used to detect small deletions and single-nucleotide polymorphisms in assays where template is in abundance. Unlabeled probes have not been applied to low-level target detection, however.**

Methods: **Herpes simplex virus (HSV) was chosen as a model to compare the unlabeled probe method to an in-house reference assay using dual-labeled, minor groove binding probes. A saturating dsDNA dye (LCGreen® Plus) was used for real-time PCR. HSV-1, HSV-2, and an internal control were differentiated by PCR amplicon and unlabeled probe melting analysis after PCR.**

Results: **The unlabeled probe technique displayed 98% concordance with the reference assay for the detection of HSV from a variety of archived clinical samples (n = 182). HSV typing using unlabeled probes was 99% concordant (n = 104) to sequenced clinical samples and allowed for the detection of sequence polymorphisms in the amplicon and under the probe.**

Conclusions: **Unlabeled probes and amplicon melting can be used to detect and genotype as few as 10 copies of target per reaction, restricted only by stochastic limitations. The use of unlabeled probes provides an attractive alternative to conventional fluorescence-labeled, probe-based assays for genotyping and detection of HSV and might be useful for other low-copy targets where typing is informative.**

Real-time PCR allows for the amplification, quantification, and detection of nucleic acid in a single-tube reaction *(1, 2)*. Detection of target can be achieved using synthetic oligonucleotide probes or with the addition of a double-stranded DNA (dsDNA)[3] binding dye. Further specificity may be added by including a postamplification melting analysis step to confirm target when using a dsDNA binding dye or certain types of probes *(3–8)*. One method of real-time PCR detection and confirmation uses an unlabeled probe in combination with asymmetric amplification. Unlabeled probes possess no fluorescent moiety; rather, they produce a characteristic melting signature when used with a dsDNA binding dye, such as LCGreen® Plus (Idaho Technology). In its simplest form, the unlabeled probe technique generates 2 melting curves, 1 from the dissociation of the unlabeled probe off single-stranded DNA (ssDNA) generated by asymmetric PCR and 1 from the homozygous dsDNA amplicon. Unlabeled probe and amplicon melting analysis have been used to detect small deletions and single-nucleotide polymorphisms in human *RET* and factor V genes, among others *(5, 9–12)*. Human genomic DNA is an ideal target for unlabeled probe assays since target copy number is rarely limited, but the use of unlabeled probes in assays that require low copy number sensitivity has not been reported.

This report describes the limits of copy number detection, typing accuracy, and melting reproducibility with the use of unlabeled probes. Herpes simplex virus (HSV) was chosen to develop an unlabeled probe assay based on the availability of quantified DNA targets for HSV-1 and HSV-2, characterized clinical samples, the necessity of low-level detection *(13–16)*, and an established in-house reference assay *(17)*.

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[3] Nonstandard abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; HSV, herpes simplex virus; IC, internal control; C_T, crossing threshold.

Materials and Methods

CLINICAL SAMPLES, HSV, AND INTERNAL CONTROLS

We deidentified 182 residual clinical samples submitted to ARUP for HSV testing after the Health Insurance Portability and Accountability Act of 1996, in accordance with University of Utah Institutional Review Board protocol number 7275, which covers research conducted at ARUP Laboratories. Samples were extracted using a 96-well DNA extraction set (Qiagen) following the manufacturer's protocol. The internal control (IC), a *Caenorhabditis elegans*::green fluorescent protein transcriptional fusion plasmid *(17)*, was added to AVL lysis buffer (Qiagen) to obtain an expected concentration of 1×10^2 copies/μL in eluted nucleic acid.

HSV genomic DNA standards purified from HSV-1 (1×10^4 DNA copies/μL, Macintyre strain, ABI) and HSV-2 (1×10^4 DNA copies/μL, ABI) were used as quantified control standards. We subcloned 180-bp glycoprotein D amplicons from the ABI quantified HSV-1 and HSV-2 DNA into pCR® II-TOPO® (Invitrogen) following the manufacturer's protocols. These clones, designated pHSV-1 and pHSV-2, confirmed the sequence of the glycoprotein D gene of the ABI-quantified HSV-1 and HSV-2 and determined the range of detection for the unlabeled probe method.

HSV REFERENCE ASSAY

For comparison, we used an in-house reference assay, an Epoch minor groove binding probe-based assay (Nanogen) *(17)*. The minor groove binding probe was 100% complementary to a region of the glycoprotein D gene in HSV-1 and -2. Samples were run on an ABI 7900 real-time PCR instrument as described by Stevenson et al. *(17)*. Because the reference assay does not discriminate between HSV-1 and -2, only the presence or absence of HSV was tested.

