For *in vitro* diagnostic use (Rx only)

Intended use

The *Sentosa*® SQ HIV-1 Genotyping Assay is a next generation sequencing (NGS)-based *in vitro* diagnostic (IVD) test intended for use in detecting HIV-1 genomic mutations (in the protease, reverse transcriptase and integrase regions of the pol gene), as an aid in monitoring and treating HIV-1 infection. This test is used in adjunct to the therapeutic management of patients diagnosed with HIV-1 Group M infection with viral loads of at least 1,000 RNA copies per mL in EDTA plasma specimens.

The *Sentosa*® SQ HIV-1 Genotyping Assay is used in conjunction with the *Sentosa*® SX Virus Total Nucleic Acid Plus (4x24) and *Sentosa*® SX IA Template Prep kits on the *Sentosa*® SX101 instrument, and *Sentosa*® SQ301 Sequencing instrument and *Sentosa*® SQ 318 Chip kit.

Results should be used in conjunction with other available laboratory and clinical information and are not intended for use as an aid in the diagnosis of infection with HIV or to confirm the presence of HIV infection, or for screening donors of blood, plasma or human cells, tissues and cellular and tissue-based products (HCT/Ps).

Summary and explanation of the test

HIV / Acquired Immunodeficiency Syndrome (AIDS) is a global pandemic. An estimated > 35 million people worldwide are infected with HIV[1]. There are two genetically distinct types of HIV: HIV-1 and HIV-2. Although both infect humans, HIV-1 is the major cause of AIDS globally. In the absence of specific treatments, approximately half of the people infected with HIV-1 develop AIDS within ten years.

HIV-1 is a small (about 120 nm), enveloped, positive-sense single-stranded RNA virus of the family *Retroviridae*[2]. The HIV-1 genome is about 9750 bases long and consists of many open reading frames[3,4]. HIV-1 is divided into a major group (M) and three minor groups. Group M is subdivided further into subtypes A to K[5]. HIV-1 is transmitted horizontally, e.g., through sexual contact, exposure to infected body fluids or tissues; and vertically e.g., from mother to child during pregnancy, delivery or breastfeeding[6].

AIDS is defined as either a CD4+ T cell count below 200 cells per µL or the occurrence of specific diseases in association with HIV-1 infection. The most common initial conditions that alert to the presence of AIDS are pneumocystis pneumonia (40%), cachexia in the form of HIV wasting syndrome (20%) and esophageal candidiasis. Other common signs include recurring respiratory tract infections[7]. Bacteria, viruses, fungi or parasites that are normally controlled by the immune system may cause opportunistic infections. Presently, available tests for diagnosing HIV / AIDS include ELISA and RT-PCR tests.

Vela Diagnostics' *Sentosa*® SQ HIV-1 Genotyping Assay provides an NGS-based *in vitro* diagnostic test to determine the viral genetic variants in patients diagnosed with HIV-1 infection. Genotyping and variant calling information can help to guide treatment regimens and is therefore relevant for the management of HIV-1 infected patients[8,9].

The *Sentosa*® SQ HIV-1 Genotyping Assay is an automated assay performed on the *Sentosa*® SX101 instrument, with an off-board RT-PCR amplification using the Veriti® Dx 96-Well Thermal Cycler, 0.2 mL instrument. *Sentosa*® SQ HIV-1 Genotyping Assay comprises five automated stages: (1) Sample preparation / RT-PCR, (2) Library preparation, (3) Isothermal template preparation, (4) Sequencing, (5) Report generation by the *Sentosa*® SQ Reporter.

Principles of the Assay

The *Sentosa*® SQ HIV-1 Genotyping Assay is a ready-to-use kit for automated NGS library preparation using the *Sentosa*® SX101 instrument, for the genotyping of HIV-1. The *Sentosa*® SQ HIV-1 Genotyping Assay consists of the *Sentosa*® SQ HIV-1 Genotyping Reagents and the *Sentosa*® SQ Virus Testing Solutions. The *Sentosa*® SQ HIV-1 Positive Control Kit comprises HIV-1 Positive Control (PC) required in this assay.

The *Sentosa*® SQ HIV-1 Genotyping Reagents kit contains four sets of reagents and solutions. Each set is used for the preparation of 24 libraries (22 HIV-1 libraries, one HIV-1 PC and one system control (SC)) for one run.

Sample preparation

HIV-1 nucleic acids are extracted from plasma sample automatically using the *Sentosa*® SX101 instrument. Briefly, the samples are collected in 1.5 mL *Sentosa*® SX Safe-Lock Tubes and loaded onto the *Sentosa*® SX101 worktable together with reagents from *Sentosa*® SX Virus Total Nucleic Acid Plus Kit and *Sentosa*® SQ HIV-1 Genotyping Reagents for extraction and RT-PCR amplification, respectively.

Extraction

Using the *Sentosa*® SX101 instrument, the samples are automatically processed using reagents from the *Sentosa*® SX Virus Total Nucleic Acid Plus Kit to release and isolate the nucleic acids.

