Clinical Evaluation of the
Sentosa® SA HSV1/2 Qualitative PCR Test

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Sponsor: **Vela Research Singapore Pte. Ltd.**
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Clinical sites:	TBD

Study Synopsis

Sponsor:	Vela Research Singapore Pte. Ltd.
Device:	*Sentosa*® SA HSV1/2 Qualitative PCR Test
Title of study:	Clinical Evaluation of the *Sentosa* SA HSV1/2 Qualitative PCR Test
Number of sites:	3 external US sample collection sites and 3 testing sites; may be same sites.
Study objective:	The purpose of this study is to evaluate the performance of the *Sentosa* SA HSV1/2 Qualitative PCR Test. Precision of results and concordance of results with a reference assay will be evaluated.
Study population & sample size:	This study will utilize residual samples from male and female patients with signs and symptoms of oral or genital HSV infections. Genital samples will include internal and external genital lesions such as those collected from lesions of the anus, buttocks, vagina, labia, or penis. Oral samples will include those collected from lip, gum, and mouth lesions. For concordance testing, the sample size is based on historical study design and estimates of the expected prevalence of HSV1 and/or HSV2 in the population enrolled. For Precision/Reproducibility testing, the sample size follows the CLSI EP 5-A2 (2004) guidance document.
Study plan:	Samples may be tested at the same facility at which they were obtained, or may be shipped to a central laboratory for storage and redistribution to ensure adequate sample numbers at all testing sites. Testing will be done using the *Sentosa* SA HSV1/2 Qualitative PCR Test and the ELVIS® HSV ID and D^3 Typing Test System (reference assay).
Statistical analysis plan:	Clinical sensitivity will be the ratio of test assay to reference assay positives. Clinical specificity will be the ratio of test assay negatives to reference assay negatives. Ratios will be presented with 95% confidence intervals. Precision/Reproducibility testing: Analysis will be done following the CLSI EP 5-A2 guidance document.
Expected study duration:	1 - 4 months

Clinical Evaluation of the *Sentosa*® SA HSV1/2 Qualitative PCR Test

Table of Contents

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1. Introduction

1.1 Background

Herpes Simplex viruses type 1 (HSV1) and type 2 (HSV2) are ubiquitous double-stranded neurotropic DNA viruses of the *Herpesviridae* family. Both HSV1 and HSV2 cause incurable lifelong infections. Infection results from inoculation of the virus through abraded skin or mucous membranes. Both viruses may remain latent for long periods and cause recurrent episodes of symptomatic disease.

HSV1 is transmitted primarily via oral secretions and non-genital contact. This results predominantly in infections of the oropharynx, face, eyes and central nervous system. In recent years the frequency of genital HSV1 infections has increased due to a lower rate of oral infection during prepubescent childhood, rendering individuals susceptible to genital infection in later life, and a rise in frequency of oro-genital contact.[1,2] Nevertheless, seroprevalence studies show up to 80% of children infected with HSV1 by adulthood, with the highest rates among those in poor socioeconomic groups.[3]

In contrast with HSV1, infection with HSV2 is usually the result of sexual transmission. In the United States, 20-25% of the population has antibodies to HSV2 by the age of 40, and there are at least 50 million people with genital herpes.[3] In the majority of cases, symptoms are mild or unrecognized and the infection remains undiagnosed, although intermittent shedding of infectious virus into the genital tract still occurs. As a result, most transmission of genital herpes occurs through sexual contact with persons who are asymptomatic or unaware they are infected. Infection with HSV2 is more common in women than in men. HSV2 infection is linked to an increased risk of sexually transmitted human immunodeficiency virus (HIV).[4] While transmission of the herpes virus to neonates *in utero* or intrapartum is rare, the consequences of such infections are severe and frequently fatal.

Classical primary genital herpes infection is preceded by localized pain or tingling, frequently accompanied by fever, malaise and inquinal lymphadenopathy. Within days, vesicles appear on the labia minora, introitus and urethra meatus of women and on the shaft and glans of the penis in men. The perineum and perianal areas may also be affected, as may the upper thighs and buttocks. Cervical lesions also occur frequently in women.

Primary infection with HSV1 cannot be distinguished clinically from that caused by HSV2, and the lesions may also be confused with those caused by other sexually transmitted diseases. As a consequence, laboratory testing is required for definitive diagnosis to reduce symptoms and hasten the healing of lesions. In addition, because the recurrence of HSV1 infections and subclinical shedding are less frequent than for HSV2, determination of the etiology of infection and typing of the virus is useful in the

assessment of prognosis and counseling.[1,2,5,6] Historically, the preferred method of diagnosis of herpes infection has been viral isolation in tissue culture followed by type-specific immunofluorescent detection. The enhanced sensitivity, robustness, and rapid time to results of amplification methods for the detection of viral DNA are leading to their increasingly widespread adoption.[1,3,6]

The *Sentosa*® SA HSV1/2 Qualitative PCR test is a polymerase chain reaction (PCR)-based qualitative *in vitro* diagnostic test for detection and typing of Herpes simplex virus (HSV) DNA using genital or oral samples. The assay includes a set of oligonucleotide primers and dual-labeled, linear, sequence specific hydrolysis probes for use in real-time PCR assays on the *Sentosa* SA201. The *Sentosa* SA201 instrument is a real-time PCR cycler with 5 channels. The *Sentosa* SA HSV1/2 Qualitative PCR assay uses 3 of the 5 available channels.

