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A rapid and simple isothermal nucleic acid amplification test for detection of herpes simplex virus types 1 and 2

Hyun-Jin Kim[a,1], Yanhong Tong[a,1], Wen Tang[a], Louisito Quimson[b], Vicki A. Cope[c], Xiaojing Pan[a], Aurelie Motre[a], Richard Kong[a], Jian Hong[a], Debbie Kohn[b], Nancy S. Miller[d], Melinda D. Poulter[e], Huimin Kong[a], Yi-Wei Tang[c,*], Belinda Yen-Lieberman[b]

[a] BioHelix Corporation, Beverly, MA 01915, United States
[b] Cleveland Clinic, Cleveland, OH 44195, United States
[c] Vanderbilt University Medical Center, Nashville, TN 37232, United States
[d] Boston Medical Center, Boston, MA 02118, United States
[e] University of Virginia Health System, Charlottesville, VA 22908, United States

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ABSTRACT

Background: A simple and rapid IsoAmp® HSV assay has been developed for qualitative detection of herpes simplex virus (HSV) types 1 and 2 from genital lesions. Sample preparation involved a simple dilution step and the diluted specimens were directly added to the device and amplified by isothermal helicase-dependent amplification (HDA). Amplification products were then detected by a DNA strip embedded in a disposable cassette without any instrument. The total test turn-around time is less than 1.5 h from specimen processing to result reporting.
Objectives: To evaluate the analytical and clinical performance of the IsoAmp® HSV assay as well as the robustness and reproducibility of the assay.
Study design: The analytical sensitivity of the IsoAmp® HSV assay was determined using both HSV-1 and HSV-2. Clinical performance was evaluated using 135 frozen specimens collected from patients with suspected HSV infection in genital area.
Results: The analytical sensitivity of the assays was 5.5 and 34.1 copies/reaction for HSV-1 and HSV-2 respectively with a 95% confidence interval. When the herpes viral culture was used as the reference standard, the clinical sensitivity and specificity of the IsoAmp® HSV assay were 100.0% and 96.3% respectively. The inter-laboratory reproducibility achieved an overall 97.5% agreement by testing a total of 80 blinded HSV-1 samples among five laboratories.
Conclusion: Adequate analytical and clinical performance of the IsoAmp® HSV assay was demonstrated. This assay is simple to perform and has acceptable inter-laboratory reproducibility.

1. Background

Herpes simplex viruses (HSVs) are a common human pathogen found worldwide that cause a variety of diseases, including viral meningitis, skin infection, genital herpes, and neonatal herpes.[1] Neonatal herpes virus infection is often severe, if not fatal.[2] HSV transmission can result from direct contact with infectious secretions from either symptomatic or asymptomatic individuals. HSV has two different types: HSV-1 is generally associated with infections of the oro-pharynx; whereas HSV-2 is primarily associated with genital and neonatal infections.[1] However, data from Europe and Canada showed that more cases of neonatal herpes are associated with HSV-1 infection than HSV-2.[3] In the U.S., about one out of six individuals at 14–49 years of age have had genital HSV-2 infection (http://www.cdc.gov/std/herpes/stdfact-herpes.htm). The proportion of primary genital infections due to HSV-1 is increasing, from 10% in 1983 to 32% in 1995.[4,5]

Conventional and modified cell cultures remain the test of choice to detect HSV in mucocutaneous, genital and ocular lesions. The Enzyme-Linked Virus Inducible System (ELVIS®, Diagnostics Hybrids Inc., Athens, OH) has been widely used in clinical laboratories to detect the presence of HSV-1 or HSV-2.[6–8] The transgenic shell vial cell culture-based test takes a minimum of 16 h for results and it can only be performed in a laboratory setting with cell culture facility. Numerous molecular assays have been reported to

* Corresponding author at: Molecular Infectious Disease Laboratory, Vanderbilt University Hospital, 4605 TVC, Nashville, TN 37232-5310, United States.
Tel.: +1 615 322 0126; fax: +1 615 343 8420.
E-mail address: yiwei.tang@vanderbilt.edu (Y.-W. Tang).
[1] These two authors contributed equally.

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Table 1
HDA primers and probes.