PCR set-up

The *Sentosa*® SQ HIV-1 Genotyping Reagents comprises a reagent plates and contains components required for RT-PCR amplification. The *Sentosa*® SX101 instrument automatically combines the reagents from the reagent plate and tubes that were loaded onto the *Sentosa*® SX101 worktable. The resultant master mix is subsequently dispensed into the *Sentosa*® SX Barcoded PCR Plate 96 where it is mixed with the isolated nucleic acids.

Amplification

RT-PCR is performed using the Veriti® Dx 96-Well Thermal Cycler, 0.2 mL instrument. A recombinant high-fidelity DNA polymerase and gene-specific primers in the HIV-1 Reagent Plate of the *Sentosa*® SQ HIV-1 Genotyping Reagents are used to amplify the target DNA sequences using a 1-step RT-PCR approach.

Library Preparation

The amplified PCR products in the *Sentosa*® SX Barcoded PCR Plate 96 are loaded onto the *Sentosa*® SX101 worktable and undergoes a library preparation procedure. A series of automated chemical and enzymatic processes using reagents provided in the *Sentosa*® SQ Virus Testing Solutions normalizes, shears and ligates barcodes to the PCR products. After the run, a DNA library in a 1.5 mL LoBind tube is ready for template preparation.

Isothermal Template Preparation and Chip-loading

The 1.5 mL Lo-bind Tube containing the collected DNA library is loaded onto the *Sentosa*® SX101 worktable together with the *Sentosa*® SX IA Template Prep Kit, and the *Sentosa*® SQ 318 Chip (on the *Sentosa*® SQ 318 Chip Rack). Here, the DNA library is rapidly templated to Ion Sphere™ Particles (ISPs) at a single temperature. Positive ISPs carrying the DNA templates are then enriched automatically. Next, the enriched ISPs are primed for sequencing and finally loaded onto the *Sentosa*® SQ 318 Chip Kit automatically by the *Sentosa*® SX101 instrument. The positive ISPs are then ready for sequencing on the *Sentosa*® SQ301 Sequencer.

Sequencing

Proceed to sequencing on the *Sentosa*® SQ301 Sequencer within 15 minutes after the end of the *Sentosa*® SX101 instrument run. For more information, please refer to "Procedure" section.

Report generation

The *Sentosa*® SQ Reporter generates three (3) reports:

1) Quality Control Report which provides overall run performances.
2) Laboratory Test Report which uses a proprietary bioinformatics pipeline to compare sequences detected by the *Sentosa*® SQ301 Sequencer with reference HIV sequences and the Stanford University HIV Drug Resistance Database to identify genotypes and mutations.
3) HIV Drug Resistance Interpretation which reports to the physician a list of drug-resistance mutations detected and resistance / susceptibility to drugs according to the Stanford University HIV Drug Resistance Database.

Quality Control Procedures

The *Sentosa*® SQ HIV-1 Genotyping Assay contains a HIV-1 System Control (SC) and an Extraction Control (EC). The primer mix provided in this kit is designed for the specific amplification of SC and EC in addition to targeted HIV-1 sequences.

EC is added to all samples - functioning as a control for the nucleic acid extraction procedure. It is also used to monitor PCR inhibition.

NOTE: The presence of EC does not compromise the analytical detection limit of the assay.

SC functions as a no template control (NTC). SC is a non-HIV-1 RNA (control), thus it is able to function as a NTC through monitoring instrument / workflow issues, such as reagent and / or environmental contamination by HIV-1 nucleic acids.

The HIV-1 PC is a recombinant non-infectious alpha-virus containing the target HIV-1 nucleic acids. It is used to control for extraction, amplification, sequencing and detection. The PC mimics a patient specimen with a HIV-1 viral load of 5000 copies/mL and contains 10 representative drug resistance markers (M46I, I50L and L90M in Protease; M41L, K103N, M184V and M230L in Reverse Transcriptase; T66A, E92Q and Y143C in Integrase) at 20% variant frequency in a pooled plasma matrix.

Reagents

Sentosa® SQ HIV-1 Genotyping Reagents kit is comprised of the following:

Item	Cap color	Description	Quantity	Volume / tube
HIV-1 RP (24)	N/A	HIV-1 Reagent Plate	4 plates	N/A
SC	Red	HIV-1 System Control	4 tubes	730 µL
EC	Yellow	Extraction Control	4 tubes	200 µL

CONTROL SC: Linearized IVT-RNA with HIV-1 sequences (<0.01%) in buffer.

EC**:** Linearized plasmid with sequence amplified by extraction control primers and probe (<0.01%) in buffer with carrier RNA (polyA).