PRIMERS AND PROBE

Oligonucleotides, manufactured by Integrated DNA Technologies, were desalted and resuspended in 1× TE (10 mmol/L Tris, pH 8.0, 1 mmol/L EDTA). Primers for HSV were numbered from the ATG start codon of glycoprotein D gene. Primers used for HSV asymmetric real-time PCR were 458F and 603R. The unlabeled oligonucleotide probe (HSVP) was designed with 100% complementarity to HSV-2 and modified with a 3′ amino C6 modification to prevent DNA polymerase extension *(18)*. We used HSVFS and HSVRS to amplify HSV from clinical samples for sequencing and HSVnest for sequencing to confirm HSV type. We designed primers ICF and ICR to amplify the IC. Primer and probe sequences are summarized in Table 1.

REAL-TIME PCRS AND MELTING ANALYSIS

PCR amplification was performed in a LightCycler® (Roche Diagnostics). Real-time PCR and unlabeled probe melting analysis reactions contained 1× Roche FastStart DNA Hybridization Mix (includes dNTPs with dUTP and 1 mmol/L $MgCl_2$), 0.1 μmol/L forward primer 458F, 0.5 μmol/L reverse primer 603R, 0.5 μmol/L probe HSVP, 0.15 μmol/L forward primer ICF, 0.15 μmol/L reverse primer ICR, 0.8× LCGreen Plus, an additional 2 mmol/L $MgCl_2$, 0.25 units uracil–DNA glycosylase, and 1 μL DNA, in a total volume of 10 μL. Cycling and postamplification melting analysis were performed using the following conditions at a programmed transition rate of 20 °C/s: [55 °C$^{(5:00)}$ + 95 °C$^{(10:00)}$ + (95 °C$^{(0:01)}$ + 60 °C$^{(0:01)}$ + 72 °C$^{(0:10)}$) × 55 cycles + 95 °C$^{(0:00)}$ + 40 °C$^{(0:20)}$ + 50 °C→95 °C at 0.5 °C/s + 40 °C$^{(0:00)}$]. We used an arbitrary crossing threshold (C_T) cutoff of 50 cycles to determine whether a sample was positive. After LightCycler amplification and melting analysis, samples were placed at 4 °C for ≥10 min before high-resolution melting analysis using an HR-1™ instrument (Idaho Technology). The HR-1 uses the same capillaries as the LightCycler and is capable of continuous, high-resolution melting and fluorescence data acquisition *(19)*. For HR-1 melting analysis, samples were removed from 4 °C and melted from 60 °C to 95 °C at 0.3 °C/s. We analyzed HR-1 melting data by use of Idaho Technology High Resolution Melting Analysis software (version 1.5f).

DETECTION AND TYPING OF HSV BY UNLABELED PROBE AND AMPLICON MELTING

We blinded 182 previously assayed clinical HSV samples and tested them using unlabeled probes. Samples were amplified and typed by low-resolution melting in a LightCycler. All samples were subsequently melted in the HR-1 to confirm type. To assess genotypes for HSV-1 or -2, unlabeled probe and amplicon melting were required. A subset of indeterminate samples was reamplified if we observed ambiguous HSV typing by melting analysis. The

Table 1. Oligonucleotides used for real-time PCR amplification, detection, and sequencing.

Name	Sequence, 5′→3′	Use
ICF	ACAAGATCTCCGGTAGAAAAAATGAG	IC forward
ICR	CGGCCGGGACAACTCCAGTGAAAAGTTCTTC	IC reverse
458F	CCCATCCGAACGCAG	HSV forward
603R	CTGTGTAATCTCCGTCCAGTCGTTTATCTTCAC	HSV reverse
HSVP	ATTCCTGATGCACGCCCCCGCCTTCGAGACCGCGGGT-C6	Unlabeled probe
HSVFS	GGACCCGTTCCAGCCCCCCAGCATCCCGATCAC	HSV sequencing
HSVRS	AGCGGGGGAGCATCCCGATGCTGTC	HSV sequencing
HSVnest	GCCTGCCGCAGCGTGCTCC	HSV sequencing

2nd amplification of indeterminate samples was performed as previously described, but without IC primers, and melted on the HR-1 to verify HSV type.

