EXPERT REVIEWS

Near instrument-free, simple molecular device for rapid detection of herpes simplex viruses

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The first near instrument-free, inexpensive and simple molecular diagnostic device (IsoAmp® HSV, BioHelix Corp., MA, USA) recently received US FDA clearance for use in the detection of herpes simplex viruses (HSV) in genital and oral lesion specimens. The IsoAmp HSV assay uses isothermal helicase-dependent amplification in combination with a disposable, hermetically-sealed, vertical-flow strip identification. The IsoAmp HSV assay has a total test-to-result time of less than 1.5 h by omitting the time-consuming nucleic acid extraction. The diagnostic sensitivity and specificity are comparable to PCR and are superior to culture-based methods. The near instrument-free, rapid and simple characteristics of the IsoAmp HSV assay make it potentially suitable for point-of-care testing.

Keywords: genital lesion • helicase-dependent amplification • herpes simplex virus • isothermal nucleic acid amplification • point-of-care testing • sexually transmitted disease • vertical flow test strip

Market forces are driving a transformation in molecular diagnostics towards low-cost, low-throughput systems for on-demand, near-patient testing. This transformation is driven by the need for faster turnaround time nucleic acid amplification test (NAAT) systems to screen patients for colonization by methicillin-resistant *Staphylococcus aureus* (MRSA). This need is driving some hospitals to adopt lower-throughput, automated instrument platforms such as the GeneXpert® (Cepheid, CA, USA) on site rather than sending out tests to reference laboratories. In the veteran administration system, rapid turnaround MRSA screening is credited with lowering healthcare-associated MRSA infections by 59% [1]. Other PCR-based NAAT platforms are being developed for modest throughput laboratories (e.g., IQuum's LIAT™ technology and Idaho Technology's FilmArray™ technology) [2,3]. Interestingly, the performance of the FilmArray multiplex assay was demonstrated to be superior to the more traditional multiplex assay xTAG RVP (Luminex Corporation, Toronto, Canada) [4,5]. Integrated molecular test platforms will aid physicians in getting a differential diagnosis of influenza infections more rapidly to reduce the threat of an epidemic. However, these alternative US FDA-cleared platforms remain costly compared with nonmolecular diagnostic test systems. For example, published list prices for the FilmArray are $49,500 for the instrument and US$129/test for the device. In this article, we review helicase-dependent amplification (HDA) and other isothermal NAAT systems that have also recently been FDA cleared that bring the cost of NAAT in line with other point-of-care testing technologies.

Methodology

A nearly instrument-free isothermal NAAT platform called IsoAmp® HSV (Biohelix Corp., MA, USA) was recently cleared by the FDA [6,7]. IsoAmp technology is based on thermophilic HDA chemistry (tHDA) (Figure 1) [8]. As tHDA uses Bst DNA polymerase, an enzyme more tolerant to amplification inhibitors than the Taq polymerase used in PCR-based platforms, much simpler sample processing work-flows can be used in IsoAmp assays than in high-throughput molecular diagnostic systems relying on PCR. HDA uses only two oligonucleotide primers to initiate the exponential DNA synthesis needed to reach high analytical sensitivity. In addition, by using a different ratio of the two amplification primers, HDA can yield a surplus of one of the two strands of

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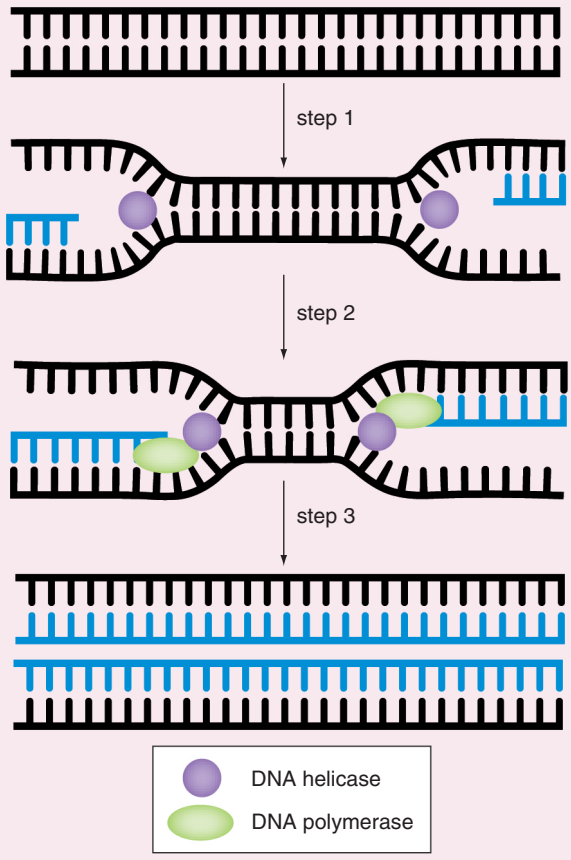