Warnings and precautions

General / Safety precautions

- For *In Vitro* Diagnostic Use.
- Use sterile pipette tips with filters.
- During manual steps, ensure that the tubes are closed when possible to avoid contamination.
- **Do not mix components from kits with different lot numbers.**
- All reagents should be thawed completely before use.
- All reagents are designed for single-use only. Do not use the remaining reagents from previous runs.
- The products contain substances at concentrations that require hazardous labeling in accordance with Directive 1272/2008/EC. We recommend handling all chemicals with caution. Refer to the Material Safety Data Sheet (MSDS) (PS103469) for more information.
- Wear personal protective equipment.
- Discard samples and waste according to the local safety regulations.
- All relevant documents (refer to the "Resources" section) should be read thoroughly before performing the assay.
- For safety information on the instruments, please refer to the relevant instrument user manual.

Laboratory precautions

- All samples and waste should be considered potentially infectious. Clean and disinfect all work surfaces thoroughly with disinfectants recommended by local authorities.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats and eye protection when handling samples and chemicals; kit reagents.
- Clean and decontaminate work area and instruments, including pipettes, with commercially available decontamination products.
- Use good laboratory practices to minimize cross-contamination. If possible, perform DNA library preparation and template preparation in segregated areas. Laboratory disinfectant[i] can be used for regular disinfection procedure to prevent contamination.
- Avoid microbial and nuclease contamination of reagents when removing aliquots from reagent bottles. Use sterile disposable pipette tips.
- Handle infectious materials according to the local regulations.
- A primary source of contamination is library DNA from previously processed samples. Always keep the library DNA enclosed in the specified containers. Aerosol-barrier tips are required for all pipetting steps. Change gloves frequently.
- To avoid environmental contamination by PCR amplicons, do not remove the PCR seal after amplification.
- Wash hands thoroughly after handling samples and kit reagents.

Storage

Sentosa® SQ HIV-1 Genotyping Assay & *Sentosa*® SQ HIV-1 Positive Control Kit

- Store at -25° to -15°C.
- Do not use beyond the expiration date.

Sentosa® SQ HIV-1 Genotyping Reagent

- Store at -25° to -15°C.
- Avoid multiple freeze-thaw cycles.
- Do not use beyond the expiration date.

Instruments

The *Sentosa*® SQ HIV-1 Genotyping Assay is performed on the *Sentosa*® SX101, the Veriti® DX 96-Well Thermal Cycler, 0.2 mL and the *Sentosa*® SQ301 Instruments. The relevant *Sentosa*® SX application must be installed in the *Sentosa*® SX software prior to performing the assay. Refer to the *Sentosa*® SQ HIV-1 Genotyping Assay User Manual (PS103472) for detailed operating procedures for the test.

For detailed information on software and instrument operations, refer to the *Sentosa*® SX101 User Manual (PS102963) and the *Sentosa*® SQ301 Platform – Sequencing User Guide (PS102958) for detailed procedures.

Specimen collection, handling and storage

The *Sentosa*® SQ HIV-1 Genotyping Assay is validated for use with EDTA plasma specimens.

Collection of whole blood and plasma

- It is recommended to collect whole blood in lavender-top tube(s) (EDTA-treated that are stored at room temperature (approximately 15°C to 25°C).
- After collection of whole blood sample(s), gently invert the tube(s) for 8 to 10 times.
- Store tube(s) upright at room temperature (up to two hours) before subjecting them to centrifugation.
- Retrieve plasma from whole blood via centrifugation of tube(s) at 1,900 x *g* for 10 minutes at 2 to 8°C (to remove cells from plasma).

Storage of plasma

[i] DNA / RNase AWAY Decontamination Reagent (Cat. No. 7010 / 7002) from Thermo Fisher Scientific is recommended.

- Transfer the supernatant (plasma) to clean, polypropylene tube(s) using a pipette, while ensuring 2 to 8°C when handling the sample(s).
- If the plasma specimen(s) are to be stored for lab testing, store the specimen(s) at 2 to 8°C for up to a maximum of 1 week. The plasma specimen(s) can be aliquoted into 1.5 mL tubes and stored at -20°C up to one month.

NOTE:

- Inadequate or inappropriate specimen collection, storage or transport is likely to yield false negative results.
- Avoid multiple freeze-thaw cycles.

Procedure

Materials provided

Sentosa® SQ HIV-1 Genotyping Assay

Materials and equipment required but not supplied with the assay

Equipment / software	Vela item no.
Pipettes (adjustable)	N/A
Vortex mixer	N/A
Bench top centrifuge	N/A
Sentosa® SX101 instrument	400089
Sentosa® SQ301 (120V) and accessories	690026
PX1™ PCR Plate Sealer	N/A
Veriti® Dx 96-Well Thermal Cycler, 0.2 mL	N/A
Sentosa® SX software	460018
Sentosa® SQ Suite	480174
Sentosa® SQ Reporter v 2.0	690237