Primers and probes	Sequences and modifications[a]
GBF1	5′-TTCAAGGAGAACATCGCCCCGTACAA-3′
Bio-GBR1	5′-Biotin-TAAACTGGGAGTAGCGGTGGCCGAAC-3′
HSV-FI	5′-ATGTACTACAAAGACGT-FAM-3′
HSV-Dig	5′-ATGTACTACAAAGACGT-digoxN-3′
HSVIC-Dig	5′-CAAAGAGTCCGCAC-digoxN-3′

[a] FAM, 6-carboxyfluorescien; digoxN, digoxigenin NHS ester.

detect and differentiate HSV-1 and HSV-2 with high sensitivities and specificities.[7–9] Recently, a PCR-based test for the direct detection of HSV-1 and HSV-2 from vaginal lesion specimens was cleared by the Food and Drug Administration.[8] However, the MultiCode® HSV-1&2 assay requires extraction of nucleic acid from the specimen and expensive real-time PCR instrumentation.

Helicase-dependent amplification (HDA) is an isothermal nucleic acid amplification method that relies on the use of a DNA helicase to unwind double-stranded nucleic acids.[10,11] The amplified DNA can then be applied on to a vertical-flow strip embedded in a disposable cassette for rapid detection of the organism. This method has been used for the rapid amplification and detection of methicillin-resistant *Staphylococcus aureus* in positive blood cultures,[12] *Clostridium difficile* in stool specimens from hospitalized patients with diarrhoea[13] and HIV-1 in plasma in low-resource settings.[14]

2. Objectives

Develop a user friendly, rapid isothermal nucleic acid amplification test (IsoAmp® HSV assay) for the detection of HSV-1 and HSV-2 on samples collected from genital lesions. Evaluate the performance characteristics of IsoAmp® HSV assay including analytical sensitivity and specificity, clinical sensitivity and specificity as well as intra- and inter-laboratory reproducibility.

3. Study design

3.1. IsoAmp® HSV assay development

A pair of primers, GBF1 and Bio-GBR1, was used to amplify the HSV glycoprotein B (gB) gene. Primer Bio-GBR1 was labeled with biotin at its 5′ end. To generate biotin-labeled single-stranded amplicon for probe hybridization, asymmetric HDA was performed with a primer ratio of 90 nM for primer Bio-GBR1 to 30 nM for primer GBF1. Probes for the HSV target, HSV-FI and HSV-Dig, were labeled with 6-carboxyfluorescien (FAM) and digoxigenin NHS ester (digoxN) at their 3′ ends respectively. A competitive internal control (IC) was developed to monitor specimen inhibition and reaction failure. The same primer pair (GBF1 and Bio-GBR1) was used to amplify the IC sequence and the IC amplicon which was detected by probe HSVIC-Dig labeled with digoxN at its 3′ end. All oligonucleotides were purchased from Integrated DNA Technology, Inc. (Coralville, IA). The HDA primers and probes used in this study are listed in Table 1.

3.2. IsoAmp® HSV assay procedure

HDA reactions were performed using an IsoAmp® HSV assay (BioHelix Corporation, Beverly, MA) including three steps. (1) Sample-preparation – 25 μl of viral transport medium containing genital lesion swab specimens was diluted with 1 mL dilution buffer. After mixing, 25 μl of each diluted specimens was transferred to a 0.2 mL thin-wall Amplification tube (Fig. 1). (2) Isothermal amplification – The master mix was prepared by mixing

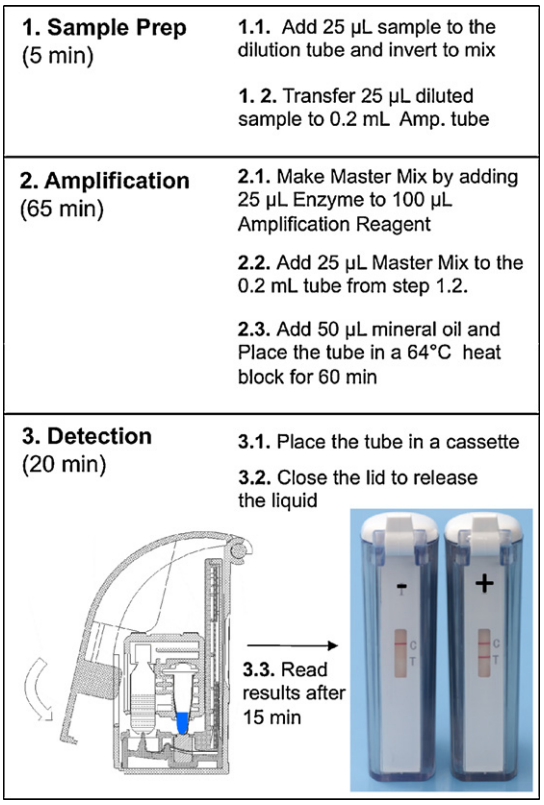

Fig. 1. IsoAmp® HSV assay workflow.