Figure 1. Helicase-dependent amplification mechanism. In step 1, the helicase enzyme loads on to the template and traverses along the target DNA, disrupting the hydrogen bonds linking the two strands. Exposure of the single-stranded target region by helicase allows primers to anneal. In step 2, the DNA polymerase extends the 3′ ends of each primer using free deoxynucleotides to produce two DNA replicates. In step 3, the two replicated DNAs independently enter the next cycle of helicase-dependent amplification, resulting in exponential amplification of the target sequence.

DNA, such as to allow for the use of hybridization probes to confirm the legitimacy of the NAAT amplification products without a denaturation heating step. By enabling the use of probes under isothermal conditions (65°C), HDA allows for the inclusion of competitive internal controls (CIC) in its assays. The CIC is a spike-in-template DNA that shares the same primer binding sequence as the target sequence, but has a different internal sequence such that it can be detected with a separate probe in BioHelix-designed NAAT. The use of a CIC allows test operators to distinguish between true negative assay results from invalids owing to the presence of amplification inhibitors found in some clinical specimens. The IsoAmp technology offers an additional advantage by performing on simply-diluted crude specimens, which significantly shortens the test-to-result time to within 1.5 h.

The diagnostic platform developed by BioHelix for the IsoAmp herpes simplex virus (HSV) test uses a vertical flow test strip to detect amplification products. The primer present in excess in the reaction mix has a 5′ end biotin-label, while the probes have 3′ end-labels to prevent extension during DNA synthesis (i.e., either fluorescein [FITC] or digoxigenin [DIG]) [6]. The probe–amplicon complex formed by the hybridization of the probe to the biotin-labeled single-stranded DNA is detected through sandwich immunoassays using anti-FITC, anti-DIG and anti-DNP antibodies striped onto a vertical flow test strip, and a streptavidin–latex conjugate applied to the test strip. The CIC amplicon is detected by a 3′-end DIG-labeled probe while the HSV amplicon is detected by a 3′-end FITC-labeled probe. The cartridge (i.e., BESt™ cassette) contains a knife that cuts the amplification tube when the cartridge is closed, such that users will not contaminate their laboratories with amplification products [9,10]. Lateral flow is an established method in point-of-care testing, but the integration of isothermal amplification, a closed cartridge, and vertical-flow detection represents an innovative use of this technology.

Sensitivity & reproducibility

The analytical sensitivity and specificity of HDA is comparable to that of PCR. A significant number of publications reporting the performance of HDA assays have appeared recently [6,7,9–16,101]. Table 1 lists the analytical sensitivities of some of these tests performed using the IsoAmp platform, as well as the analytical sensitivities of tests currently under development for commercial release. The analytical sensitivity reached by most HDA assays is comparable to that reached by other commercial molecular tests. In addition, reverse transcription HDA is faster with RNA targets than with DNA targets because a linear amplification of repeated cDNA synthesis off the RNA takes place in parallel with an exponential amplification of the DNA [12].

The limit of detection of the IsoAmp HSV test was determined using two representative strains of HSV-1 (McIntyre & HF) and HSV-2 (G & MS) that were serially diluted to five concentrations, and tested in replicates of ten using three reagent lots. The concentration of the virus particles, expressed in 50% tissue culture infective dose ($TCID_{50}$) in the 510(k) summary, were 1.1 × 105 for HSV-1 and 1.1 × 104 for HSV-2, while the limits of detection were expressed in copies/assay [6]. The 95% CIs for detecting the aforementioned $TCID_{50}$ quantities of HSV-1 and HSV-2 were 88.65–100.00% and 88.65–100.00%, respectively (510[k] summary K111951). $TCID_{50}$ values are innately more variable in that they measure viable virus quantities, while nucleic acid amplifications measure total DNA load in a sample. Using the sample work flow illustrated in Figure 2 and the analytical sensitivity in Table 1, we estimate the sensitivity of the IsoAmp HSV assay at 8800 viral particles/ml for HSV-1, and at 54,560 viral particles/ml for HSV-2. The sensitivity data was confirmed with 20 isolates of HSV-1 and HSV-2, and interlaboratory reproducibility of IsoAmp HSV achieved an overall 97.5% agreement by testing a total of 80 blinded HSV-1 samples among five laboratories [6].