Accessory / consumable / reagent	Vela item no.
Sentosa® SX Virus Total Nucleic Acid Plus Kit	300395
Sentosa® SQ HIV-1 Positive Control Kit	300729
Sentosa® SX IA Template Prep Kit	690233
Sentosa® SQ Sequencing Kit	690194
Sentosa® SQ 318 Chip Kit	300300
Sentosa® SQ 318 Chip Rack	400233
Sentosa® SX NGS Starter Kit	400105
Sentosa® SX Safe-Lock Tubes (1000)	400031
Sentosa® SX Partition 50 µL Filter Tips (960)	400026
Sentosa® SX Partition 1000 µL Filter Tips (960)	400025
Sentosa® SX Non-partition 50 µL Filter Tips (960)	400224
Sentosa® SX Non-partition 1000 µL Filter Tips (960)	400223
Sentosa® SX 100 mL Reservoir (50)	400027
Sentosa® SX 30 mL Reservoir (50)	400028
Sentosa® SX Deepwell Plates 96/2000 µL (20)	400068
Sentosa® SX Biohazard Bag (100)	400033
Sentosa® SX Magnetic Plate	400090
Sentosa® SX Barcoded PCR Plate 96	400100
Sterile pipette tips with filters	N/A
Pierceable Foil Heat Seal	N/A
0.5 mL / 1.5 mL DNA low binding tubes	N/A
100% Ethanol (molecular biology grade)	N/A
DNase / RNase-free water	N/A
Disinfectant	N/A

Important points before starting

- The operator must be familiar with operating the *Sentosa*® SX101, Veriti® Dx 96-Well Thermal Cycler, 0.2 mL, *Sentosa*® SQ301 instruments and the *Sentosa*® Workflow Assistant, *Sentosa*® SQ Suite and *Sentosa*® Reporter SQ software. Please refer to the respective user manuals supplied with the instruments for operating instructions.
- Ensure that the specified heat sealing program is created on the PX1™ PCR Plate Sealer instrument, and the specific PCR program is created on the Veriti® Dx 96-Well Thermal Cycler, 0.2 mL before use. Please refer to the respective user manuals for operating instructions, and the detailed procedure for the parameter settings.
- Ensure that all reagents of the *Sentosa*® SX Virus Total Nucleic Acid Plus Kit are not precipitated before use. If precipitates are observed, dissolve by incubating the reagent tube(s) / bottles(s) in a water bath (≥ 37°C).
- Prepare fresh 80% Ethanol (molecular biology grade) prior to use.
- Ensure that the samples are completely thawed and briefly centrifuged before use.
- All reagents are designed for single-use only. Do not use the remaining reagents from previous runs.
- Do not discard the *Sentosa*® SQ HIV-1 Genotyping Reagents box, the HIV-1 Reagent Plate primary packaging (ziplock bag) and the *Sentosa*® SX Virus Total Nucleic Acid Plus Kit box as the operator needs to scan the 2-D barcode on the packaging and boxes.

Sample preparation

- Transfer 730 µL of plasma sample to a 1.5 mL *Sentosa*® SX Safe-Lock Tube.

  NOTE:

  - For frozen plasma sample, completely thaw the sample and equilibrate to room temperature (approximately 15°C to 25°C). Vortex the sample for 5 seconds followed by brief centrifugation at 1,200 x *g*. Transfer the supernatant to a 1.5 mL *Sentosa*® SX Safe-Lock Tube.
  - Sample stored at 4°C should be equilibrated to room temperature. Vortex the sample for 5 seconds followed by brief centrifugation. Transfer the sample to a 1.5 mL *Sentosa*® SX Safe-Lock Tube.

Reagent preparation for nucleic acid extraction

Prepare the reagents from the *Sentosa*® SX Virus Total Nucleic Acid Plus Kit as follows:

- Virus Plus A1 tubes containing Proteinase K solution require gentle inversion followed by brief centrifugation. Virus Plus A2 tubes containing magnetic beads require thorough vortexing before use to ensure proper resuspension.
- **Prior to use, Virus Plus A3 (lyophilized carrier RNA) must be reconstituted with 125 µL of Virus Plus A4 (carrier RNA buffer) followed by brief centrifugation.**
- **Slowly pipette 120 µL of Virus Plus AF (antifoaming agent) into Virus Plus B1 (lysis buffer), followed by gentle swirling of Virus Plus B1 until the content becomes homogenous in visual appearance.**
- Mix the buffers in the bottles by gentle swirling, ensuring no foam or bubbles are present.
- The components of *Sentosa*® SQ HIV-1 Genotyping Reagents and HIV-1 PC from the *Sentosa*® SQ HIV-1 Positive Control Kit should be completely thawed before use.
  - Ensure that no air bubbles are present at the bottom of the HIV-1 RP (Reagent Plate).
  - Ensure that the HIV-1 RP is kept on ice until the beginning of the run.
  - Briefly spin the HIV-1 RP to collect the contents at the bottom of the wells.
  - Ensure that the HIV-1 EC, SC and PC are completely thawed followed by brief centrifugation before use.
- The components of *Sentosa*® SQ Virus Testing Solutions should be equilibrated at room temperature (approximately 15°C to 25°C) for at least 10 minutes prior to the run.
  - **Mix the CB1 (Clean Up Beads) and SB (Normalization Beads) tubes thoroughly to ensure the complete re-suspension of the beads prior to use.** The content should be homogenous in visual appearance.