SEQUENCING

We reamplified 129 positive and negative HSV samples, as determined by amplicon and unlabeled probe melting analysis, using primers HSVFS and HSVRS for sequencing analysis. Reaction conditions consisted of 1× CoralLoad [Qiagen, proprietary, contains Tris·Cl, KCl, $(NH_4)2SO_4$, 1.5 mmol/L $MgCl_2$, orange dye, red dye; pH 8.7 (20 °C)], an additional 2 mmol/L $MgCl_2$, 0.3 μmol/L forward primer HSVFS, 0.3 μmol/L reverse primer HSVRS, 0.5 mmol/L dNTPs, 1 units Qiagen *Taq* DNA polymerase, and 2 μL of the extracted clinical sample in a final volume of 40 μL. Cycling was performed in an ABI 9700 using the following conditions: $94\ °C^{(3:00)}$ + [$94\ °C^{(0:10)}$ + $65\ °C^{(0:30)}$ + $72\ °C^{(0:45)}$] × 45 cycles. We performed postamplification analysis by use of a 2% agarose gel to observe products. Excess primers and nucleotides were removed using a Qiagen PCR Cleanup set following manufacturer's protocol. All positive amplification products were submitted to the ARUP sequencing facility for dye terminator sequencing with primer HSVnest. Some HSV-positive samples could not be adequately amplified with primers HSVFS and HSVRS and were sequenced from the 458F and 603R recovered amplicon.

DYNAMIC RANGE OF HSV DETECTION AND TYPING

Plasmids pHSV-1 and pHSV-2 were quantified by $A_{260/280}$ measurements to estimate copy number. We compared dilution series of each plasmid to ABI-quantified standards diluted to 1000 copies of HSV-1 or HSV-2 per reaction. We adjusted plasmid concentrations to the ABI-quantified source at 1000 copies per reaction, so the C_Ts were within 0.5 of the quantified control. To determine the dynamic range, we performed a serial dilution of both plasmids at 1:10 dilutions from 1×10^9 to 1 copy of target per reaction.

Results

HSV ASSAY DEVELOPMENT

Universal primers 458F and 603R were designed to amplify a 153-bp region of the HSV glycoprotein D gene. A consensus alignment of the glycoprotein D gene for types 1 and 2 is shown in Fig. 1A [accession X14112 and U12182 *(20, 21)*]. The amplicon included 8 verified sequence variations between HSV-1 and -2, resulting in a mean 1.0 °C difference in amplicon melting temperature based on LightCycler melting peak data (Table 2). A 37-base oligonucleotide probe, HSVP, designed with 100% complementarity to HSV-2 and 5 mismatches to HSV-1, was chosen for unlabeled probe analysis. The use of a single probe allowed for 2 distinct melting profiles depending on the HSV type amplified. The mean melting temperature of the probe when HSV-1 was present in the sample

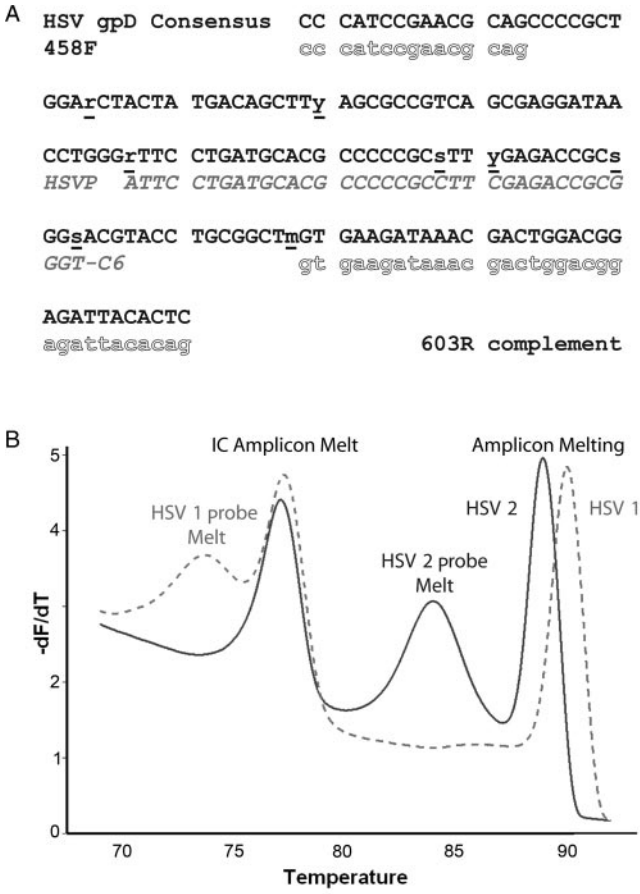

Fig. 1. Consensus alignment of HSV glycoprotein D gene and high resolution unlabeled probe melting analysis of HSV.

