The Journal of Molecular Diagnostics, Vol. 14, No. 6, November 2012

http://dx.doi.org/10.1016/j.jmoldx.2012.05.005

Development and Comparison of a Rapid Isothermal Nucleic Acid Amplification Test for Typing of Herpes Simplex Virus Types 1 and 2 on a Portable Fluorescence Detector

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We have developed a rapid and simple molecular test, the IsoGlow HSV Typing assay, for the detection and typing of herpes simplex virus (type 1 and 2) from genital or oral lesions. Clinical samples suspended in viral transport mediums are simply diluted and then added to a helicase-dependent amplification master mix. The amplification and detection were performed on a portable fluorescence detector called the FireFly instrument. Detection of amplification products is based on end-point analysis using cycling probe technology. An internal control nucleic acid was included in the amplification master mix to monitor the presence of amplification inhibitors in the samples. Because the device has only two fluorescence detection channels, two strategies were developed and compared to detect the internal control template: internal control detected by melting curve analysis using a dual-labeled probe, versus internal control detection using end-point fluorescence release by a CPT probe at a lower temperature. Both have a total turnaround time of about 1 hour. Clinical performance relative to herpes viral culture was evaluated using 176 clinical specimens. Both formats of the IsoGlow HSV typing assay had sensitivities comparable to that of the Food and Drug Administration–cleared IsoAmp HSV (BioHelix Corp., Beverly MA) test and specificity for the two types of HSV comparable to that of ELVIS HSV (Diagnostic Hybrids, Athens, OH). ***(J Mol Diagn 2012, 14:569–576; http://dx.doi.org/10.1016/j.jmoldx.2012.05.005)***

Two herpes simplex viruses (HSV), HSV-1 and HSV-2, are common and contagious viruses that cause blistering and sores on the mouth, lips, or genitals. As such, genital herpes is one of the most prevalent sexually transmitted diseases (STDs). The pattern of recurrence of genital infection is substantially different for the two types, with recurrences and asymptomatic viral shedding being much more common in patients infected with HSV-2.[1–3] Therefore, typing this virus is important, as it can influence prognosis and counseling of patients.

The IsoAmp HSV assay, recently cleared by the Food and Drug Administration (FDA), provides a rapid and simple near-patient diagnostic for the detection of HSV in genital and oral lesion specimens from symptomatic patients.[4] The assay combines helicase-dependent amplification (HDA) technology, and a single-use, handheld detection device (BESt cassette) for end-point detection.[5–7] The overall assay process consists of sample preparation by a one-step dilution, isothermal nucleic acid amplification, and detection on the disposable cassette. Results are available within 1.5 hours. However, the assay does not differentiate between HSV-1 and HSV-2. Although the BD ProbeTec Herpes Simplex Viruses (HSV-1 and -2) Q^x amplified DNA assay, and the EraGen MultiCode-RTx HSV 1&2 kit provide HSV typing resolution, these assays depend on expensive instruments with large countertop footprints that may not be suitable for near-patient diagnostics or resource-limited settings. This creates a substantial market need for a low-cost, point-of-care HSV typing assay.

In this study, we developed a rapid HSV typing assay called IsoGlow HSV Typing, by modifying the IsoAmp HSV test to adapt it to the FireFly instrument (BioHelix, Beverly, MA). The FireFly instrument offers an option for portable and cost-efficient fluorescence detection. With on-board computer software, the device can be used for both real-time and end-point data acquisition. Although some isothermal amplification technologies have been adapted to the similar device recently,[8] this is the first report of it being used for detecting HDA products. As this instrument has only two detection channels (FAM and TAMRA), and its dynamic range is narrower than that of the much more expensive real-time PCR instrument, we had to devise two novel strategies for the detection of two

Supported by NIH awards 2R44AI066487-03 and 5R44AI066487-04.

Accepted for publication May 25, 2012.

Disclosure: All authors are employees of BioHelix Corporation, which manufactures the FireFly instrument used in these studies.

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analytes (HSV-1 or HSV-2), and an internal control (IC). In both strategies, cycling probe technology (CPT) was used for end-point detection of the HSV products amplified by HDA. CPT uses a chimeric DNA-RNA-DNA probe that is cut by RNase H when it is bound to its complementary target sequence.[9] In our assay, we use a thermostable RNase H2 (BioHelix Corp.) that specifically cleaves chimeric probes containing a single RNA monomer when it is bound to a target DNA. Hou and colleagues[10,11] used this type of RNase H2 for genotyping single nucelotide polymorphisms with a microplate reader. In one strategy, we used a postamplification melting curve analysis to detect this control, while in another strategy we detected fluorescence released from a CPT probe after amplification, at a different temperature than that used during amplification (hereafter defined as two-step CPT). The relative performance of these two strategies was compared with a set of 176 clinical samples.