Workflow overview

Nucleic acid extraction and PCR set-up, library preparation and template preparation via isothermal amplification are performed with the *Sentosa*® SX101 instrument. PCR is performed with the Veriti® DX 96-Well Thermal Cycler, 0.2 mL while sequencing is performed on the *Sentosa*® SQ301 Sequencer. Data analysis and interpretation are performed with the *Sentosa*® SQ Reporter software. The whole workflow and run plan is assisted by the *Sentosa*® Workflow Assistant software.

Nucleic acid extraction and PCR set-up

Once the samples and reagents are prepared, load them on the *Sentosa*® SX101 worktable together with the reagent plate, SC and EC tubes from the *Sentosa*® SQ HIV-1 Genotyping Reagents.

The procedure is automated using the *Sentosa*® SX101 instrument.

NOTE: Please refer to the layout as indicated by the software or the appendix in *Sentosa*® SQ HIV-1 Genotyping Assay user manual (PS103472).

Log in to the *Sentosa*® SX software. Select the "*Vela Dx*" account and choose the folder "*NGS Virology*". Run the application "*24_HIV_VirusPlusII_Pre*". Successive windows will assist in loading the prepared samples and reagents, labware and consumables on the worktable. Scanning of respective barcodes will be prompted.

On the final window, click *"Run"*.

After the run is complete, remove the *Sentosa*® SX Barcoded PCR Plate 96 (reaction plate) and apply a Pierceable Foil Heat Seal on it. Using the PX1™ PCR Plate Sealer, heat seal the plate at 170°C for 3 seconds.

Centrifuge the plate at 100 x *g* for 30 seconds.

PCR

Place the sealed reaction plate in the Veriti® Dx 96-Well Thermal Cycler, 0.2 mL. Set the PCR amplification program according to the following table.

Step	Temperature (°C)	Duration	Number of cycles
Reverse transcription (RT)	50	20 minutes	1
RT inactivation & initial denaturation	95	2 minutes	
PCR amplification cycle	95	15 seconds	3
	59 (-2 after each cycle)	30 seconds	
	72	2 minutes	
	95	15 seconds	37
	53	30 seconds	
	72	2 minutes	
Hold	10	∞	-

NOTE:

- All ramp rates are set at the maximum.
- The reaction volume is 25 µL.
- The lid temperature for the thermal cycler is 105°C.

After PCR amplification is complete, remove the reaction plate from the Veriti® Dx 96-Well Thermal Cycler, 0.2 mL and centrifuge the reaction plate at 100 x *g* for 30 seconds to collect the contents at the bottom of the plate.

Library preparation

Load the reaction plate and solutions tubes from *Sentosa*® SQ Virus Testing Solutions onto the *Sentosa*® SX101 worktable. The procedure is automated using the *Sentosa*® SX101 instrument.

NOTE: Please refer to the layout as indicated by the software or the appendix in *Sentosa*® SQ HIV-1 Genotyping Assay user manual (PS103472).

Log in to the *Sentosa*® SX software. Select the "*Vela Dx*" account and choose the folder "*NGS Virology*". Run the application "*24_HIV_LibPrep_Post*". Successive windows will assist in loading the prepared samples and reagents, labware and consumables on the worktable. Scanning of respective barcodes will be prompted.

On the final window, click *"Run"*.

After the run, aliquots of the DNA libraries are pooled into a 1.5 mL DNA low binding tube (pooled library tube).

The sample ID list file (".xml") generated by the *Sentosa*® SX software will be automatically transferred to the *Sentosa*® Workflow Assistant.

Plan a run

Clean the Sentosa® SQ301 Sequencer:

- Log in to the *Sentosa*® SQ301 Sequencer.
- On the main menu of the touchscreen user interface, press the *"Clean"* icon.
- Select either *"18 MΩ water cleaning"* or *"Chlorite cleaning"*. For further information, please refer to the *Sentosa*® NGS workflow – Sequencing User Guide (PS102958).
- Follow the prompts displayed on the *Sentosa*® SQ301 Sequencer touchscreen.

Initialize the Sentosa® SQ301 Sequencer:

- After cleaning is complete, press the *"Initialize"* icon.
- Determine the number of sequencing runs planned for the initialization.

  NOTE: Up to two sequencing runs may be performed from one initialization, if both runs are started within 27 hours after initialization.
- Scan the data matrix barcode on the box of the *Sentosa*® SQ Sequencing Reagents used for the particular run.
- Follow the prompts displayed on the *Sentosa*® SQ301 Sequencer touchscreen.