25 μl of enzyme reagent with 100 μl of Amplification Reagent prior to use. 25 μl of master mix was then added into 25 μl diluted sample in each Amplification tube followed by adding 50 μl of mineral oil. The Amplification tube was placed in a 64 °C heat block for 60 min to amplify the target DNA. (3) Instrument-free detection – After the amplification the 0.2 mL Amplification tube was placed in an amplicon cartridge of a Type II BESt™ Cassette (BioHelix Corporation, Beverly, MA) for amplicon detection.[12–14] Closing the cassette-lid forced the tube to release amplicon into a vertical-flow DNA strip for colorimetric detection without cross-contamination. The assay result can be visibly read by viewing through the detection window after 10–15 min (Fig. 1).

3.3. Viral cultures

Viral cultures for HSV were performed using Enzyme-Linked Virus Inducible System (ELVIS®) shell vial assay (Diagnostic Hybrid Inc. Athens, OH). ELVIS® HSV uses a genetically engineered host cell line (transgenic baby hamster kidney cells) that was provided as fresh confluent monolayer cultures. The BHK cells contain the promoter sequence for HSV which can be activated only by viral proteins produced by HSV type 1 or 2.[15] This viral promoter controls LacZ, the *Escherichia coli* gene for galactosidase which serves as a reporter molecule in the ELVIS HSV assay system. When an ELVIS cell is infected with HSV present in a patient sample, the reporter enzyme quickly accumulates inside of the infected cells and catalyzes the reporter to produce intense blue color after cells are fixed and the ELVIS staining buffer is added. Results can be read with an inverted microscope per the manufacturer's protocol.

3.4. Analytical sensitivity analysis

HSV-1 (9.0×10^{10} viral-particle/mL) and HSV-2 (1.6×10^{11} viral-particle/mL) viral stocks (Advanced Biotechnologies Inc, Columbia,

Table 2
Limit of detection determination of IsoAmp® HSV for HSV-1/2 detection.[a]

HSV concentration (copies/assay)	HSV-1			HSV-2		
	Number tested	Number positive	Positive rate (%)	Number tested	Number positive	Positive rate (%)
50	20	20	100.0	20	19	95.0
25	20	20	100.0	20	17	85.0
12.5	20	20	100.0	20	17	85.0
6	20	20	100.0	20	10	50.0
3	20	15	75.0	20	8	40.0
0	10	0	0.0	10	0	0.0

[a] The result of LoD studies was analyzed using Probit analysis. The LoD for HSV-1 was determined to be 5.5 copies/test. At this concentration, IsoAmp HSV assay can detect 95% of the samples with a 95% confidence interval (CI) of 4.0–9.1 copies/test. The LoD for HSV-2 was determined to be 34.1 copies/test with a 95% CI of 21.9–60.8 copies/test.

MD) were first diluted 1000-fold to 9.0×10^7 viral-particle/mL and 1.6×10^8 viral-particle/mL. The viral concentrations of the primary dilutions were determined as 5.9×10^7 copies/mL for HSV-1 and 1.3×10^8 copies/mL for HSV-2 by real-time qPCR using HSV-1 or HSV-2 specific primers and quantified HSV-1 or HSV-2 viral DNA (Advanced Biotechnologies Inc, Columbia, MD) as a standard. Serial dilutions of each HSV type, which were prepared from the primary dilutions in pooled HSV-negative clinical samples, were repeatedly tested in IsoAmp® HSV assay to generate hit rates at each concentration. The limit of detection (LoD) was calculated using Probit analysis (Minitab Inc, State College, PA).

3.5. Analytical specificity analysis

The potential cross-reactivity of the IsoAmp® HSV assay was evaluated in the presence of genomic DNA or microorganisms. Genomic DNAs were purchased from Advanced Biotechnologies Inc (Columbia, MD) including varicella-zoster virus (VZV), *Chlamydia trachomatis*, *Toxoplasma gondii*, human papillomavirus (HPV)-16, and HPV-18. Microorganisms were purchased from American Type Culture Collection (ATCC) (Manassas, VA) including *Trichomonas vaginalis* (ATCC 30001), *Mobiluncus mulieris* (ATCC 35240), *Candida albicans* (ATCC 14053), *Neisseria gonorrheae* (ATCC 21823) and *Gardnerella vaginalis* (ATCC 14018 cells). The analytical specificity was tested at concentrations ranging from 1.2×10^3 to 2.5×10^6 copies/reaction (genomic DNA) or CFU/reaction (microorganisms).