(*A*), consensus alignment of HSV glycoprotein D gene (*uppercase*) with sequence variations between HSV-1 and -2 *underlined* in *lowercase*. Primers 458F and the complement of 603R are in *outlined lowercase*, and probe HSVP is in *italic uppercase*. Probe HSVP has 100% complementarity to wild-type HSV-2 and 5 mismatches to wild-type HSV-1. (*B*), high-resolution melting profiles for wild-type HSV-1 and HSV-2 in the presence of LCGreen Plus, unlabeled probe HSVP, and IC. HSV-1 amplicon and probe melting is a *dashed line*, and HSV-2 amplicon and probe melting is shown as a *solid line*. Note that melting peak temperatures are shifted approximately 1 °C higher when melted using the HR-1 vs the LightCycler.

was 71.6 °C, because of 5 mismatches between the probe and the ssDNA complement, which lowered the melting temperature of the HSV-1/probe duplex (Table 2). The mean melting temperature of the probe when detecting HSV-2 was 83.0 °C because of its 100% identity with HSV-2 (Table 2). Representative melting analysis curves for amplicon and unlabeled probe melting analysis with IC present are shown in Fig. 1B. To ensure accurate typing for each experiment, we included ABI-quantified HSV-1 and -2 controls to confirm amplification and melting analysis. The addition of an IC plasmid monitors extraction and PCR efficiency in clinical samples. The IC primers were designed to amplify a 64-bp region of the IC control plasmid and melt at 76 °C (Fig. 1B; Table 2). Negative controls without DNA were also included in each run. Primers and probes used for the real-time detection of HSV types 1 and 2 were tested against

Table 2. Observed melting temperatures of IC amplicon, HSV-1 and -2 amplicon, and unlabeled probe HSVP/HSV ssDNA duplex.

Amplicon or probe	Duplex length, bp	Mean melting temperature, °C[a]	SD melting temperature, °C	Maximum-minimum range, °C
IC amplicon (HSV-1)	64	76.04	0.22	0.65
IC amplicon (HSV-2)	64	76.00	0.09	0.27
HSV-1 amplicon	153	88.69	0.09	0.54
HSV-2 amplicon	153	87.65	0.18	0.24
HSVP detecting HSV-1	37	71.60	0.31	0.93
HSVP detecting HSV-2	37	83.03	0.15	0.44

[a] Mean of 8 reactions performed in parallel as measured on a LightCycler.

Epstein–Barr virus, human herpes virus types 6A and 6B, varicella zoster virus, and cytomegalovirus and displayed no cross-reactivity (data not shown).

DYNAMIC RANGE

Universal primers 458F and 603R amplified HSV-1 and 2 with equal efficiency based on ABI-quantified controls and cloned plasmids pHSV-1 and pHSV-2 (Fig. 2A). The range of detection for amplicon and unlabeled probe analysis was <10 copies to 1×10^9 copies of HSV per reaction (data not shown). A representative dilution experiment for low copy number detection is shown in Fig. 2B. The unlabeled probe assay could reproducibly detect and type 10 copies of HSV per reaction. Lower copy numbers of target will not always amplify HSV but will exhibit IC amplification. The example in Fig. 2B shows HSV amplification from the same dilution series, at an expected concentration of 1 copy per reaction for pHSV-1 and -2. One set exhibits amplification; the 2nd set did not amplify HSV but displays IC amplification at the expected C_T. High-resolution melting analysis of HSV samples with positive amplification from Fig. 2B for 10 and 1 copy are shown in Fig. 2C. Samples with an estimated 10 copies of pHSV-1 or pHSV-2 exhibit the signature melting patterns expected for both amplicon and unlabeled probe melting analysis. HSV-positive samples of an estimated 1 copy show amplicon melting profiles but have weak or absent probe melting signals. The unlabeled probe method can reproducibly detect and type 10 or more copies of HSV per reaction. Amplicon melting was sufficient for the detection of HSV for some samples with <10 copies of target per reaction but was not accurate for typing because of the amplicon melting temperature shifts sometimes observed when very low copies of target were amplified (Fig. 2, B and C).