The assay kit consists of real-time PCR master mix (MM), primers and probes mixture (PPM), negative control (NC), extraction control (EC) and positive control (PC). There are two sets of primers and three sets of probes for detection of HSV1, HSV2, and extraction control nucleotides, and the amplifications of the targets in 3 channels (green, orange and red) on the *Sentosa* SA201 instrument. Output is recorded as the increase of fluorescence over time in comparison to background signal.

1.2 Principle of the procedure

Pathogen detection by PCR is based on the amplification of specific regions of the pathogen genome. In real-time PCR, the amplified product is detected via fluorescent dyes, which are usually linked to oligonucleotide probes that bind specifically to the target sequences. Real-time monitoring of the fluorescence intensities during the PCR run allows the detection of the accumulating product.

2. Description of the device

2.1 Overview

The *Sentosa* SA HSV1/2 Qualitative PCR Test workflow starts with extraction of nucleic acids from samples (genital or oral swabs in Universal Viral Transport Medium; VTM) using the *Sentosa* SX Virus Total Nucleic Acid Kit on the *Sentosa* SX101 instrument. After extraction, the instrument will set up the PCR with the extracted nucleic acids automatically in the 96-well PCR plate. Subsequently, the 96-well PCR plate is sealed and transferred to the *Sentosa* SA201 instrument for PCR amplification, followed by data analysis. Refer to the user manual for a detailed description.

2.2 Description of the test system

The *Sentosa* SA HSV1/2 Qualitative PCR Test is a ready-to-use system for the detection and differentiation of HSV1/2 DNA in genital or oral swabs in VTM. Nucleic acid extraction from clinical specimens and PCR set-up are performed on the *Sentosa* SX101, an automated liquid handling and sample preparation system. PCR is done on

the *Sentosa*® SA201 instrument. The HSV1/2 master mix contains reagents and enzymes for specific amplification of a 104 base pair (bp) fragment of the *UL30* gene common to both HSV1 and HSV2. The master mix also contains specific probes for the direct detection and differentiation of HSV1 and HSV2 amplicons in the fluorescence channels Cycling Green and Cycling Orange of the *Sentosa* SA201 instrument.

In addition, the *Sentosa* SA HSV1/2 Qualitative PCR Test contains a third set of primers/probes designed to detect an extraction control (EC) target in the fluorescence channel Cycling Red. This extraction control is used as control for the nucleic acid isolation procedure and as a PCR inhibition control. The extraction control amplification does not compromise the detection limit of the HSV1/2 PCR amplification. The test also contains a negative control (NC) and a positive control (PC) that allow the user to assess whether the PCR reaction has been performed properly.

The following is an overview of the system workflow.

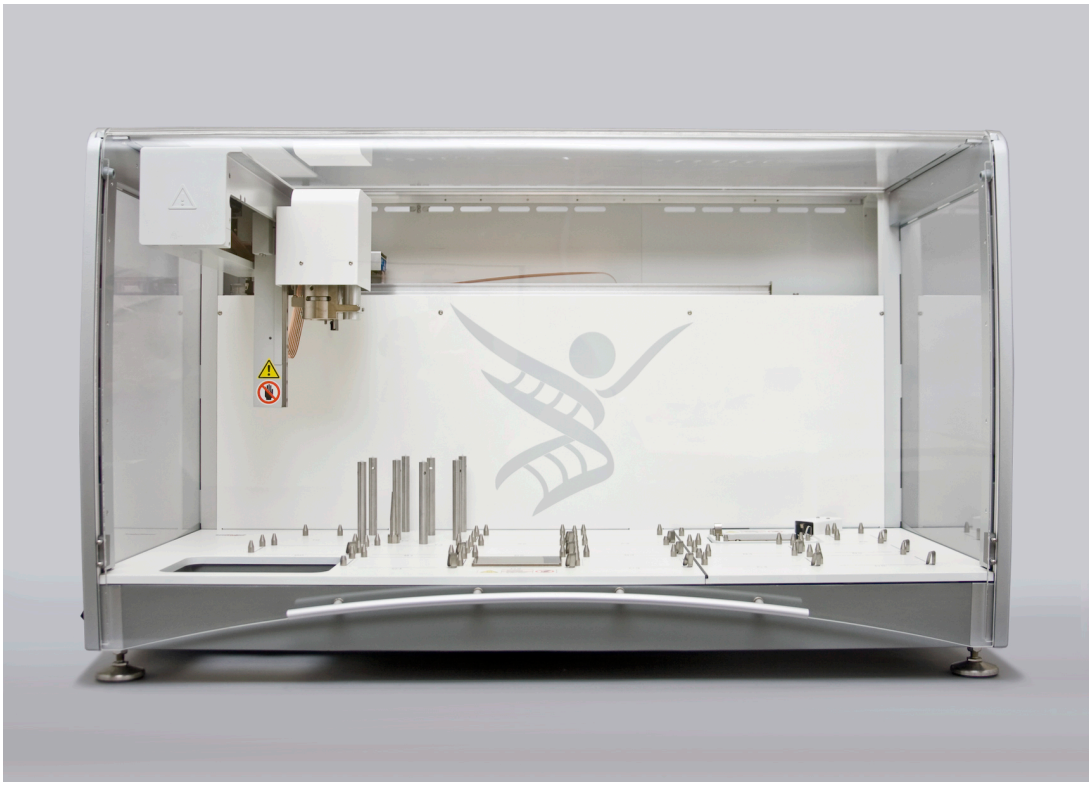
1. Prepare samples.
2. Scan samples using *Sentosa* Link, which generates an entry list file.
3. Import entry list file to *Sentosa* SX101 software.
4. Load consumables, reagents, controls and samples onto the *Sentosa* SX101.
5. Select relevant application and start the run.
6. After run is completed, remove and seal the 96-well PCR plate.
7. *Sentosa* SX101 software generates "*.smp*" file.
8. *Sentosa* Link transfers "*.smp*" file from *Sentosa* SX101 to *Sentosa* SA201 instrument.
9. Transfer the 96-well PCR plate into the *Sentosa* SA201 instrument chamber.
10. Import "*.smp*" file and define the PCR run, then start the PCR run.
11. After the run is completed, *Sentosa* SA series software analyzes result and *Sentosa* SA Reporter software interprets results.
12. *Sentosa* Link reads and displays the results.