Materials and Methods

Reagents

All of the chemicals used in this study were from Sigma-Aldrich (St. Louis, MO). Restriction endonucleases were purchased from New England Biolabs (Ipswich, MA). IsoGlow HSV enzyme reagent, IsoGlow HSV amplification reagent, and all of the other HDA reagents were from BioHelix Corp. All of the oligonucleotides were ordered from IDT (Coralville, IA).

Purified Viral DNA and Assay Standard

Quantified HSV-1 or HSV-2 viral DNA from Advanced Biotechnologies Inc. (Columbia, MD) were used as a gold standard. The concentrations of HSV-1–positive or HSV-2–positive control plasmid from IsoAmp HSV were determined and were verified by quantitative polymerase chain reaction (qPCR) using the gold standard and the same primer pairs of HDA. The qPCR was performed with DyNAmo HS SYBR Green qPCR Kit with 400 nmol/L of each primer per reaction (25 μL per reaction). The qPCR assay was performed in a ABI 7300 with the following program: 15 minutes of 95°C, and then 40 cycles of 10 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C (with fluorescence data collection), and 3 minutes of 72°C for a final extension followed by melting curve analysis. The IsoGlow HSV typing assays were initially optimized with HSV-1 and HSV-2 control plasmid, and were finally validated with quantified HSV-1 or HSV-2 viral DNA.

Collection, Shipping, and Storage of Clinical Samples

Sixty clinical samples in viral transport media were collected from Laboratory Alliance of Central New York (Syracuse, NY). A total of 116 clinical swabs suspended in vial transport medium were collected from the Cleveland Clinic (Cleveland, OH). The samples were shipped on ice for overnight delivery, and were aliquoted on receipt. Some were placed at −80°C for long-term storage, and some were placed at −20°C for short-term storage or near-term testing.

IsoGlow HSV Typing Assay

The primer pairs used in the IsoGlow HSV assay are the same as those reported for the IsoAmp HSV kit.[4] The assay was further optimized based on the strategies described in the previous publication.[12] A thermostable RNase H2 enzyme (BioHelix Corp.) was added at a concentration of 20 ng per assay. For the melting curve based detection of the IC, the IsoGlow HSV typing amplification reagent was prepared as follows: for each assay, 60 nmol/L of HSV-1 typing probe, 60 nmol/L of HSV-2 typing probe, and 60 nmol/L of HSVIC melting probe were combined with the other components in the IsoAmp HSV amplification reagent (primers and buffers). In the case of the CPT-based detection of internal control, the IsoGlow HSV typing amplification reagent were prepared the same way except that the HSVIC melting probe was replaced by 80 nmol/L of HSVIC CPT probe. In both assay formats, the internal control template was premixed in the amplification reagent.

The IsoGlow HSV typing assays were done using the following set-up: the reaction master mix was prepared by combining 40 μL of IsoGlow HSV amplification reagent, and 5 μL of IsoGlow HSV enzyme reagent per assay. A 5-μL quantity of the tested sample was mixed with the 45-μL master mix in 200-μL, thin-walled PCR tubes (Bio-Rad, Hercules, CA), and covered with mineral oil. The tubes were placed in a FireFly instrument for amplification and fluorescence detection.

Data Collection Program on FireFly Instrument

For assays in which the IC was detected by melting curve analysis, the FireFly instrument was programed to follow the profile:

- Incubate at 35°C for 1 minute with three data collections separated by 20 seconds so as to average the three readings to obtain an N_{35} value.
- Incubate at 64°C for 45 minutes with data collection every minute as an option.
- Incubate 35°C for 2 minutes with data collection every 30 seconds, such that four data points are averaged to generate the S_{35} value.
- The FireFly instrument then performs a melting curve analysis by heating the reaction to 95°C for 1 minute, cooling to 35°C, and raising the reaction to70°C at a rate of 0.1°C per second, with 1 second of fluorescence data collection every minute.

The typing results were determined by the signal-to-noise ratio F (S_{35}/N_{35}), T (S_{35}/N_{35}), and melting curve analysis from the TAMRA channel; where S represents end-point reading at the temperature in subscript, N represents initial-point reading at the temperature in subscript, F represents FAM channel, and T represents TAMRA channel.