DETECTION OF HSV IN CLINICAL SAMPLES

We blinded 110 archived clinical samples and tested them for the presence of HSV; 87 samples were from cerebrospinal fluid and 23 were from genital (6), serum/plasma (6), oral (3), and miscellaneous (8) sources. The unlabeled probe method detected 91 of 94 HSV-positive reference samples, 16 of 16 HSV-negative samples, and 3 false negatives. The 3 undetected positive samples had C_T values that would calculate to <10 copies of HSV per reaction based on the reference assay. Given that some samples were >2 years old, and HSV could not be detected, it was not clear whether the samples degraded over time or the unlabeled probe technique simply did not detect HSV. The 3 negative samples were additionally processed with sequencing primers HSVFS and HSVRS, as well as with equimolar primers 458F and 603R without the unlabeled probe. In each case, the reactions failed to produce an amplicon, indicating that the samples had degraded (data not shown).

To further support the sensitivity of the unlabeled probe method, we performed an additional study. A 2nd set of 72 HSV reference samples <3 months old was assayed using the unlabeled probe method. Thirty-one of the clinical samples were from cerebrospinal fluid, and 41 were from genital (11), serum/plasma (10), oral (5), and miscellaneous (15) sources. Examination of this 2nd set showed 100% concordance with the reference assay. The unlabeled probe method detected 45 of 45 HSV-positive reference samples, 27 of 27 HSV-negative samples, and no false negatives. Of the 45 positive clinical samples, 4 had C_T values indicating <10 copies of HSV per reaction, suggesting a sensitivity limited only by stochastic factors, similar to the reference assay.

TYPING OF HSV IN CLINICAL SAMPLES

We sequenced 103 positive HSV clinical samples to verify HSV type. We included 36 HSV-1, 68 HSV-2, and 4 indeterminates based on glycoprotein D sequencing. Indeterminate samples were positive for HSV but, because of insufficient sample quantity, could not be accurately typed by unlabeled probes or sequenced. Representative melting profiles for 15 clinical samples melted on the LightCycler and on HR-1 are shown in Fig. 3, A and B. These examples were chosen to highlight the capability of the unlabeled probe technique to detect sequence variations in the amplicon and under the unlabeled probe. Two samples, 1 HSV-1 and 1 HSV-2, displayed lower-temperature unlabeled probe melting than control HSV samples, whereas amplicon melting profiles were less affected. Sequence analysis confirmed that both samples had single-base sequence variations under the probe, a G→T at position 533 for HSV-1 and a C→T at position 551 for HSV-2. Of the 103 sequence-verified samples, 18 had at least 1 sequence variation in the amplicon, and 9 of these had a sequence variation