2.2.1 *Sentosa*® SX101

The *Sentosa* SX101 is an automated pipetting system that provides an easy-to-use workflow for nucleic acid extraction and PCR setup for up to four assays simultaneously (Figure 1).

The *Sentosa* SX101 system operates as follows: liquid (samples from the source tube) is transported via pipette tips to the destination tube. An optical sensor automatically checks the correct selection and positioning of tubes, available supplies (labware), and the position of pipette tips in the rack.

Figure 1. *Sentosa*® SX101



The *Sentosa* SX101 instrument has the following features:

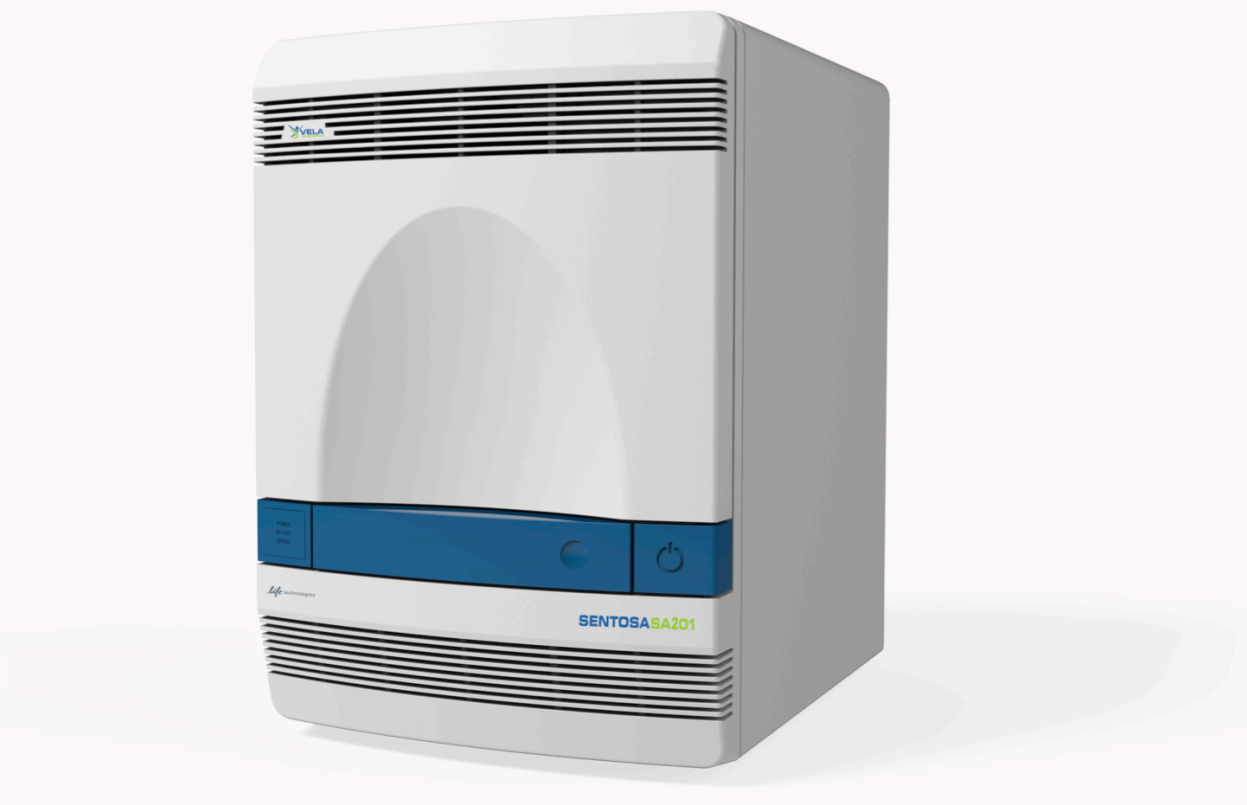
- Software-controlled thermo-mixer module for mixing, heating and cooling of samples, and for supporting the magnetic beads-based *Sentosa* SX Virus Total Nucleic Acid Kit;
- Preset *in vitro* diagnostic (IVD) protocol for the *Sentosa* SA HSV1/2 Qualitative PCR Test;
- Color matching of tube holders and reagent caps, ensuring correct placement of reagents;
- Inscribed names, providing further assurance on the correct positioning of reagents;
- Integrated workflow with barcode identification (ID) tracking functionality to ensure sample traceability from start to end of workflow;
- Barcoding of tubes containing the samples to reduce error rates due to manual input of information; and
- Optical sensor scanning of the deck before each run to ensure the correct number and types of tips and labware.