For assays in which the IC was detected with a CPT probe, the FireFly instrument was programmed to do the following profile:

- Incubate at 35°C for 1 minute, and average the value of three readings taken at 20 second intervals to get a N_{35} value.
- Incubate at 64°C for 45 minutes with data collection every minute, calculating the average of the first four readings to obtain N_{64} as well as the average of the last four readings to obtain S_{64}.
- Incubate at 35°C for 1 minute without data collection, to allow for binding the maximum number of IC probes.
- Incubate at 46°C for 10 minutes. Fluorescence is not read at 46°C because of differences in background at different temperatures.
- Incubate 35°C for 2 minutes with data collection every 30 seconds, such that four data points are averaged to generate the S_{35} value.

The final results were determined by the T (S_{64}/N_{64}), F (S_{35}/N_{35}), and T (S_{35}/N_{35}) ratios; where S represents endpoint reading at the temperature in subscript, N represents initial-point reading at the temperature in subscript, F represents FAM channel, and T represents TAMRA channel.

Statistical Data Analysis to Determine Cutoff

The fluorescence signal-to-noise ratios obtained from positive samples and negative samples were grouped using "Data analysis" in the ToolPak functions of Microsoft (Seattle, Washington) Excel 2010 software. The functions of "Histogram" and "Descriptive statistics" were used for data distribution analysis. The mean and SD of signal-to-noise ratios (S/N) were calculated to determine the corresponding cutoff. Generally, the cutoff was set as the mean of S/N from negative samples plus at least three to 10 times the value of the SD on the S/N from negative samples. The extent of the separation of the S/N for positive samples versus that of negative samples was determined empirically with training samples, and varied for each probe.

Feasibility Study with Clinical Samples

A 5-μL quantity of viral transport medium was mixed together with HSV dilution buffer. Five microliter of the diluted sample was mixed together with the 45-μL master mix prepared as described in the previous section. The tubes were placed in a FireFly instrument for amplification and detection. The results were reported based on the predetermined decision algorithms. The algorithm for the assay with IC detection with melting-curve analysis used the equation F (S_{35}/N_{35}) $\geq$ 2.5 or T (S_{35}/N_{35}) $\geq$ 2.5, to report positive samples. Only samples that failed to yield ratios greater that the aforementioned cutoffs were subjected to melting-curve analysis. In those cases, if a detectable fluorescence peak was observed between 47°C and 55°C during the melting-curve profile with the TAMRA channel, the sample was reported as being HSV negative; otherwise, it was reported as an invalid test. In assays where the IC was detected with a two-step CPT, the equation F (S_{35}/N_{35}) $\geq$ 2.5 or T (S_{64}/N_{64}) $\geq$ 1.5 was used to distinguish positive samples for either HSV-1 or HSV-2. Only if none of the ratios passed the cutoff was the ratio of T (S_{35}/N_{35}) considered. If T (S_{35}/N_{35}) $\geq$ 1.8, the sample was reported as "HSV negative." Samples that failed to meet the aforementioned cutoff were reported as having yielded invalid test results.

Results

Implementation of Cycling Probe Technology with HDA

To adapt HDA detection to the FireFly fluorescence reader, several detection platforms have been developed and evaluated on the simple device. The CPT presented the most promise than the other target-specific probes described in the previous publication, including isothermal TaqMan and molecular beacon.[13] CPT is a simple signal amplification method with flexible detection choices. The CPT probe can be designed with a Tm as close to, or higher than, the temperature of isothermal amplification for real-time detection. The probe can also be designed with a Tm lower than the amplification temperature for postamplification detection or end-point analysis. Moreover, the HDA assay combined with CPT probe had a higher signal-to-noise ratio, a faster speed and a better sensitivity than that combined with TaqMan probe or molecular beacon probe (data not shown). Therefore, we decided to implement CPT with HDA (CPT-HDA) for developing a rapid and sensitive HSV typing assay.

In this report, the CPT probes for the HSV-1 and HSV-2 targets were designed to coincide with the location of two polymorphic sites within the amplicon for typing purpose. As shown in Table 1, the probes have a Tm near the 64°C HDA incubation temperature, such as to allow them to bind to the corresponding complementary sequence efficiently, and to be cleaved by the RNase H2 enzyme. The presence of the two polymorphisms between the type-specific probes makes the detection specific enough that the probes do not cross-hybridize to the other target. Figure 1 shows an example of the performance of the assay on the FireFly instrument. The FAM channel detects the amplification and specific cleavage of the CPT probe for HSV-2 target, while the TAMRA channel detects the corresponding signal for HSV-1 tar-