Clinical profile

The clinical performance of the IsoAmp HSV was compared with that of the ELVIS® HSV ID/Typing Test System (Diagnostic Hybrid, Inc., OH, USA) [17,18]. The sample work flow recommended

Table 1. Analytical sensitivity and speed of helicase-dependent amplification assays under development at BioHelix.

IsoAmp® Test	IsoAmp analytical sensitivity†	Other test analytical sensitivity	Incubation duration (min)	Development stage	Ref.
HSV	5.5 HSV-1, 34.1 HSV-2	23 HSV-1, 84 HSV-2	60	US FDA cleared 2011	[6,7]
C. diff	20	460	60	FDA submission 2012	[11]
TB	2	500	30	Research use only	[15]
GBS	10	750	30	FDA submission 2013	[Tong Y *et al.*, Unpublished Data]
NG	20	362	30	CE release 2012	[16]
CT	1	20	30	CE release 2012	[Tong Y *et al.*, Unpublished Data]
MRSA	50	250	60	FDA submission 2012	[9]
HIV	50	40	60	Research use only	[10]
Malaria	50	NA	60	Research use only	[Li Y *et al.*, Unpublished Data]

†Copies per reaction as determined by quantitative culture (GBS, NG and MRSA) or quantitative PCR (HSV, *C. diff*, TB, CT, HIV and malaria) of stocks.
C. diff: *Clostridium difficile*; CT: *Chlamydia trachomatis*; GBS: *Streptococcus agalactiae*; HSV: Herpes simplex virus; MRSA: Methicillin-resistant *Staphylococcus aureus*; NA: Not applicable; NG: *Neisseria gonorrhoeae*; TB: *Mycobacterium tuberculosis*.

in the package insert is illustrated in Figure 2. This workflow was validated on several universal viral transport media from Remel, Bartels and Becton, Dickinson and Co. The workflow consists of diluting 25 µl of the viral transport media 40-fold in a dilution buffer containing the CIC, transferring 25 µl of the dilute samples and 25 µl of 2X Amplification Mastermix to a 0.2-ml amplification tube, heating to 64°C for 60 min, after which the tube was placed in the BESt cassette according to the instructions supplied by the manufacturer (IsoAmp® HSV Assay package insert. BioHelix Corp., MA, USA [2011]). The result of the test is read 15 min after the cassette is closed. Table 2 summarizes the clinical performance data obtained with both oral and genital lesion swabs collected from 994 patients using the IsoAmp HSV, and ELVIS HSV ID/Typing Test System. These samples consisted of 962 prospective specimens (803 genital and 159 oral) and 32 retrospective specimens (15 genital and 17 oral) collected and tested at Boston Medical Center (MA, USA), the Cleveland Clinic (OH, USA), the University of Virginia Health System (VA, USA), Vanderbilt University Medical Center (TN, USA) and the Laboratory Alliance of Central New York (NY, USA). Among the prospective samples, 309 specimens were positive and 593 specimens were negative by both methods. A total of 60 samples gave discrepant results that were resolved using bidirectional DNA sequencing of PCR amplicons. HSV was detected in 42 of the 49 discordant IsoAmp-positive/ELVIS-negative samples. By contrast, bidirectional sequencing of discordant IsoAmp-negative/ELVIS-positive samples detected HSV in only five of 11 samples. When the results of this discrepant sample analysis are taken into consideration, the sensitivity and specificity of IsoAmp HSV was 98.6 and 98.8%, while ELVIS was 88.2 and 99%, respectively [7].