Plan a run on the Sentosa® Workflow Assistant:

- Log in to the *Sentosa*® Workflow Assistant software.
- Click "*Create*" on the applicable plan run under the "*To Be Planned"* section.
- Key in the "*Tube Label*" and "Template Kit Barcode" in their respective fields and press / click "*Send".*
- Log in to the *Sentosa*® SQ Suite in the web browser and take note of the "*Run Short Code*" e.g. *"6YMFY*", and proceed to the *Sentosa*® SQ301 sequencer that was initialized previously.

  NOTE: Ensure that *Sentosa*® SQ Suite v5.6 is installed.
- On the touch-screen LCD screen of the *Sentosa*® SQ301 sequencer, press *"Run"* and follow the instructions in the interface. Scan / Manually key in the "*Run Short Code*" (from the *Sentosa*® SQ Suite) and press "*Next*" to continue.
- Follow the prompts displayed on the *Sentosa*® SQ301 Sequencer touchscreen and prepare the *Sentosa*® SQ318 Chip accordingly.

  NOTE: Please refer to the *Sentosa*® SQ HIV-1 Genotyping Assay user manual (PS103472) for more information.

Template preparation (Isothermal Amplification)

Load the tubes from the *Sentosa*® SX IA Template Prep Kit onto the *Sentosa*® SQ 318 Chip Rack on the *Sentosa*® SX101 worktable. Ensure that the pooled library tube and *Sentosa*® SQ318 Chip are also loaded onto the Chip Rack. The procedure is automated using the *Sentosa*® SX101 instrument.

NOTE: Please refer to the layout as indicated by the software or the appendix in *Sentosa*® SQ HIV-1 Genotyping Assay user manual (PS103472).

Log in to the *Sentosa*® SX software. Select the "*Vela Dx*" account and choose the folder "*NGS Virology*". Run the *"IA_ES_ChipLoading"* application. Successive windows will assist in loading the prepared samples and reagents, labware and consumables on the worktable. Scanning of respective barcodes will be prompted.

On the final window, click *"Run"*.

After the run is complete, the sample will be loaded onto the *Sentosa*® SQ 318 Chip.

Sequencing

Proceed to sequencing on the *Sentosa*® SQ301 Sequencer within 15 minutes after the end of the *Sentosa*® SX101 instrument run. The *Sentosa*® SQ Sequencing Kit will be required in the sequencing procedures. Refer to the *Sentosa*® SQ301 Platform – Sequencing User Guide (PS102958) for more details.

Start the run:

- Remove excess liquid from the *Sentosa*® SQ 318 Chip.

  NOTE: Please refer to the *Sentosa*® SQ HIV-1 Genotyping Assay user manual (PS103472) for more information.
- Press the *"Keyboard"* button next to the *"Top barcode"* field. Using the barcode scanner attached to the sequencer, scan the barcode located on the top of the newly loaded chip. Press *"OK"*.

- Remove the used chip and secure the loaded chip in the chip clamp of the *Sentosa*® SQ301 sequencer.
- Press *"Next"* on the touchscreen to calibrate the loaded chip. Visually inspect the chip for leaks before closing the instrument cover. Close the lid when prompted to do so.
- When the calibration is complete (approximately 1 minute), the touchscreen will indicate whether the calibration was successful.
- The *Sentosa*® SQ301 Sequencer will automatically begin the sequencing run. The sequencing run takes approximately 4.5 hours to complete.
- When the run is complete, the touchscreen will return to the main menu. Review the run data using the *Sentosa*® SQ Suite Browser.

Data interpretation

After successful completion of template preparation by the *Sentosa*® SX101 instrument and sequencing by the *Sentosa*® SQ301 Sequencer, *Sentosa*® SQ Suite software performs primary analysis (signal processing and base-calling) on the raw sequencing data generated by *Sentosa*® SQ301 Server. After primary analysis, the data is transferred to *Sentosa*® SQ Reporter for secondary analysis and report generation.

If the sequencing run passes *Sentosa*® SQ301 primary analysis criteria, the data will be automatically transferred and analyzed in *Sentosa*® SQ Reporter and reported under the "*Pending Decision*" tab. If the run fails *Sentosa*® SQ301 primary analysis criteria, the data will be reported under the "*Sequencing Failure*" tab.

Click the "*View Run Statistics*" button to access the details for the respective runs. Quality Control (QC) criteria can be accessed under the *"QC Report"* tab for each sample. QC criteria are divided into two main groups: Run Quality Controls and Sample Quality Controls.

All criteria can have "*PASSED*" or "*FAILED*" status. If any of the Run Quality Controls criteria fails then the whole run is invalid. If any of the Sample Quality Controls criteria fails then the particular sample(s) is invalid. Some criteria can have *"WARNING"* status meaning that the data is valid but needs attention. The details are described in "Results" section.