Table 1. IsoGlow HSV Probes

Probes	Sequences and modifications
HSV-1 typing probe	5′-/5TYE563/CGTCACCGTTTcG CAGGTGTG/3BHQ_1/−3′
HSV-2 typing probe	5′-/56-FAM/CGTGACcGTGTCG CAGG/3BHQ_1/−3′
HSVIC melting probe	5′-/5TYE563/CAAAGAGTCCG CAC/3BHQ_1/−3′
HSVIC CPT probe	5′-/5TYE563/TACGAAGGCGA cAAA/3BHQ_1/−3′

Lower case represents RNA base.
Underlines highlight the polymorphisms between HSV-1 and HSV-2.

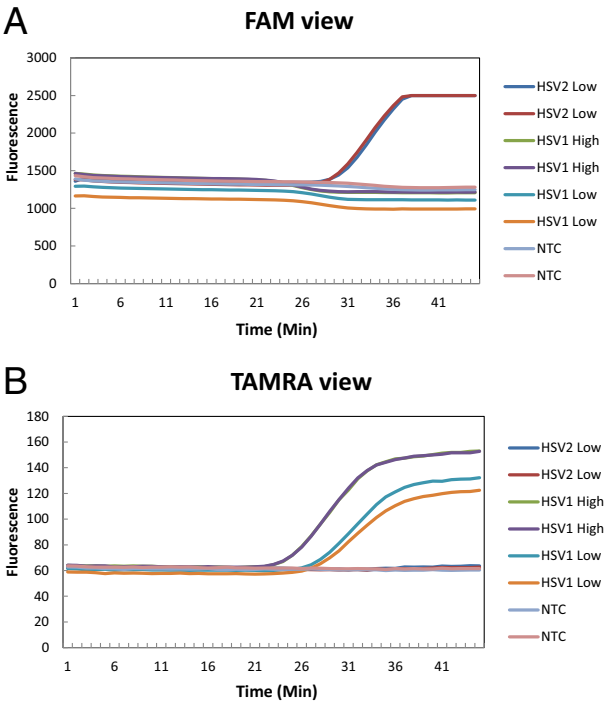

Figure 1. Real-time detection HSV-1 versus HSV-2 on the Firefly instrument by implementing CPT together with HDA. Fluorescence emission intensity versus time is charted for HSV-2 detection using the FAM channel (**A**), and for HSV-1 detection in the TAMRA channel (**B**). The reactions charted with the lines labeled "HSV2 low" contained 50 copies/reaction of HSV-2 DNA, lines labeled HSV1 high contained 5000 copies per reaction of HSV-1 DNA, the lines labeled HSV1 low had 50 copies per reaction of HSV-1 DNA, and the lines labeled NTC (non-template control) contained no HSV DNA. All test results shown were performed in duplicate.

get. There is no cross-hybridization or cross-cleavage noise. This is the first report of CPT probes being used in combination with HDA.

Development of IsoGlow HSV Typing Assay on the FireFly Instrument, with Internal Control Determined by Melting Curve Analysis

One of the key advantages of HDA over other isothermal amplification technologies is its ability to use an IC to detect the presence of amplification inhibitors often found in clinical specimens. When such inhibitors are present in a sample collected from an infected patient, assays that lack an IC test could result in a misdiagnosis of the patient; however, when assays that have an IC are performed on samples with inhibitors, the usual result in an invalid test. Indeed, an invalid test is a preferred outcome compared to a false negative result. Performing an HSV typing assay on any commercial real-time PCR instrument is relatively straightforward because these instruments typically have more than four fluorescence detection channels. However, the compact FireFly instrument has only two detection channels such that there is no third channel to accommodate IC detection. To solve the limitations from the device, we devised two strategies to detect the IC: IC determined by melting curve analysis versus IC determined by two-step CPT.

The first strategy was developed based on the capability of melting curve analysis by the device. The IC probe, HSVIC melting probe, was designed as a dual-labeled probe, with a Tm of around 51°C without RNA bases. Therefore, the IC probe cannot bind to the target at the amplification temperature or be cleaved by the RNase H2 enzyme at any temperature. The amplified IC products were detected with the IC probe by performing a melting curve after the 64°C incubation. An example of melting curve analysis of internal control is shown in Figure 2. The reaction mix was optimized and finalized as described in *Materials and Methods*.