Cost–effectiveness

Currently, the most widely used method for detecting HSV in oral and genital swab samples is ELVIS HSV ID/Typing Test System. As this method requires the implementation of shell vial culture in the client laboratory, most hospitals typically send swabs collected from suspected HSV-infected lesions to reference laboratories [17–19]. The typical charge for this service is near the Center for Medicare Services 2011 reimbursement for HSV virus isolation and typing (i.e., CPT code 87255 is $47.66, and CPT 87273 and 87274 are $16.88, respectively), leaving no profit for the hospital sending the test to the reference laboratory for culture. By contrast, the IsoAmp HSV assay is priced the same as other 'free standing' cGMP manufactured nucleic acid reagents (i.e., excluding reagent lease equipment rental costs), and the $200 dry block incubator is provided free of charge to clients. In view of the minimal hands on time of the assay ($7 in estimated cost of labor), low instrument amortization cost ($0), and the average cost of validated nucleic acid amplification analyte-specific reagents excluding instrument amortization (~$25) [20], adoption of the FDA-cleared IsoAmp HSV test by laboratories that currently send out HSV testing would turn HSV into a profit center, assuming the CPT code for HSV molecular testing by amplification using a probe is applied (CPT 87529 is $49.39).

Alternative tests

Table 3 lists the current FDA-cleared tests for detecting HSV in oral or genital lesions. ELVIS HSV ID is a shell vial-based diagnostic test that uses genetically engineered baby hamster kidney cells that express β-galactosidase when infected with either HSV-1 or HSV-2 [17–19]. Infection by HSV also results in the formation of HSV-type-specific proteins that can be detected microscopically using fluorescent labeled HSV-type-specific antibodies. The turnaround time for the ELVIS HSV ID is 1020 min (17 h) compared with 75 min for the IsoAmp HSV test. When the ELVIS HSV ID test is sent out to a reference laboratory that performs shell vial culture, the difference in turnaround time can be even greater.

The sensitivity of molecular tests for HSV detection in lesions is clearly superior to that of ELVIS based culture (i.e., all are

Table 2. A clinical performance of the IsoAmp® HSV test versus ELVIS® HSV ID/typing test system.

Swabs	Assay result	Reference result			n/N sensitivity, % (95% CI)	n/N specificity, % (95% CI)
		POS	*NEG*	*Total*		
Genital lesion swabs						
IsoAmp® HSV Assay†	POS	264	35‡	299	264/272: 97.1% (94.3–98.5%)	496/531: 93.4% (91.0–95.2%)
	NEG	8§	496	504		
	Total	272	531	803		
Oral lesion swabs						
IsoAmp HSV Assay†	POS	45	14¶	59	45/48: 93.8% (83.2–97.9%)	97/111: 87.4% (79.9–92.3%)
	NEG	3#	97	100		
	Total	48	111	159		

†Data taken from 510(k) summary # K111951?.
‡Thirty five samples were tested using bidirectional sequencing analysis. Sequence analysis detected HSV target in 29 of the 35 discordant samples (six HSV-1, 23 HSV-2) identified as HSV-positive by the IsoAmp® HSV Assay (Biohelix Corp.). Sequence analysis did not detect HSV in six of the discordant samples.
§Eight samples were tested using bidirectional sequencing analysis. Sequence analysis did not detect HSV target in four of the eight samples identified as HSV-negative by the IsoAmp HSV Assay. Sequence analysis did detect HSV in four samples (two HSV-1, two HSV-2).
¶Fourteen samples were tested using bidirectional sequencing analysis. Sequence analysis detected HSV target in 13 of the 14 discordant samples (12 HSV-1) identified as HSV-positive by the IsoAmp HSV Assay. Sequence analysis did not detect HSV in one of the discordant samples.
#Three samples were tested using bidirectional sequencing analysis. Sequence analysis did not detect HSV target in two of the three samples identified as HSV-negative by the IsoAmp HSV Assay. Sequence analysis did detect HSV in one of the discordant samples.
HSV: Herpes simplex virus; NEG: Negative; POS: Positive.