2.2.2 *Sentosa*® SA201

Sentosa SA201 is a real-time nucleic acid amplification and detection system that measures nucleic acid signals and converts them to comparative quantitative readouts using fluorescent detection of dual-labeled, linear, sequence specific hydrolysis probes.

The thermocycler is in 96-well format and the 5-colour detection system enables the use of a wide range of fluorophores (dyes). Ten dyes are calibrated as part of the standard installation of the Sentosa® SA201: FAM, SYBR Green I, JOE, VIC, NED, TAMRA, Cy3, ROX, Texas Red and Cy5. The *Sentosa*® SA HSV1/2 Qualitative PCR Test will use the following four dyes: FAM, JOE, ROX and Quasar 670. Quasar 670 is used as a substitute for Cy5 in the *Sentosa*® SA HSV1/2 Qualitative PCR Test. A calibration procedure will be provided by Vela for the use of Quasar 670, which will be part of the standard recommended calibration procedure for this test. The *Sentosa* SA201 is shown in Figure 2.

Figure 2. *Sentosa* SA201



2.3 Workflow

The *Sentosa* SA HSV1/2 Qualitative PCR Test workflow starts with extraction of nucleic acids from samples (genital or oral swabs) using the *Sentosa* SX Virus Total Nucleic

Acid Kit on the *Sentosa* SX101 instrument. After extraction, the instrument automatically sets up the PCR with the extracted nucleic acids in the 96-well PCR plate. Subsequently, the 96-well PCR plate is sealed and transferred to the *Sentosa* SA201 for PCR amplification, followed by data analysis.

Drivers are installed on the *Sentosa*® Link to connect the *Sentosa* SX101 instrument and the *Sentosa* SA201. This creates a user environment that links the SX101 and the *Sentosa* SA201 to facilitate automatic workflow to export results in a LIS/LIMS-compatible format.

2.4 Calibrators and controls

The control set for the *Sentosa* SA HSV1/2 Qualitative PCR Test is provided in the assay kit for each set of assay reagents. One positive and one negative control must be included for each batch of samples and for new reagent kits. Controls must be positioned according to the *Sentosa* SX101 Instrument User's Manual. The HSV positive control is a mixture of two linearized plasmids and monitors for substantial reagent failure, as well as instrument and workflow, and inhibition due to instrument or workflow failure. The HSV negative control monitors for reagent and/or environmental contamination.

The extraction control (EC) oligonucleotide is used to confirm the validity of the extraction process. The EC is supplied as a liquid in extraction tubes and can be loaded directly onto the *Sentosa* SX101 platform. During the automated procedure, the EC is added directly into lysis buffer for PC, NC and sample extraction. At the end of the extraction process, the EC fluorescence is monitored by the instrument and an automated algorithm is applied to both the EC and the HSV specific signals to report sample results as positive, negative, or EC failure.

2.5 Reference assay

The reference assay to be used in this evaluation is the ELVIS®HSV ID and D³ Typing Test System (K091753), manufactured by Diagnostic Hybrids. The product code for this assay system is OQO.

3. Intended Use

The *Sentosa* SA HSV1/2 Qualitative PCR Test is a multiplex, real-time PCR-based qualitative *in vitro* diagnostic test for detection and typing of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) DNA from male external and internal genital lesions, female external (anogenital) and internal (vaginal) lesions, or external and internal oral lesions. The test is intended for use as an aid in the diagnosis of HSV infection in symptomatic patients.

Warning: The *Sentosa* SA HSV1/2 Qualitative PCR Test is not FDA cleared for use with cerebrospinal fluid (CSF). The assay has not been established for antiviral therapy and is not intended for prenatal screening.

4. Study materials

4.1 Materials supplied by the sponsor

Vela Research USA will supply the following reagents and equipment and its associated software to each testing site:

- *Sentosa* SX101 instrument for extraction and PCR set-up;
- *Sentosa* SA201 for real time nucleic acid amplification and detection;
- *Sentosa* Link software;
- *Sentosa* SA201 Series software;
- *Sentosa* SA Reporter;
- *Sentosa* SX Virus Total Nucleic Acid Kit;
- *Sentosa* SA HSV1/2 Qualitative PCR Test;
- *Sentosa* SX Safe-Lock Tubes;
- *Sentosa* SX Partition 50µL Filter Tips;
- *Sentosa* SX Partition 1000µL Filter Tips;
- *Sentosa* SX 100mL Reservoir;
- *Sentosa* SX 30mL Reservoir;
- *Sentosa* SX Deepwell Plates 96/2000µL
- *Sentosa* SX Rack for 96 1.5/2.0mL Screw Tubes;
- *Sentosa* SX Microplates 96/V;
- *Sentosa* SX Biohazard Bag;
- *Sentosa* SX PCR Foil, adhesive;
- *Sentosa* SX Thermoblock PCR 96;
- Sentosa SX Dispensing tool TM 50
- Sentosa SX Thermoblock PCR 96
- MicroAmp® Splash-free Support Base;
- MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL;
- MicroAmp® Optical Adhesive Film.