The data collection program was designed as follows. Since the dynamic detection range of the TAMRA channel of the device is wider than that of FAM channel, the IC probe uses a TAMRA reporter, the same detector as the HSV-1 probe. Dual-labeled probes have a lower background noise at a lower temperature because of quencher effects when in the random-coil configuration.[14] Therefore, to avoid misclassifications, such as a negative result for a positive result, we maximized the S/N by collecting data at a lower temperature (eg, 35°C) before (N_{35}), and after (S_{35}) a 45-minute amplification at 64°C. The ratios of fluorescence intensities collected from the FAM channel F (S_{35}/N_{35}), and from the TAMRA channel T (S_{35}/N_{35}) were calculated. If the ratio passed a

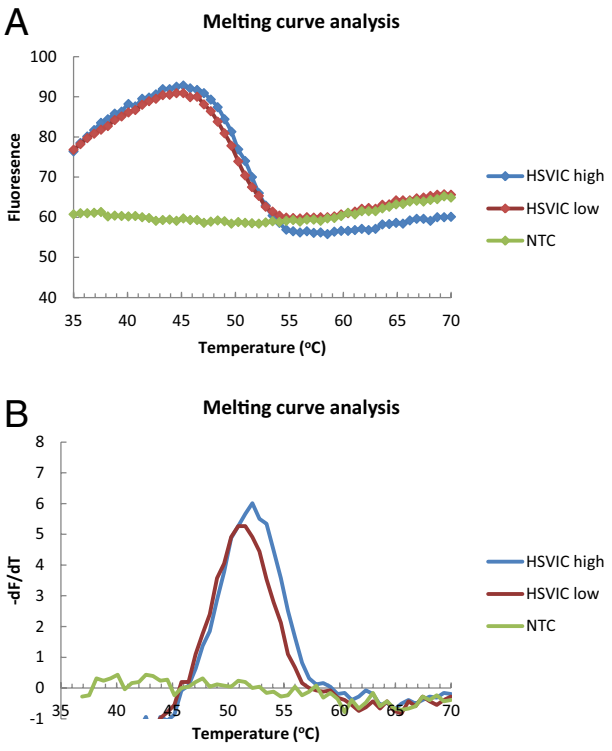

Figure 2. Melting curve-based IC detection. **A:** Fluorescence intensity charted against the temperature of the reaction. **B:** First derivative of the rate of change in fluorescence intensity as a function of the rate of change in temperature (dF/dT) charted against temperature. The Tm of IC probe is approximately 51°C. The line labeled HSVIC high represents an assay performed with 5000 copies of input of HSVIC DNA template in the reaction. The line labeled HSVIC low corresponds to an assay performed with 700 copies of HSVIC DNA template per reaction. In the IsoGlow HSV Typing assay, we add 700 copies of HSVIC DNA template. The line labeled NTC is a non-template control containing no HSVIC DNA.

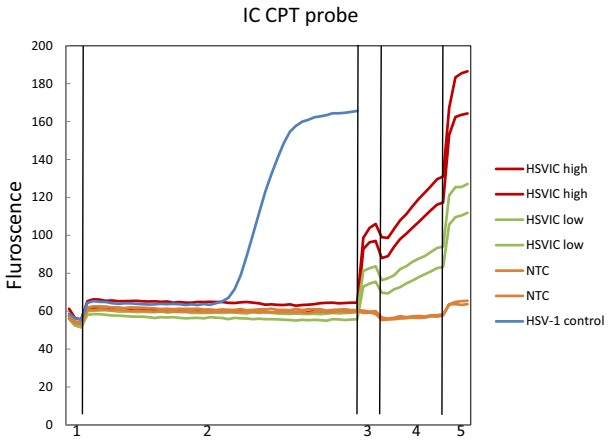

Figure 3. Example of the two-step CPT strategy for detecting the IC. The chart shows the fluorescence intensity of an assay containing 5000 copies/reaction of HSV-1 DNA, labeled HSV-1 control, as well as different copies of internal control at multiple temperatures. The lines labeled HSVIC high and HSVIC low are from assays performed with 5000 copies and 700 copies of the IC, respectively. Data collection stages are divided into five stages. In stage 1, incubation was at 35°C for 1 minute with data collection every 20 seconds. In stage 2, incubation was at 64°C for 45 minutes with data collection every 1 minute. In stage 3, incubation was at 35°C for 1 minute with data collection every 20 seconds. In stage 4, incubation was at 46°C for 10 minutes with data collection every minute. In stage 5, incubation was at 35°C for 2 minutes with data collection every 30 seconds.

cutoff determined with a set of training samples (described below), it was reported as "positive" for the corresponding analyte. Tests that failed to reach the cutoff of both channels were determined by a melting curve analysis at the TAMRA channel. When a detectable fluorescence peak was observed between 47°C and 55°C, a sample was reported as an "HSV negative" sample; otherwise, it was reported as an "invalid test".