greater than 98% while ELVIS is 83.7% when discrepant results are resolved with bidirectional sequencing or PCR) (Table 3). The MultiCode-RTx HSV 1 & 2 (Eragen Biosciences) [21] and PROBETEC HSV (Becton Dickinson) are the FDA-cleared NAAT-based diagnostic alternatives to the IsoAmp HSV assay. The MultiCode-RTx test is cleared for vaginal lesion swab specimens from symptomatic female patients as an aid in the diagnosis of genital herpes infection requiring a 2-h nucleic acid extraction procedure before PCR amplification. PROBETEC HSV is a strand displacement amplification assay used with the Becton Dickinson Viper™ system. This assay is cleared for clinician-collected external anogenital lesion specimens to help diagnose symptomatic patients with HSV1 and HSV2 infections. Incorporated in the automatic Viper platform, it is designed for high-throughput laboratories while IsoAmp and MultiCode-RTx cater to lower throughput needs of 8–20 samples per day. With the turnaround time of 2.5 h, the PROBETEC HSV can be centralized in reference laboratories with hundreds of specimens batched. Although the IsoAmp HSV assay does not differentiate between subtypes HSV-1 and HSV-2, it is cleared for detecting HSV in both male and female oral and genital lesions.

Conclusion

The simple sample work flow recommended in the IsoAmp HSV package insert is a major contributor to its rapid turnaround time, and factors in the cost–effectiveness of the platform for moderate and low-throughput laboratories. The fact that the FDA categorized the IsoAmp HSV test as having a moderate degree of complexity will allow for its implementation in smaller hospitals such that these institutions will no longer have to send HSV testing out to reference laboratories. The analytical sensitivities of a wide range of HDA assays are comparable to those reported for other molecular tests. The use of probes and CIC templates in HDA tests allow for both the moderate multiplex detection of analytes [22], as well as the detection of nucleic acid amplification inhibitors that are often present in clinical specimens [11]. The aforementioned characteristics make the IsoAmp platform ideal for infrequent tests in sentinel hospitals and clinics that do not want to invest in costly instrumentation.

Expert commentary

The IsoAmp HSV test is part of a new trend in molecular diagnostics towards near instrument-free and simple sample-preparation workflows. This trend will continue in the near future as cost–benefit considerations gain greater importance among hospital administrators. We anticipate that the spread of more cost-effective molecular tests with simple sample processing workflow will be focused on detecting infectious diseases that are present in abundance in clinical specimens such as to negate the need for extensive nucleic acid extraction. In addition, these assays will tend to target repeated sequences in the pathogen's genome in order to maximize sensitivity using the aforementioned minimally processed

Table 3. Alternative US FDA-cleared herpes simplex virus tests for genital or oral lesions.

Test	Analytical sensitivity† HSV-1 ($TCID_{50}$)	Analytical sensitivity† HSV-2 ($TCID_{50}$)	Clinical specificity HSV-1 and -2 (%)	Clinical sensitivity HSV-1 and -2 (%)	Use label sample type†	Turnaround time‡ (h)	Instrument footprint
IsoAmp® HSV	1.1 × 105	1.1 × 104	98.8§	98.6§	Both genital and oral, men and women, no typing	1.25	0.07 cubic feet
MultiCode® HSV	2 × 103	6.4 × 101	99.2§	99.4§	Only vaginal, typing	4	10.5 cubic feet
Probetec™ HSV	2.5 × 101	2.6 × 102	98.1§	99§	Anogenital men and women, typing	2.5	16.5 cubic feet
ELVIS® HSV	8.5	8	98.8¶	83.7¶	Both genital and oral, men and women, typing	17	Basic cell culture facility is needed

†Analytical sensitivity and use label taken from 510(k) summaries # K091753 for ELVIS HSV, K103798 for Probetec HSV, K100336 for MultiCode HSV and K111951 for IsoAmp HSV.
‡Turnaround times for sample to result were taken from company's product literature.
§Clinical sensitivity and specificity was adjusted using bidirectional sequencing-based discrepancy resolution data reported in 510(k) summaries # K100336 for MultiCode HSV, K111951 for IsoAmp HSV, while clinical sensitivity and specificity was adjusted using concordance of PCR and SDA in 510(k) summary # K103798 for Probetec HSV.
¶Clinical sensitivity and specificity of ELVIS HSV is calculated from the data in 510(k) summary # K111951 for IsoAmp HSV, and K100336 for MultiCode HSV where ELVIS HSV was the reference method.
HSV: Herpes simplex virus.

clinical specimens. Finally, we expect the application of this decentralized testing paradigm will be focused on diseases that present either a public health/disease containment imperative (e.g., sexually transmitted diseases, respiratory viruses and tuberculosis) or diseases with an urgency for prompt treatment to avoid patient complications (e.g., *Clostridium difficile*).