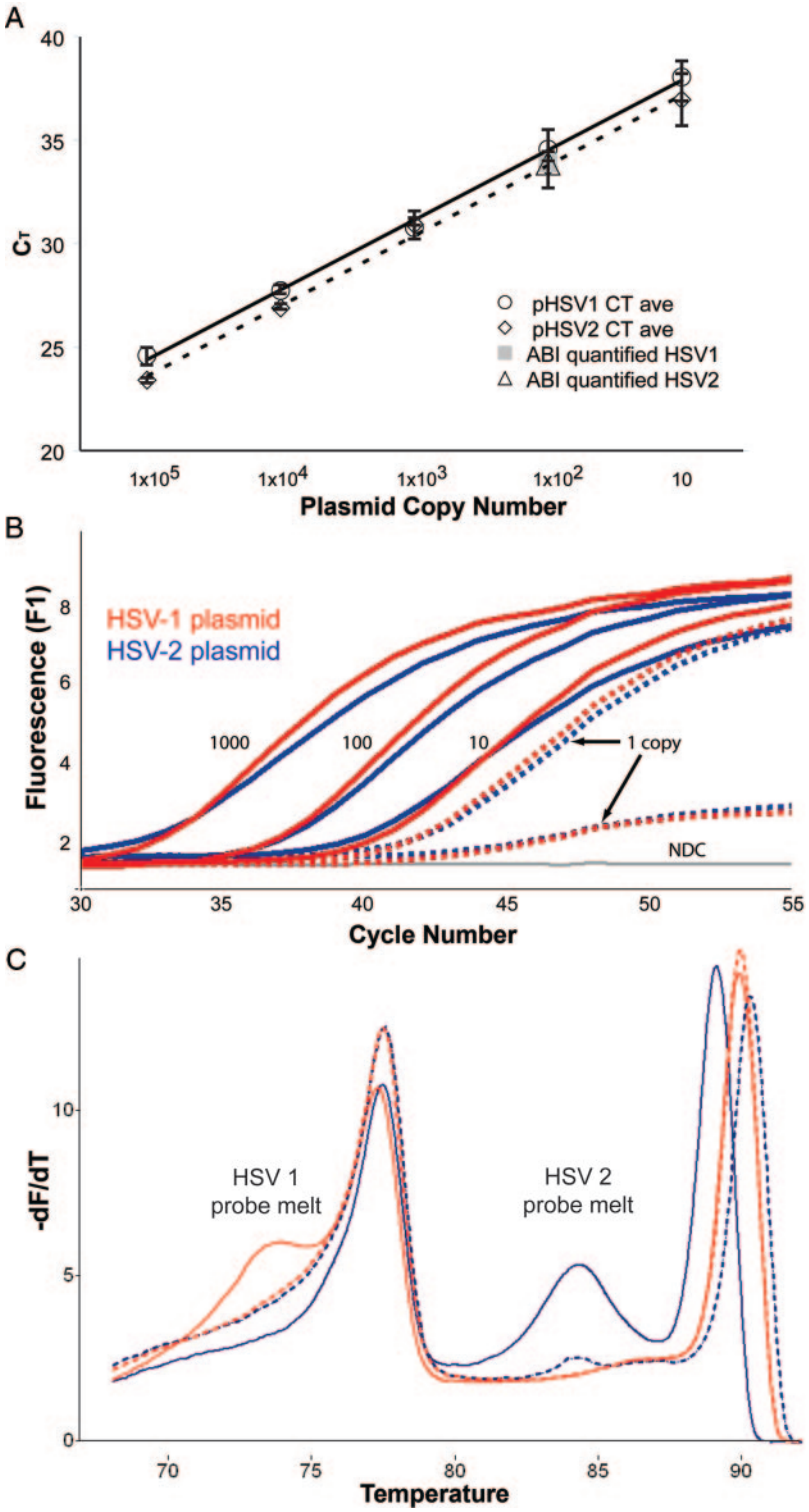

Fig. 2. Universal primer amplification efficiency and low-level detection and typing of HSV.

(*A*), copy number amplification reproducibility of primers 458F and 603R. Amplification of plasmids pHSV-1 (*open circle*) and pHSV-2 (*open diamond*) were tested against ABI-quantified HSV-1 (*black square*) and HSV-2 (*open triangle*) to confirm copy number of plasmids for dynamic range experiments. Primers 458F and 603R amplify HSV-1 and HSV-2 with equal efficiency based on C_T comparison (all reactions run in triplicate). *Error bars* are SD of replicates at a given concentration. (*B*), LightCycler amplification plot of 1×10^3, 1×10^2, 10, and replicates of 1 copy (*dashed lines*) of HSV-1 (*red*) and HSV-2 (*blue*). Single copies of HSV-1 or -2 may be amplified, if present in the sample, but stochastic limitations make detection unreliable at <10 copies per reaction. The *lower set of dashed lines* represents single-copy samples that amplified the IC but not HSV. The *upper set of dashed lines* represents single-copy HSV amplification. A no-DNA control is shown in *gray*. (*C*), high-resolution melting analysis of positive single copy (*dashed lines*) and 10 copies (*solid lines*) HSV-1 (*red*) and -2 (*blue*) from Fig. 3B. Ten copies of HSV can be typed by amplicon and unlabeled probe melting analysis, whereas estimated single-copy amplification reactions cannot always be typed by probe or amplicon melting.

under the probe. Of the 18 samples with sequence variations in the amplicon, only 1 sample had an amplicon melting profile that was altered by >0.5 °C (data not shown). Of the 9 samples with a sequence variation under the probe, all displayed aberrant probe melting profiles compared with controls. However, all samples were still accurately typed based on high-resolution probe melting and amplicon melting.

TYPING OF SAMPLES WITHOUT IC

Of the 103 HSV samples typed using unlabeled probes, 18 samples initially could not be accurately typed because of