To determine the assay cutoff for each target (HSV1, HSV2, IC), a set of training samples were used to evaluate the assay. Serial dilutions of HSV-1 and HSV-2 standards (as low as 30 copies/assay), and equal mixtures of HSV-1 and HSV-2 standards (competitive inhibition study) were used as positive training samples. Samples of dilution buffer or MiliQ water were used as negative training samples. The F (S_{35}/N_{35}) and T (S_{35}/N_{35}) were calculated, and then statistically analyzed. A histogram was used to view the distribution of F (S_{35}/N_{35}) and T (S_{35}/N_{35}) data from a group of known positive and negative samples. The mean and SD were used for cutoff determination based on the data distribution, as described in *Materials and Methods*. From the statistical analysis, the cutoff was determined to be a ratio of 2.5 for both detectors. When there was no HSV target in the sample, only the internal control was amplified with the ratio of T (S_{35}/N_{35}) in the range of 1.5–2. The increased signal was from the probe hybridization to the target only, similar to that of a molecular beacon probe.[14]

The analytical sensitivity study showed that the developed assay has a LoD of 10 copies per assay for either HSV-1 or HSV-2 target. Competitive inhibition was not observed at any of the concentrations tested (from high to as low as 30 copies per assay) when the concentrations of HSV-1 and HSV-2 were equal. However, when HSV-1 was present at more than 500 copies per assay alongside 50 copies of HSV-2, there was competitive inhibition of the amplification of HSV-2. A similar phenomenon was observed for the test with a 10-fold excess of HSV-2 versus HSV-1.

Development of IsoGlow HSV Typing Assay on the Firefly Instrument, Internal Control Is Determined by Two-Step CPT

Using a melting curve to detect the IC adds 20 minutes to the test procedure when it is performed by the FireFly instrument. In contrast, detecting the IC with a CPT probe that binds at a lower temperature can require less time, because the assay can be optimized independent of the device. In the second strategy, the IC was detected using a CPT probe with a lower Tm than that of the HSV target-specific probes. The lower Tm of the probe does not allow it to bind to the IC amplicon during the 64°C incubation of isothermal amplification stage. However, when the incubation temperature is dropped to a lower temperature (eg, 35°C) after amplification, the IC probe can bind to the IC template. The incubation temperature of the reaction is then raised to a higher temperature (eg, 46°C) to facilitate the cleavage of the bound IC probe by the thermostable RNase H2.

To make the two-step CPT detection feasible, the Tm of the IC probe must be at least 10°C below that of the analyte

Table 2. Performance of IsoGlow HSV Assay on 176 Clinical Samples in Comparison to ELVIS Shell Vial Assay: IC Determined by Melting Curve Analysis

	HSV type 1 comparison results			HSV type 2 comparison results		
	Reference method			Reference method		
	Positive	Negative	Total	Positive	Negative	Total
IsoGlow HSV assay						
Positive	36	0	36	36	0	36
Negative	0	140	140	0	140	140
Total	36	140	176	36	140	176
	Value	95% CI		Value	95% CI	
Sensitivity	100%	90.4%–100.0%		100%	90.4%–100.0%	
Specificity	100%	97.3%–100.0%		100%	97.3%–100.0%	

Table 3. Performance of IsoGlow HSV Assay on 176 Clinical Samples in Comparison to ELVIS Shell Vial Assay: IC Determined by Two-Step CPT

	HSV type 1 comparison results			HSV type 2 comparison results		
	Reference method			Reference method		
	Positive	Negative	Total	Positive	Negative	Total
IsoGlow HSV assay						
Positive	36	2	38	36	1	37
Negative	0*	138	138	0	139	139
Total	36	140	176	36	140	176
	Value	95% CI		Value	95% CI	
Sensitivity	100.0%	90.4%−100.0%		100%	90.4%−100.0%	
Specificity	98.6%	94.9%−99.6%		99.3%	96.1%−99.9%	

*One of the HSV1 positive samples showed as close to the cutoff of T (S_{64}/N_{64}). Some tests slightly pass the cutoff, and some tests show as negative.

probe (data not shown). The IC probe, HSVIC CPT probe, has a Tm neighboring 50°C, and contains an RNA base (Table 1). Therefore, the IC probe cannot bind to the target to be cleaved by RNase H2 at the amplification temperature. After amplification, the temperature was lowered to a temperature that matches the melting temperature of the IC probe, or even to a lower temperature (such as 35°C) to increase the probe binding efficiency. Figure 3 shows an example of IC amplification, and detection by the data collection program. It shows that there is no signal from IC probe cleavage during amplification stage, and 10-minute cleavage at 46°C can increase the final signal-to-noise ratio (T (S_{35}/N_{35}). The reaction mix was optimized, and finalized as described in *Materials and Methods*.