3.6. Intra- and inter-laboratory reproducibility

Five laboratories participated in the inter-laboratory reproducibility study, including four clinical laboratories plus BioHelix. A panel of blinded samples was prepared at BioHelix by diluting inactivated HSV-1 virus (Zeptometrix, Buffalo, NY) with M4 viral transport medium. Four different viral concentrations were made: (1) high (450 copies/reaction); (2) medium (150 copies/reaction); (3) low (50 copies/reaction); and (4) blank (0 copy/reaction test). Each laboratory tested each concentration four times in a five-day period plus positive and negative controls.

Table 3
Performance of IsoAmp® HSV assay on 135 clinical specimens in comparison to the ELVIS shell vial assay.

	Reference method		Total
	Positive	Negative	
IsoAmp® HSV			
Positive	60	5	65
Negative	0	70	70
Total	60	75	135
Sensitivity	100%		
Specificity	96.3%		

3.7. Tolerance for variation

To test the tolerance for variation of the HSV assay, each critical component (i.e. dNTPs, Mg^{2+}, primers, etc.) was tested at three concentrations, the target concentration, 10–30% higher, and 10–30% lower. IsoAmp HSV assays were carried out with three concentrations of the testing component on three substrates, HSV-1 virus (3× LoD), HSV-2 virus (3× LoD), and internal control (IC) in 6 replicates for each condition. If all 6 replicates pass the variation tolerance test for a critical component, the tolerance range for this critical component is verified. If 1 (or more) reactions fail, the tolerance range will be reduced and the experiment will be repeated until it passes the narrowed test range.

4. Result

4.1. Robustness of the IsoAmp® HSV assay

The robustness of the IsoAmp® HSV RUO kit was evaluated by testing the HSV-1 assay positive control (a single-copy HSV-1 gB gene cloned in a plasmid), HSV-2 assay positive control (a single-copy HSV-2 gB gene cloned in a plasmid), and negative control (TE buffer) in 100 replicates. All 100 replicates per each HSV-1 (150 copies/reaction) and HSV-2 assay positive controls (300 copies/reaction) showed positive test results. Out of 100 replicates tested for negative control, 99 replicates showed negative test results and one tested false-positive. Therefore, the invalid rates of IsoAmp® HSV assay for positive and negative assay controls were 0% and 1%, respectively.

We also evaluated the IsoAmp® HSV assay on tolerance for variation in critical components and procedure. The variation tolerance study showed that the assay can tolerate minimal at 10% variation on dNTPs, $MgSO_4$, primers, probes concentrations, 20% variation on enzymes, and 30% variation on IC control and positive controls based on the observations that the HSV assay was able to detect both HSV-1 and HSV-2 at three times the LoD in the presence of these variations. The assay performed well with 10% variation in assay procedures including diluted sample input, enzyme reagents input and ±1 °C incubation temperature variation.

4.2. Analytical sensitivity and specificity

For HSV-1, virus dilutions were tested in IsoAmp® HSV assay at 50, 25, 12.5, 6 and 3 copies/assay. Each run tested the HSV-1 in 5 different concentrations in duplicates as well as one negative and positive control each. A total of 20 replicates were tested per each concentration. However, the positive rate was too high to estimate the LoD in the first series dilution experiment (Table 2). Therefore, the LoD study was repeated with lower concentrations of HSV-1 (6, 3, 1.5, 0.8 copies/reaction) and hit rates from 83% for the 6 copies/reaction to 17% for the 0.8 copies/reaction were observed. Probit analysis on combined data on HSV-1 showed that the ana-

Table 4
Inter-laboratory reproducibility of HSV-1 detection.

HSV-1 concentration[a]	Number tested	Site 1	Site 2	Site 3	Site 4	Site 5
High	20	4 (100.0)[b]	4 (100.0)	4 (100.0)	4 (100.0)	4 (100.0)
Medium	20	4 (100.0)	4 (100.0)	4 (100.0)	4 (100.0)	3 (75.0)[c]
Low	20	4 (100.0)	4 (100.0)	4 (100.0)	4 (100.0)	4 (100.0)
Blank (matrix only)	20	4 (100.0)	4 (100.0)	4 (100.0)	4 (100.0)	3 (75.0)[d]

[a] High, medium, low, and matrix only indicated 450, 150, 50, and 0 copies/reaction of HSV-1 concentration respectively. Each run included 2 positive and 2 negative controls.
[b] Number positive (%).
[c] One was detected indeterminate.
[d] One blank matrix was tested positive.

lytical sensitivity for HSV-1 was estimated at 5.5 copies/test with a 95% confidence interval (CI) between 4.0 and 9.1 copies/test. Similar experiments conducted with IsoAmp® HSV assay on HSV-2 showed an estimated LOD at 34.1 copies/test with a 95% CI between 21.9 and 60.8 copies/test (Table 2).