Limitations

- The product is to be used by trained personnel only.
- Detection of HIV-1 is dependent on the number of viral particles collected. Optimal performance of this test requires appropriate specimen collection, storage, and transport to the test site (refer to the **SPECIMEN COLLECTION, HANDLING AND STORAGE** section of this package insert).
- This test is for use with plasma specimens collected from whole blood samples stored in lavender-top tube(s) (EDTA-treated). Performance characteristics of other specimen types have not been established.
- The assay is designed to detect HIV-1 Group M subtypes and recombinant viral sequences. However, as the HIV-1 genome is highly mutable, there will always be a small possibility that some strains of HIV-1 will react poorly with the assay, especially if random mutations occur within the primer binding sites.
- Detection of a genomic drug resistance mutation may not correlate with phenotypic gene expression.
- This test does not detect mutations in HIV-1 Group O or HIV-2 as performance with these has not been established.
- The lack of detection of a drug-resistance mutation does not preclude the possibility of genetic mutation. The treating physician should use other laboratory tests and clinical information to manage the patients.
- This test will not detect genetic mutations outside the scope of this assay (see details for Gag mutations on page 8).
- Mutation drug resistance identification was made by comparing sequences generated to the Stanford University HIV Drug Resistance Database v8.5.

Expected results

Run QC Metric	Range
Run Throughput (bp)	Optimal ≥ 60,000,000 Min ≥ 125,000
Loading (%)	≥ 15.00
Invalid Reads (%)	≤ 10.00
Invalid Bases (%)	≤ 10.00
HIV-1 Positive Control Sample	PC sample QC passed (see below)
System Control / Extraction Control Median Coverage	≥ 50
No Template Control	HIV-1 Reads ≤ 833 or 0.001 * Control Amplicon Reads whichever is higher

HIV-1 PC QC Metric	Expected Results
HIV-1 PC Sample Mutations	≥ 9 expected mutations detected
Integrase Assembly	Median Cov. ≥ 1000 Min Cov. ≥ 50
Protease / RT Assembly	

Sample QC Metric	Range
Sample Throughput (bp)	Optimal ≥ 2,500,000 Min ≥ 125,000
Integrase Assembly	Median Cov. ≥ 1000 Min Cov. ≥ 50
Protease / RT Assembly	
Control Amplicon Median Coverage	≥ 50

Specific performance characteristics

Analytical sensitivity

The analytical limit of detection (LOD1) is defined as the lowest concentration at which ⩾ 95% of tested replicates showed presumptive positive for the detection of PR, RT and IN genomic targets of HIV-1.

LOD1 is 1000 copies/mL for HIV-1 subtypes A, B, C, D, F, G, H, J & K.

LOD2, defined as the minimum variant frequency of the targeted mutations that can be detected at LOD1 (1000 copies/mL), was determined to be 20%.

LOD3, defined as the lowest viral load when the variant frequency of 5% and 10% that can be correctly detected in ⩾ 90% of HIV-1 Group M in human plasma samples, was determined to be 15000 copies/mL and 5000 copies/mL, respectively.

Analytical reactivity and specificity

The *Sentosa*® SQ HIV-1 Genotyping Assay is able to detect clinical samples from strains that belonged to all HIV-1 Group M subtypes (A, B, C, D, F, G, H, J and K) at LOD1 (1000 copies/mL). The following microorganisms and interfering substances at the maximum concentrations tested do not interfere with the assay.

List of unreactive organisms

Organism tested	Max Concentration
Human immunodeficiency virus type 2 (HIV-2)	1 x 10^6 copies/mL
Human T-lymphotropic virus type I (HTLV-I)	115.18 S/CO
Human T-lymphotropic virus type II (HTLV-II)	20 Ct
Cytomegalovirus (CMV)	1 x 10^6 copies/mL
Epstein Barr virus (EBV)	1 x 10^6 copies/mL
Hepatitis B Virus (HBV)	1 x 10^6 copies/mL
Hepatitis C Virus (HCV)	1 x 10^6 copies/mL
Mycobacterium tuberculosis	1 x 10^6 copies/mL
Mycobacterium intracellulare	1 x 10^6 copies/mL
Mycobacterium avium	1 x 10^6 copies/mL
Candida albicans	1 x 10^6 copies/mL
Pneumocystis jirovecii	1 x 10^6 copies/mL
Homo sapiens, genomic DNA	1 µg