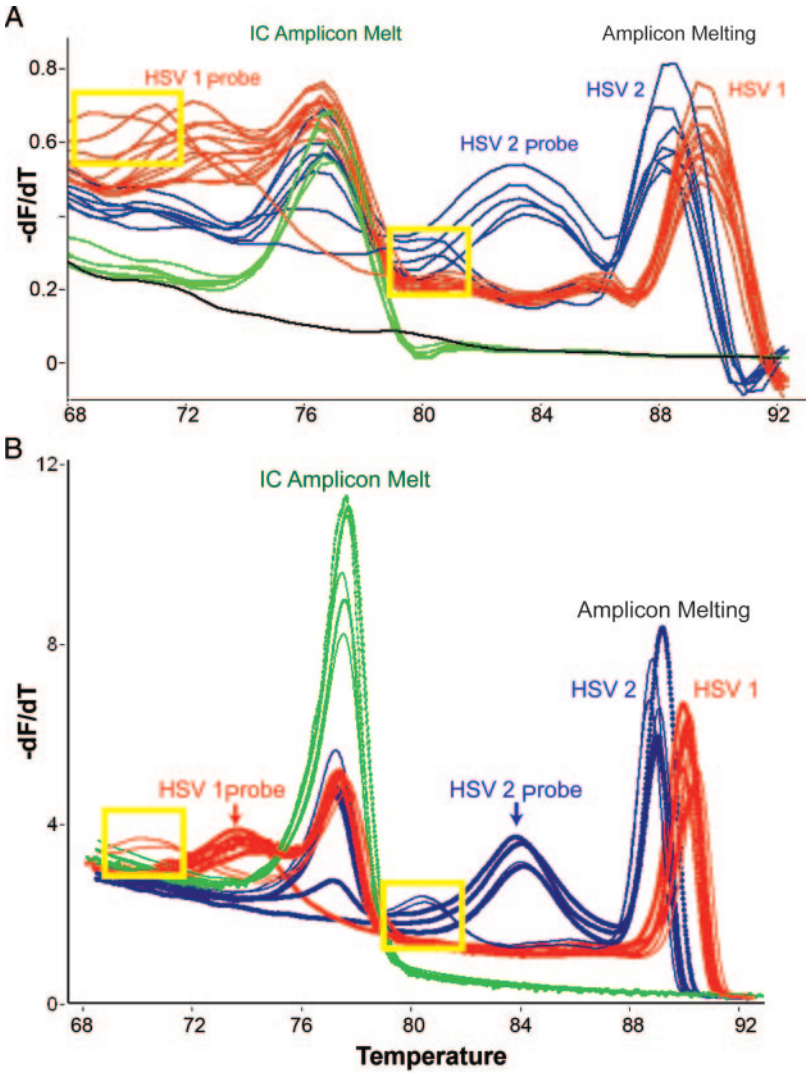

Fig. 3. Detection of HSV sequence variations in clinical samples by melting analysis.

(*A*), LightCycler derivative melting plot of 15 clinical samples. The majority of samples show signature melting profiles for HSV-1, HSV-2, or IC. Two samples, 1 HSV-1 and 1 HSV-2 (each shown in replicate), exhibit aberrant unlabeled probe melting profiles (*yellow boxes*). (*B*), high-resolution derivative melting of the same clinical samples shown in (*A*). Two samples (in replicate), 1 HSV-1 and 1 HSV-2 (*yellow boxes*), show aberrant unlabeled probe melting profiles easily distinguishable from signature melting profiles for HSV-1 and -2.

aberrant melting profiles or weak probe signals. The exclusion of the IC primers improved HSV amplification and detection and, for some samples, allowed for accurate typing by unlabeled probe melting. Nine indeterminate samples had aberrant unlabeled probe melting that was reproducible without the IC primers. These samples possessed sequence variations under the unlabeled probe as previously described. Of the remaining 9 indeterminate samples, 2 were HSV negative (C_T >50), 2 were HSV-2, and 5 displayed amplicon melting but no unlabeled probe melting transition, presumably because of insufficient ssDNA amplification. These 5 were considered HSV$^+$ (see Table 3), and only 1 could be sequenced.

Discussion

Unlabeled probe assay design is similar to other fluorescence-labeled probe techniques but requires some unique considerations. The use of LCGreen Plus with unlabeled probes is necessary for genotyping (*7*). Because LCGreen Plus emits a single spectrum, IC, amplicon, and probe melting profiles should not overlap too much in temperature. Primer concentrations must be accurately quantified to ensure reproducibility of low-level copy detection between oligonucleotide syntheses. Asymmetric primer ratios are required to produce ssDNA for unlabeled probe annealing. We tested a number of asymmetric ratios between 1:2 and 1:20. As primer ratios approach equimo-

Table 3. Comparison of unlabeled probe HSV typing to glycoprotein D gene sequencing.