No cross-reactivities were observed when VZV (4×10^5 copies/reaction), *C. trachomatis* (3.6×10^5 copies/reaction), *T. gondii* (6.1×10^3 copies/reaction), HPV-16 (6.1×10^3 copies/reaction), HPV-18 (1.2×10^3 copies/reaction), *T. vaginalis* (2.5×10^6 CFU/reaction), *M. mulieris* (2.5×10^6 CFU/reaction), *C. albicans* (6.1×10^3 CFU/reaction), *N. gonorrheae* (6.1×10^3 CFU/reaction) and *G. vaginalis* (6.1×10^3 CFU/reaction) were tested by the IsoAmp® HSV assay.

4.3. Clinical sensitivity and specificity

One hundred and thirty-five un-linked frozen specimens collected by swabs (Microbiology Lab, Cleveland Clinic) from lesions on patients with suspected HSV infection in genital area were tested with IsoAmp HSV assay. These samples consisted of 60 positive and 75 negative samples determined by HSV culture method (ELVIS® shell vial assay; DHI, Athens, OH). IsoAmp® HSV showed a 100% sensitivity and 96.3% specificity when compared to the reference method (Table 3).

4.4. Inter-laboratory reproducibility

A total of 80 samples spiked with different concentrations of HSV-1 virions were tested in five sites (blinded). One site reported an indeterminate result on medium HSV-1 concentration specimen which probably was due to detection cassette failure. The same site also reported one false positive on a negative matrix. An overall 97.5% agreement was observed by testing a total of 80 blind-coded HSV-1 samples between five laboratories (Table 4).

5. Discussion

The IsoAmp® HSV assay for genital herpes combines a simple sample-prep (dilution-only), an isothermal HDA amplification, and a self-contained disposable amplicon detection device for rapid diagnosis of HSV-1 and HSV-2. As it does not require any special instrumentation, or complicated specimen handling, the assay enables more laboratories to perform rapid nucleic acid test for diagnosis of HSV infections without culture or expensive real-time PCR instrumentation. This study showed that the IsoAmp® HSV assay has 100% clinical sensitivity and 96.3% specificity for the detection of HSV from genital herpes when compared to the reference culture method. Good inter-laboratory reproducibility was observed in five sites with all positive and negative controls worked as expected. Based on the analytical sensitivity data, the IsoAmp® HSV assay demonstrated significantly lower LoD for HSV-1 (5.5 copies/test) than that of HSV-2 (34.1 copies/test). This LoD difference (HSV-1 from HSV-2) is probably caused by variation in the gB sequence since there are three single nucleotide polymorphisms (SNPs) between the two primers. Other factors such the accuracy of the quantification method may also contribute to the difference of the LoDs between HSV-1 and HSV-2.

The preliminary evaluation of the IsoAmp® HSV assay on clinical samples showed a sensitivity of 100%. The IsoAmp® assay also detected 5 culture-negative samples. Further investigation revealed that these 5 IsoAmp-positive/culture-negative samples were repeatedly tested positive by nucleic acid amplification test on another HSV target sequence (UL30, DNA polymerase). It is likely that IsoAmp® HSV assay has higher detection sensitivity than ELVIS® culture and therefore it was able to detect low-viral load and/or non-viable herpes simplex viruses. No cross-reactivities were observed when other related microorganisms including VZV, *C. trachomatis*, *T. gondii*, HPV-16, HPV-18, *T. vaginalis*, *M. mulieris*, *C. albicans*, *N. gonorrheae* and *G. vaginalis* were tested by the IsoAmp® assay.

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Ethical approval

This study received approval from the institutional review boards in all participant sites.

Conflict of interest

Hyun-Jin Kim, Yanhong Tong, Wen Tang, Xiaojing Pan, Aurelie Motre, Jian Hong, and Huimin Kong are employees of BioHelix Corporation, the commercial manufacturer of the IsoAmp® HSV assay. Yi-Wei Tang is a scientific advisory board member of BioHelix Corporation.

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