4.2 Equipment and Materials supplied by the clinical site

The clinical site will provide all miscellaneous laboratory supplies, including nitrile gloves, 1% (v/v) sodium hypochlorite, pipettes capable of transferring 0.5 mL, pipette tips with filters, a vortex mixer, a bench top centrifuge, 96-well PCR plate centrifuge, and saline (0.9% NaCl).

In addition, the clinical site will ensure there is adequate bench space for all instrumentation supplied by Vela Research USA, maintained at required conditions as stated in the Instructions for Use. Also, the clinical site will ensure there is adequate

secure storage space for all supplies used in the clinical evaluation, including refrigerated space as needed.

The clinical laboratories that participate in this study as testing sites will be required to have the reference assay installed and qualified for use, with appropriate quality assurance procedures in place.

4.3 Investigational product accountability

The clinical investigator at each site, or a responsible designee, must maintain an inventory record of all materials and supplies received from the sponsor. All investigational device materials and supplies may be used only for the purposes stated in this protocol. Forms will be supplied to the sites for inventory control, including receipt, usage/disposition, and return of all investigational materials and supplies. Loss of investigational materials or supplies due to wastage or other reason must be documented with the name of the responsible individual, date, and quantity.

4.4 Investigational product disposal or return

Upon completion or termination of the study, all unused investigational materials and supplies must be returned to the sponsor or disposed of at the clinical site. Disposal requires written authorization from the sponsor. Return and/or disposal must be documented as described in section 4.3, above.

5. Sample acquisition

5.1 Sample inclusion criteria

Samples may be included in the assay validation if they meet the following criteria:

- Sample was taken from a lesion from an internal or external oral or genital site.
- Sample was submitted to a clinical laboratory for the purpose of testing for the presence of HSV1 or HSV2.
- The following information about the patient from which the sample was taken is available:
    - presumptive diagnosis or signs and symptoms causing assay requisition,
    - site of lesion,
    - age at time of sample collection or date of birth, and
    - sex.
- Samples were collected in universal viral transport media, using a plastic shaft swab made of either polyester, cotton, rayon or Dacron.
- There is sufficient residual sample to perform both test and reference assays.

5.2 Sample exclusion criteria

If a sample meets any of the following criteria, it must not be included in the assay validation:

- Sample leaked during shipment or storage prior to assay.
- Sample has undergone more than 1 freeze-thaw cycle before testing;
- Sample eluent is not clear after centrifugation (refer to section 6.3.1).
- Sample ID is missing or ambiguous.
- Sample is collected using alginate calcium swab.
- Sample handling and storage requirement in section 5.4 not followed

5.3 Sample collection

The samples used in the study will be residual material from female and male genital samples collected for clinical HSV1/2 testing from anal, buttock, vaginal, labial, or penile lesions, or oral swab samples collected from lesions of the lips, gums or mouth. The specific lesion site will be identified on the case report form for each sample.

Each sample will be de-identified to ensure there is no link to patient identity, and labeled with a study ID according to the following scheme:

XX-ZZZ, where XX represents the clinical site (e.g., 01, 02), and ZZZ represents the consecutively numbered sample acquired at each clinical site.

Sample barcode labels must be printed out and attached to the tube.

The total sample collection period of this study is 1 to 2 months. The target number of samples is 585 oral samples and 495 genital samples.

The clinical sample set may be enriched with contrived positive samples where virus is spiked into either negative clinical matrix or VTM.

5.4 Sample handling and storage

All samples should be treated as infectious biological material. Study ID must be securely affixed to the study sample; if the study ID is missing or ambiguous the sample may not be used in the study. Samples to be used in this study must be segregated from all other samples, in separate racks, both during testing and when stored.

- Store at room temperature (15°C to 25°C) if samples will be processed for *Sentosa* testing (i.e., extraction) within 4 hours after accession in the lab.
- Store at 4°C if samples will be processed for *Sentosa* testing within 72 hours of accession in the lab.
- Store at -70°C or lower temperature for long-term storage (up to 1 year).
- Only fresh samples (stored at 4^{o}C) will be tested with ELVIS within 72 hours of accession in the lab.

- Testing via *Sentosa*® SA HSV1/2 Qualitative PCR Test should be completed on samples that have undergone similar storage conditions to ELVIS or no more than 1 freeze thaw cycle.

Temperature logs for all storage facilities will be maintained, including room temperature for days on which assays are run.

6. Study plan

6.1 Overview of protocol

The clinical evaluation will be conducted at three (3) clinical sites, on three (3) separate testing systems. In total, at least three (3) lots of test kits will be used in the evaluation; approximately equal numbers of samples will be tested on each of the three lots.

For concordance evaluation, approximately 585 samples from oral lesions and 495 samples from genital lesions will be tested. The study will use residual samples collected for clinical testing from patients with clinical evidence of HSV1/2 infection. The sample size is based on prevalence of HSV1 and HSV2 in oral and genital swabs.