Five-year view

In the longer term, we expect the development of completely instrument-free molecular diagnostic assay systems will become a commercial reality. For example, a NAAT that exploits exothermic chemical heating in a platform called noninstrumented nucleic acid amplification (NINA) to perform LAMP malaria assays was recently reported [23]. Such heating is commonly used in military ready-to-eat meal packages. Although such truly instrument-free assay systems are mostly intended for distribution in the developing world, we believe isothermal NAAT that rely on exothermic chemical heating, and disposable detection devices, such as the BESt cassette, may eventually find their way to US and European domestic markets as pressures from health insurers and national healthcare systems drive reimbursements down. The prototype NINA device described by LaBarre *et al.* targets a 65°C reaction temperature, and maintains the reaction vessel at this temperature for approximately 1 h (i.e., the ideal incubation conditions for IsoAmp assays) [23]. As the CaO (i.e., quicklime; Science Stuff, Inc., TX, USA, Cat # C1450) heating

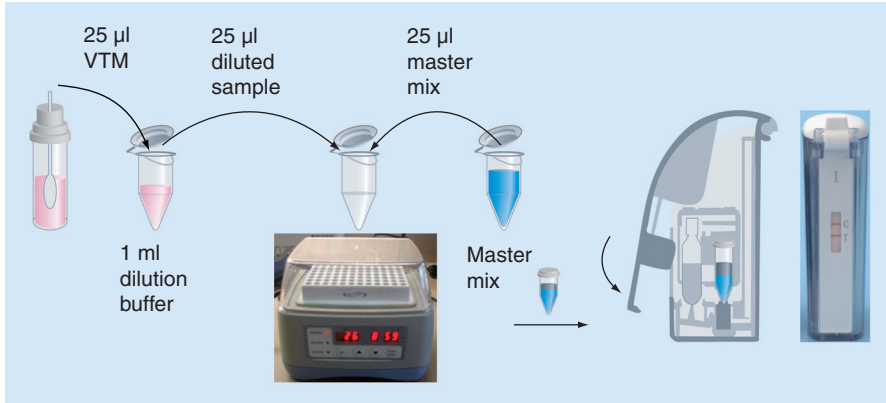

Figure 2. Sample processing workflow for IsoAmp® HSV. A genital or oral lesion swab is suspended in 2 ml of VTM. A total of 25 µl of the VTM is diluted 40-fold in a dilution buffer containing 800 copies of the competitive internal control. A total of 25 µl of the dilute sample is combined with 25 µl of 2× amplification master mix in a 0.2-ml nucleic acid amplification test tube, and heated to 64°C for 60 min, after which the tube is placed in the BESt cassette. The handle of the BESt cassette is closed and a knife located at the bottom of the cassette cuts the amplification tube, releasing the amplicon in the cassette without contaminating the laboratory with amplification products. A vertical flow test strip inside the cassette allows for the detection of the amplicon after a 15-min incubation.
VTM: Viral transport medium.

element in the NINA incubator is consumed during the assay, it is likely to be reviewed as a disposable reagent by the FDA rather than as an instrument.

Although the application of molecular diagnostics to the doctor's office or to the home is gated by significant regulatory obstacles, we believe that low-cost, near instrument-free assay systems such as IsoAmp HSV, and fully instrument-free systems such as NINA are likely to fit more easily in point-of-care testing than costly automated systems. We anticipate that automated sample-to-result systems will eventually get CLIA-waived; however, the acceptance by cost-constrained physicians will be limited, and home use of such systems is very unlikely. Home use of molecular tests faces even greater regulatory challenges but home collection of samples is already being evaluated for *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* [24–27].

Financial & competing interests disclosure

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Key issues

- The IsoAmp® HSV Assay (Biohelix Corp.) is a US FDA-cleared *in vitro* molecular diagnostic device for use in the detection of herpes simplex virus in genital and oral lesion specimens.
- The IsoAmp HSV Assay possesses a total test-to-result time of less than 1.5 h by incorporating an isothermal helicase-dependent amplification technique and omitting the time-consuming nucleic acid extraction.
- The near instrument-free, rapid and simple characteristics of the IsoAmp HSV assay make it potentially suitable for point-of-care testing.

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Website

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