Because the FAM channel of the FireFly instrument has a narrow detection range, the S/N is much higher at 35°C than at 64°C. Therefore, the data collection for HSV-2 is performed at 35°C even though there was no other probe sharing the FAM channel. For HSV-2 and the IC, the noise data (N_{35}) was collected at 35°C before amplification, and the signal data (S_{35}) was collected at 35°C after amplification and the IC cleavage step. For HSV-1, the noise data (N_{64}) were collected using the first four readings at 64°C and the signal data (S_{64}) was collected at 64°C using the last 4 readings in the amplification incubation. The ratio of F (S_{35}/N_{35}) and T (S_{64}/N_{64}) were calculated. If the ratios passed the cutoff, it was reported as "positive" for the corresponding detector. Only if none of the ratios passed the determined cutoff, was the ratio of T (S_{35}/N_{35}) considered. If this ratio passed the IC cutoff, it was reported as "HSV negative" sample; otherwise, it was reported as "invalid test."

The cutoff values for the assays using the IC CPT probe were determined using a similar approach as that used for the IC melting probe assays. Moreover, the IC cutoff was verified using blank tests where T (S_{35}/N_{35}) was collected in assays with no internal control template in the amplification mix. The blank tests were performed on clean samples (MiliQ water) or pooled negative clinical samples. Based on the statistical analysis, the cutoff was determined as the following: the cutoff of F (S_{35}/N_{35}) is 2.5, the cutoff of T (S_{64}/N_{64}) is 1.5, and the cutoff of T (S_{35}/N_{35}) is 1.8. Similar analytical sensitivity and competitive inhibition results were observed for this second detection strategy.

Evaluation of Two IC Detection Strategies with Clinical Samples

To compare the clinical performance of the two IC detection strategies, a preliminary clinical study was performed on 176 frozen clinical specimens; ie, oral or genital swabs suspended in viral transport medium. Of the 176 samples, 36 were HSV-1 positive, 36 were HSV-2 positive, and 104 were HSV negative, as determined by the ELVIS HSV test obtained from Diagnostic Hybrids. A total of 116 of the samples were also tested with the FDA cleared, IsoAmp HSV molecular assay. We found complete agreement between the two assays when the melting-curve–based IC detection method was used to detect negative samples with the IsoGlow HSV test.

A summary of the clinical study data are shown in Table 2 and Table 3. Assays using the melting-curve–based IC detection strategy were in 100% agreement with the ELVIS shell vial culture reference method. One of the HSV-1–positive samples showed evidence of either a low copy number input, or the presence of possible amplification inhibiting substances in the sample. Its real-time amplification signal collected at 64°C showed linear amplification instead of the usual exponential increase in fluorescence intensity. As illustrated in Table 4, this positive sample had a T (S_{35}/N_{35}) value of 5.5, which is the smallest of all of the HSV-1–positive clinical samples

Table 4. Summary of Statistical Analysis of the Signal-to-Noise Ratios (s/n) in Clinical Samples: IC Determined by Melting Curve Analysis

Detector	Range	Mean	Standard deviation
36 HSV-1−positive samples, T (S_{35}/N_{35}) cutoff = 2.5			
TAMRA (35°C)	5.5−8.3	7.2	0.5
FAM (35°C)	1.1−1.5	1.2	0.1
36 HSV-2−positive samples, F (S_{35}/N_{35}) cutoff = 2.5			
TAMRA (35°C)	1.3−1.5	1.3	0.1
FAM (35°C)	4.5−8.8	7.2	0.7
104 HSV-negative samples, IC determined by melting curve analysis			
TAMRA (35°C)	1.4−2.4	1.8	0.2
FAM (35°C)	1.0−2.2	1.1	0.2

Table 5. Summary of Statistical Analysis of Signal-to-Noise Ratio (s/n) from Clinical Study: IC Determined by Two-Step CPT