If required, due to low prevalence, the clinical sample set may be enriched with contrived positive samples where virus is spiked into either negative clinical matrix or VTM.

6.2 Assay validation

6.2.1 Familiarization

Familiarization testing is done to ensure that personnel at the clinical sites who will be performing the assay are appropriately trained. When a clinical site is initiated, a panel of 5 samples will be assayed in 4 replicates on the same day by one technician at each site. The sponsor will provide this sample panel and the expected result ranges. The sponsor will review the results; if the panel samples fall within the expected result ranges, the personnel at that site may proceed to assay the precision/reproducibility samples using the *Sentosa*® HSV1/2 Qualitative PCR testing system and the reference assay.

6.2.2 Precision and reproducibility

Precision and reproducibility will be evaluated with 3 lots of *Sentosa* SA HSV1/2 Qualitative PCR test kits; all lots will be used at all sites to evaluate lot-to-lot variation. The blinded Precision/Reproducibility Test Panel includes negative and positive quality control materials and a Precision/Reproducibility Sample Panel containing the following:

- a negative sample
- 1.5xLoD HSV1;

- 1.5xLoD HSV2;
- 3xLoD HSV1; and
- 3xLoDHSV2.

This sample panel plus the quality control materials will be run 20 times at each site.

Samples in this Precision/Reproducibility Sample Panel will not be assayed using the reference assay. Each sample (positive and negative samples) will be tested in replicates of 3 and each control (positive control and negative control) will be tested in replicates of 2 in one run and two runs will be carried out daily over 5 days (total test points per sample: 3 replicates x 1 run/day/operator x 2 operators x 5 days x 3 sites = 90). Intra-assay reproducibility will be calculated for within-run values. Between-day, between-run, between-lot, between-operator and between-site variance, as well as total variance, will be calculated. Statistical analysis will be done following the CLSI EP5-A2 guideline. Refer to protocol PL-457 for more details. Protocol PL-457 may be found in the appendix.

6.2.3 Concordance

For concordance testing, a minimum of 585 oral samples and 495 genital samples will be collected at 3 clinical sites. Each site will test at least 195 oral samples and 165 genital samples.

Samples will be tested using the investigational device and the reference assay in parallel (testing initiated on the same day from the same sample) by a qualified technician who has been specifically trained to participate in the study. Results will be sent to the study manager for review; the clinical site will be notified if any samples must be retested. Residual sample may be stored after initial testing following storage requirements in section 5.4 until retest notification has been received.

Samples that yield discordant results between the investigational and reference methods will be re-tested by Vela Research USA, using different PCR primers and bidirectional sequencing. Results from discrepant testing will be reported as a footnote in the package insert. Only samples containing sufficient volume will be retested for discrepant analysis.

Any sample left after all testing is completed should be frozen at -70^{o}C for shipment to Vela Research USA.

6.3 Test procedure

Refer to the *Sentosa*® SX101 System Instrument User's Manual for specific instructions for operating and maintaining the components of the system. The optimum environmental conditions for the *Sentosa* SX101 are 18°C - 27°C and 20 – 85% relative humidity.

6.3.1 Sample processing procedure

Sample material present on a swab will be eluted by vigorous agitation (i.e., vortexing) of the transport tube. The swab should then be discarded as biohazardous waste. Only clear sample eluent may be used as inoculum. If visual precipitates are observed, the eluent should be centrifuged at 4000 rpm for 10 minutes; the clear supernatant should be collected as inoculum. If centrifugation does not exclude precipitate the sample will be considered contaminated.

All frozen samples (sample eluent) must be allowed to thaw completely at room temperature and must be mixed by inversion.

During the procedure 250 µL of sample is transferred into a fresh 1.5 mL *Sentosa*® SX Safe-Lock Tube with proper label, in a biosafety cabinet, for sample loading of *Sentosa* SA HSV1/2 Qualitative PCR Test.

6.3.2 Procedure of *Sentosa* SA HSV1/2 Qualitative PCR Test.

Refer to the reference user manual of *Sentosa* SA HSV1/2 Qualitative PCR Test for detailed work instructions.

6.3.3 Control values for valid results

For the test to be valid, the negative control must be negative for fluorescence channels cycling green and cycling orange and positive for fluorescence channel cycling red, and the positive control must be positive for fluorescence channels cycling green and cycling orange

Results interpretation

For **Negative control**

Negative control (NC)			Interpretation
Green	**Orange**	**Red***	
-	-	+	Proceed to PC
+	-	+/-	Run invalid
-	+	+/-	
+	+	+/-	
-	-	-	Run invalid

For **Positive control**

Positive control (PC)			Interpretation
Green	**Orange**	**Red***	
+	+	+/-	Proceed to Samples*
+	-	+/-	Run invalid
-	+	+/-	
-	-	+/-	

For **Samples**

Samples			Interpretation
Green	**Orange**	**Red***	
-	-	+	HSV1 and HSV2 DNA not detected, extraction control positive
-	+	+/-	HSV2 DNA detected*
+	-	+/-	HSV1 DNA detected*
+	+	+/-	HSV1 and HSV2 DNA detected*
-	-	-	Sample invalid

*Fluorescence channel Cycling Red can be negative due to competition with target channels.

6.3.4 Retest Criteria

If results of any test run are invalid due to failure of a positive or negative control failure, or if instrument specifications such as temperature or humidity conditions are out of specification, repeat testing will be performed.