	Unlabeled probe typing			
Sequence typing	**HSV-1**	**HSV-2**	**HSV$^+$**	**Total**
HSV-1	35	0	1	36
HSV-2	0	68	0	68
HSV^{+a}	0	0	4	4
Total	35	68	5	108

[a] HSV$^+$ samples were not typed by unlabeled probes because of weak probe signal but were detected by amplicon melting.

lar, amplification is enhanced, as shown by C_T analysis. Improved amplification is offset by weaker unlabeled probe signals due to lower levels of ssDNA produced during cycling. Conversely, high asymmetric ratios improve the unlabeled probe signal, but significantly diminish low-level sensitivity. For assays that do not require low-level sensitivity or where target copy number is not limited, an asymmetric ratio of 1:10 is a good starting point for assay development. An asymmetric ratio of 1:5 was optimum for low-level HSV detection and typing, but this ratio will vary depending on target and amplicon length.

Equimolar concentrations of primers 458F and 603R were examined to determine whether amplicon melting alone could detect and accurately type HSV. Equimolar primers could detect HSV with 100% concordance but were not consistently accurate for typing when sequence variations were present or at lower levels of target DNA. Very low levels of target amplicon may exhibit variable melting temperatures, outside the expected variation for a given amplicon (Fig. 2B). Variability of amplicon melting temperatures at low copy numbers is not entirely understood and may be amplicon specific. Multiplexed HSV-1– or -2–specific primers were examined to determine whether type-specific amplification could accurately type HSV by amplicon melting alone. In our experience, 3′ mismatches were not sufficient to specifically amplify HSV-1 or -2 by real-time PCR, and the issue was not pursued (data not shown). However, experimentation with different-length amplicons or a different locus may indicate that amplicon melting alone is sufficient for HSV typing using universal primers.

Inconclusive typing of HSV can occur when sequence polymorphisms are present under a fluorescence-labeled probe *(22)*. The use of a dsDNA binding dye and unlabeled probe allows for a confirmatory amplicon melt, which permits HSV typing when sequence polymorphisms are present as long as reaction target copy numbers are >10. Below 10 copies per reaction, stochastic effects limit assay reproducibility, a problem common to many assays that can only be solved by increasing reaction volumes or by concentration of sample before amplification.

The range of HSV detection and typing using the unlabeled probe method was 10 to 1×10^9 copies per reaction. Ten copies of HSV per reaction may be detected, but not reproducibly typed, and concentrations of $>1 \times 10^9$ copies per reaction were not tested. The universal 458F and 603R amplified HSV-1 and -2 with equal efficiency throughout the dynamic range, based on C_T.

HR-1 melting analysis improves melting signal to noise and decreases baseline fluctuations compared with the LightCycler *(19)*. For samples in which target copy number is >100 per reaction, HR-1 melting is advantageous to accurately type the target. When the target copy number is <10 copies per reaction, or when samples display aberrant melting profiles, a 2nd amplification reaction without IC primers may be performed to improve the HSV amplicon and unlabeled probe melting signal. This reflex reaction was performed 10% of the time to verify type or to confirm that the sample contained HSV. Aberrant melting curves were observed approximately 5% of the time, most often in positive samples. In comparison, the reference assay, which initially processes samples as single reactions, would have a repeat rate of 74% based on the number of positive clinical samples processed, since all positive samples with a C_T >30 cycles are reanalyzed to confirm an HSV-positive sample *(17)*.

The unlabeled probe method displayed 100% detection concordance when samples were not degraded. Typing positive samples was successful in 103 of 104 samples. The missed sample, sequence verified to be HSV-1, was detected by amplicon melting but could not be accurately typed owing to a lack of unlabeled probe signal. Another clinical sample reproducibly showed aberrant melting profiles that did not correlate to HSV-1 or -2. Gel analysis of the amplicon showed an unknown band at 50 bp. A no-DNA control is regularly included and produces no primer dimers based on melting and agarose gel analysis, suggesting the band was not primer dimer. Further analysis of the band was not pursued, and the sample was considered HSV negative by both the unlabeled probe and reference assay.

Unlabeled probes can detect low copy numbers of HSV from clinical samples with sensitivity similar to that of fluorescence-labeled, probe-based assays. The unlabeled probe method detected HSV from a variety of clinical samples with an overall 98% concordance to the reference assay and correctly typed 99% of HSV-1– or -2–positive samples, even when sequence variations were present. Typing is most accurate with high-resolution melting, although approximately 81% of HSV samples were genotyped by LightCycler melting analysis alone. Unlabeled probes are an attractive alternative to conventional fluorescence-labeled, probe-based assays. Genotyping, low-level detection of target, and ICs to monitor extraction, amplification, and detection can all be achieved with unlabeled probes.

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