Detector	Range	Mean	Standard deviation
38 HSV-1−positive samples, T (S_{64}/N_{64}) cutoff = 1.5			
TAMRA (64°C)	1.6–3.0	2.5	0.3
FAM (35°C)	1.1–1.5	1.2	0.1
37 HSV-2−positive samples, F (S_{35}/N_{35}) cutoff = 2.5			
TAMRA (64°C)	1.0–1.4	1.1	0.1
FAM (35°C)	6.6–7.6	7.1	0.2
101 HSV-negative samples, T (S_{35}/N_{35}) cutoff = 1.8			
TAMRA (64°C)	1.0–1.4	1.0	0.1
TAMRA (35°C)	1.9–4.8	3.0	0.5
FAM (35°C)	1.0–2.1	1.2	0.2

tested. As shown in Table 5, this sample also showed more variability during repeated tests where the IC was detected by the two-step CPT probe. Indeed, some of the tests were scored as HSV negative whereas others were scored as positive, but with T (S_{64}/N_{64}) of 1.6, which was much lower than the other positive samples. Three of the ELVIS shell vial culture negative samples showed as HSV-1 or HSV-2 positive only by the method with two-step CPT. The corresponding amplification signal at 64°C showed late amplification curve, suggesting they might be HSV positive with low virus titer. Table 6 compares the performance between the IsoGlow HSV and IsoAmp HSV assays on the 116 clinical samples tested by both assays.

Comparing Table 4 and Table 5, there is no obvious difference for the data of HSV-2 positive samples between the two strategies. Table 4 and 5 also shows the distribution of S/N ratios seen in the clinical study. For both methods, the cutoff can clearly separate the positive samples from the negative ones. It fully demonstrates that the proposed decision algorithms based on the initial analytical study is feasible. Although the clinical study is modest in scale, it clearly indicates that either strategy for detecting the IC is feasible, the strategy with melting curve analysis has greater tolerance to amplification inhibitors present in certain clinical samples, because the mean signal-to-noise ratios between positive samples and negative samples are more widely separated.

Discussion

This study is the first to report an HSV typing assay using a portable device with only two fluorescence detection channels. It also uses the unique two-step strategy to detect more analytes than would otherwise be possible if only one analyte were detected with each channel. Although both two-step methods can increase the multiplex capacity of the device, the use of melting curve analysis for IC detection is more robust in the presence of amplification inhibitors. However, the two-step CPT method can be more sensitive in some cases. With the two-step CPT format, the assay signal-to-noise ratio is significantly increased by the combination of exponential nucleic acid amplification and linear signal amplification. So far, the CPT detection format has the best performance for HDA of all evaluated real-time detection formats based on target-specific probes. The optimal improvements on the assay itself dramatically reduce the requirements for instrumentation. Therefore, the simple portable fluorescence reader can be easily applied for HDA amplification and detection. With the IsoGlow platform, the overall process of nucleic acid amplification, detection and reporting can be integrated for automation. This allows it to be applied not only for small set data analysis (ie, FireFly instrument) but also for high-throughput data analysis (ie, 96 or more reactions on any commercially available fluorescence detector).

In summary, the turnaround time for the IsoGlow HSV typing assay has been shortened to approximately 1 hour, with a hands-on time of less than 5 minutes, providing a rapid nucleic acid test for diagnosis of HSV infections without the limitation of expensive instruments. This study demonstrates the feasibility of using HDA combined with end-point analysis on a miniaturized, low-cost fluorescence detector for rapid HSV detection, and typing. The FireFly instrument is just one choice of portable fluorescence detectors. This isothermal assay based on HDA can be adapted to other formats of miniaturized detectors or integrated minisystems, enabling the technology to have a great potential for application in decentralized and/or resource-limited settings.

Acknowledgments

We thank Marcia Degilio, Russell Rawling, Paul Granato (Laboratory Alliance of Central New York, LLC, Liverpool, New York), Debbie Kohn, and Belinda Yen-Lieberman (Cleveland Clinic, Cleveland, OH) for providing clinical samples. We thank Xiaojing Pan and Huyn-jin Kim for providing proteins and buffers.

Table 6. Comparison IsoGlow HSV with IsoAmp HSV Assays with 116 Clinical Samples

	HSV positive*		HSV negative	
	Agreement	95% CI	Agreement	95% CI
IsoGlow HSV, IC determined by melting curve	32/32 (100%)	89.3%−100.0%	84/84 (100%)	95.6%−100.0%
IsoGlow HSV, IC determined by two-step CPT	32/32 (100%)	9.3%−100.0%	81/84 (96.4%)	90.0%−98.8%

*IsoAmp HSV does not provide typing information. The IsoAmp HSV assays are 100% (95% CI = 96.8%–100.0%) agreement with ELVIS shell vial culture assays with these 116 clinical samples.

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