Other conditions that require repeat testing include:

- machine failure;
- presence of an error message or flag in the machine;
- wrong sample tested;
- sample invalid status;
- wrong assay or extraction kits used;
- procedure error occurred; and

- testing protocol was not followed.

6.4 Data collection and submission, and record keeping

6.4.1 Data collection and submission

Data collection will be electronic print-outs or files from laboratory instruments, and electronically or manually completed data collection instruments. Data collection instruments will be completed at the time of sample collection and will be sent electronically, if completed electronically, or by fax and/or courier (e.g., FedEx, UPS, or USPS) as agreed between the clinical site and the study manager, if completed manually. Electronic print-outs or files may be faxed or transmitted electronically to the sponsor or designee daily when testing is completed, as requested. Signed copies of the test data and any electronically or manually completed data reporting forms will be submitted via courier to the data management group.

6.4.2 Study document maintenance

Clinical study conduct must be documented. Training in study document maintenance will be done at the time of trial initiation at each clinical site. General instructions include the following:

- forms completed manually must be done in blue or black ink;
- fields must never be left blank – if information is not available or not applicable this must be noted;
- changes or corrections to documents are done by a single line strike through the incorrect information, with the corrected information initialled and dated by the person making the correction – *under no circumstances may white-out be used, the original entry must not be obscured;*
- copies of all records will be sent to the sponsor or designee as requested, original records will be kept at the clinical site;
- responsibility for ensuring accuracy, legibility, completeness, and timeliness of data lies with the clinical investigator;
- study document binders and forms will be supplied by the sponsor.

7. Statistical considerations

7.1 Sample size justification

A minimum of 585 oral samples and 495 genital samples will be collected and tested.

The clinical sample set may be enriched with contrived positive samples where virus is spiked into either negative clinical matrix or VTM.

A minimum of 73 samples per analysis group guarantees that at a minimal acceptable concordance of 95% (positive or negative) the lower bound of the 95% binomial confidence interval will be greater than 90%.

7.2 Analysis plan

Clinical sensitivity and specificity will be determined for each virus at each site. Data from female external (anogenital) and internal (vaginal) genital lesion samples will be analyzed and presented separately. The agreement will be summarized as demonstrated by Table 1.

Table 1: Agreement between Reference and Test Assays for Virus X for Subjects at Clinical Site Z.

	Reference				
		Positive	Negative	Invalid	Total
Test	Positive	a	b	d	a+b+d
	Negative	e	f	h	e+f+h
	Invalid	m	n	p	m+n+p
	Total	a+e+m	b+f+n	d+h+p	

Clinical sensitivity=100% * a/(a+e+m)

Clinical specificity=100% * f/(b+f+n). Positive concordance is defined by

$$\%Positive = a \cdot 100 / \left(a + e + i + m\right) = a \cdot 100 / N_p \ .$$

Negative concordance is defined byWilson confidence interval is calculated by:

$$\%Negative = b \cdot 100 / \left(b + f + j + n\right) = b \cdot 100 / N_n \ .$$

$$\frac{\hat{p} + \frac{1}{2n} z^2_{1-\alpha/2} \pm z_{1-\alpha/2} \sqrt{\frac{\hat{p}(1-\hat{p})}{n} + \frac{z^2_{1-\alpha/2}}{4n^2}}}{1 + \frac{1}{n} z^2_{1-\alpha/2}}$$

An exact upper and lower bound for the 95% confidence interval for positive concordance is defined by

$$\%p_{Lower} = \frac{a \cdot 100}{a + (\mathrm{N}_p - a + 1) \cdot F_{2(N_p - a + 1), 2a, 0.975}}$$

$$\%p_{Upper} = \frac{(a+1) \cdot 100 \cdot F_{2(a+1), 2(N_p - a), 0.025}}{N_p - a + (a+1) \cdot F_{2(a+1), 2(N_p - a), 0.025}}$$

Similar confidence interval formulae will be employed to calculate the 95% CI for negative concordance.

Equivalence between reference and test assays will be attained if, for both viruses, the sensitivity and specificity is above 92.5% and 93.6%, respectively, for at least two of the three sites.

7.3 Discrepant results

Extracted nucleic acids (20-30 uL) from samples that yield discordant results between the investigational and reference methods will be shipped to Vela Research USA and tested with different primers by PCR and bidirectional sequencing. If there is not sufficient sample for retesting, the discordances will be reported without further testing.

Discrepant testing results will not be used to generate sensitivity and specificity data, but will be reported as a footnote in the package insert.

8. Record and sample retention

All samples and records must be retained by the clinical investigator in condition suitable for inspection for the longest of:

- 2 years from the date of FDA clearance to market of the investigational device;
- 2 years following termination or completion of the entire clinical investigation; or
- record retention policy of the clinical site or associated IRB.

The clinical investigator will be notified by the sponsor of the date of marketing clearance or termination of the clinical investigation. Measures should be taken to prevent accidental destruction of documents and samples. Sample aliquots must be maintained at -70ºC or below and at the end of all testing must be shipped to the sponsor.

9. Training

Good clinical and laboratory practices must be followed throughout the study. Instructions for use must be followed for all assay methods used in the study.

One or more individuals will be identified at each site to be responsible for all testing with the *Sentosa*® SA HSV1/2 Qualitative PCR Test. The sponsor will install the testing system and train laboratory personnel on the instruments prior to study initiation. Only individuals trained on the *Sentosa* SA HSV1/2 Qualitative PCR Test are qualified to test samples for this study.

System maintenance as described in Operating Manuals and during training must be done. Maintenance forms will be provided and must be completed as required.

10. Regulatory Considerations

10.1 Institutional Review Board (IRB) review

This protocol and all other applicable documents will be submitted to the IRB at each participating clinical site for review and approval. The clinical investigator at each site shall obtain written confirmation from the IRB that the protocol and other applicable

documents have been approved. The study cannot commence at a clinical site until IRB approval is received.

If the clinical study extends beyond the approved time period, the clinical investigator will request renewal of the IRB approval to conduct the study. The renewal application must include all applicable documents required for the duration of the study. Written confirmation that the study approval has been renewed must be obtained from the IRB.

The clinical investigator will inform the IRB when the study is terminated and obtain written confirmation from the IRB of study closure.

10.2 Subject protection

This study will use samples collected for routine clinical care. After clinical testing is complete, residual samples will be de-identified and coded with a unique study identification number to ensure subject confidentiality. Demographic information (sex and age/date of birth) and appropriate medical history will be recorded for the samples tested for expected values and concordance. All information will be identified by the study identification number only, so that patient confidentiality is preserved. As this study is using residual clinical samples only, informed consent is not required from patients from which the samples were obtained.

10.3 Monitoring

The clinical monitor, as a representative of the sponsor, has the obligation to follow the study closely. The monitor may visit the clinical investigator and/or the study facility, in addition to maintaining necessary contact by telephone, fax, email, and letter communication. Monitoring of this study may entail, but is not limited to, review of data collection forms, regulatory documents, instrument maintenance logs, temperature charts from sample and reagent storage areas, investigational assay results, reference assay results, and staff training records.

The clinical investigator and the institution will permit trial-related monitoring, audits, IRB review, and regulatory inspection (FDA or other regulatory body) by providing direct access to study documents.

10.4 Adherence to protocol, federal regulations, GCP and GLP

The study will be conducted as described in the approved protocol and according to applicable US federal regulations and good clinical and laboratory practice (GCP and GLP). Deviations from the protocol, federal regulations, GCP, or GLP should be avoided if possible. If a deviation occurs, it must be recorded on the Protocol Deviation Form, with a description of the deviation, an explanation of why it occurred, and dated signature of the clinical investigator.

10.5 Regulatory binder maintenance

The regulatory binder will contain the following:

- contact list;
- protocol with amendments or addenda**;**
- case report forms, with instructions for completion;

- notifications of protocol amendments or addenda;
- financial disclosure statement(s);
- investigator agreement;
- investigator CV(s);
- documentation of conformance to FDA regulations on subject informed consent;
- IRB approval and correspondence;
- protocol deviation form;
- site signatures and responsibilities log**;**
- site staff CVs and training documentation;
- notes to file;
- monitoring log and reports;
- laboratory certification; and
- correspondence between the clinical site and the sponsor or the study monitor or study manager.

It is the responsibility of the clinical investigator to ensure that the regulatory binder is complete and updated as necessary. The study monitor will review the regulatory binder on all site visits.

10.6 Study handbook

A study handbook will be provided to each clinical site. The handbook will be maintained by study site personnel and will contain the following:

- test system service reports;
- test system maintenance log;
- test system troubleshooting log;
- test system calibration log;
- investigational product accountability log and return forms;
- sample receipt and shipping documents;
- sample return/retention log;
- repeat test log;
- temperature logs; and
- instrument calibration records.

11. References

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2. Gupta, R., T. Warren, A. Wald. 2007 Genital herpes. *Lancet* 370: 2127-2137.

3. Fatahzadeh, M., R.A. Schwartz. 2007 Human herpes simplex virus infections: epidemiology, pathogenesis, symptomology, diagnosis, and management. *J Am Acad Dermatol* 57: 737-763.
4. Freeman, E., H.A. Weiss, J.R. Glynn, P.L. Cross, J.A. Whitworth and R.J. Hayes. 2006 Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. *AIDS* 20: 73-83.
5. Whitley, R.J., B. Roizman. 2001. Herpes simplex virus infections. *Lancet* 357: 1513-1518.
6. Centers for Disease Control and Prevention. 2006. Sexually Transmitted Diseases Treatment Guidelines, 2006. *MMWR* 55: RR-11.

Appendix A
List of Abbreviations

ASTM	American Society for Testing Materials
bp	base pair
CLSI	Clinical Laboratory Standards Institute (previously NCCLS)
DNA	deoxyribonucleic acid
EC	extraction control
FDA	Food and Drug Administration (US)
GCP	good clinical practice
GLP	good laboratory practice
HIPAA	Health Insurance Portability and Accountability Act
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IRB	Institutional Review Board
IVD	*in vitro* diagnostic
LIS/LIMS	laboratory information system/laboratory information management system
LoD	limit of detection
NC	negative control
PBS	phosphate buffered saline
PC	positive control
PCR	polymerase chain reaction
PPM	primers and probe mixture
VTM	Universal Viral Transport Medium