List of unreactive substances

Interfering substance		Max concentration
Hemoglobin Human		0.05 mg/mL
Lipid standards - triglycerides		1.9 mg/mL
Albumin, Human Fraction V Powder		55 mg/mL
Bilirubin		0.012 mg/mL
EDTA		6.16 mM
Alpha-interferon		4.27 pg/mL
Interferon- α 2a		2580 pg/mL
Interferon- α 2b		263 pg/mL
Ribavirin		7.08 µg/mL
Ganciclovir		13.2 µg/mL
Foscarnet		0.08 mg/mL
Antimycobacterial (Rifampin)		32 µg/mL
Antimycobacterial (Isoniazid)		7 µg/mL
Antimycobacterial (Pyrazinamide)		64.6 µg/mL
Ciprofloxacin		11.8 µg/mL
Tenofovir disoproxil fumarate, TNV		0.6 µg/mL
Entecavir		19.1 ng/mL
Valacyclovir		7.55 µg/mL
Azithromycin		9.91 µg/mL
Drug pool 1	Amprenavir	12.47 µg/mL
	Darunavir Ethanolate	13.1 µg/mL
	Enfuvirtide Acetate Salt	14.8 µg/mL
	Efavirenz	10 µg/mL
Drug pool 2	Indinavir, European Pharmacopoeia	17.316 µg/mL
	Saquinavir Meslyate	11.2 µg/mL
	Lopinavir, European Pharmacopoeia	18.69 µg/mL
	Nelfinavir Mesylate Hydrate	6.7 µg/mL
	Emtricitabine, United States Pharmacopei	4.7 µg/mL
Drug pool 3	Zidovudine, European Pharmacopoeia	26.3 µM
	Didanosine, European Pharmacopoeia	12.1 µg/mL
	Stavudine, European Pharmacopoeia (Zerit)	1.276 µg/mL
	Ritonavir	38.5 µM
	Maraviroc	888 ng/mL
Drug pool 4	Lamivudine	7 ug/mL
	Abacavir Sulfate (Ziagen)	5.55 µg/mL
	Delavirdine Mesylate (Rescriptor)	100 µM
	Nevirapine	20 µg/mL

Assay Stability

The performance of the *Sentosa*® SQ HIV-1 Genotyping Assay was not affected by short term storage of reagents at ambient temperature up to 3h, and up to 10 freeze-thaw cycles.

Sample Stability

The performance of the *Sentosa*® SQ HIV-1 Genotyping Assay was not compromised when specimens were stored at ambient temperature up to 24 hours, 4°C up to 7 days, -20°C and -80°C for 60 days, and after 10 freeze-thaw cycles.

Analytical Reproducibility and Precision

Analytical reproducibility of the *Sentosa*® SQ HIV-1 Genotyping Assay was tested using a panel of HIV-1-positive samples in 30 runs across 3 test sites, where each site used 1 lot of reagents, 1 set of instruments and 2 operators. The panel of HIV-1 samples has 2 HIV-1 PC, 4 different HIV-1 subtypes at 3x LOD1 in triplicate and 3 HIV-1 mixtures, with 2 strains of viruses mixed at 5% and 95% resulting in mutations at 5%, 95% and 100% frequencies at 3x LOD3 in triplicate High analytical reproducibility was evidenced by Percent Agreement being >95%. For all tests, sample and mutation detection rates were ≥95%, and coefficient of variance (CV) of mutation frequencies measurements were <5% on average inter-assay. Intra-assay CV was <5% for >95% mutation frequencies and 16.6 – 35.96% for 5% mutation frequencies.

Reproducibility of sample and mutation detection

	Site 1		Site 2		Site 3		Percent Agreement, %
Sample	Detection	95% CI, %	Detection	95% CI, %	Detection	95% CI, %	
Run (n=10/site)	10	-	10	-	10	-	
Sample Detection (21/run)	100% (210/ 210)	(98.2, 100)	100% (210/ 210)	(98.2, 100)	100% (210 /210)	(98.2, 100)	100
5% DRM	97.0% (291/ 300)	(94.4, 98.4)	99.3% (298/ 300)	(97.6, 99.8)	97.0% (291 /300)	(94.4, 98.4)	95.01
95% DRM	100% (120/ 120)	(96.9, 100)	100% (120/ 120)	(96.9, 100)	100% (120/ 120)	(96.9, 100)	100
100% DRM	100% (60/60)	(94.0, 100)	100% (60/60)	(94.0, 100)	100% (60/60)	(94.0, 100)	100
All DRM	98.1 (471/ 480)	(98.5, 99.0)	99.6 (478/ 480)	(98.5, 99.9)	98.1 (471/ 480)	(96.5, 99.0)	96.01
HIV-1 PC Detection (2 PC/run)	100% (20/20)	(83.9, 100)	100% (20/20)	(83.9, 100)	100% (20/20)	(83.9, 100)	100
PC mutation	100% (200/ 200)	(98.1, 100)	100% (200/ 200)	(98.1, 100)	98% (196/ 200)	(95.0, 99.2)	98.69

Reproducibility and precision of detected mutation frequencies

		% CV at 5% VF				%CV at 95% VF	%CV at 100% VF
		PR	RT	IN	Overall	PR*	PR*
Inter-assay	Site-to-site Lot-to-lot Instrument-to-Instrument	5.06	3.17	5.46	4.44	0.39	0.23
Intra-assay	Site 1	26.85	31.68	18.96	28.38	3.56	0.42
	Site 2	32.60	28.34	21.79	31.40	4.19	0.25
	Site 3	28.99	35.96	16.16	30.50	3.43	0.32

*These samples did not include mutations in RT or IN

Clinical Reproducibility

Clinical reproducibility assesses the *Sentosa*® SQ HIV-1 Genotyping Assay under clinical settings. A panel of 20 HIV-1-positive samples at 4,000 copies/mL were tested over three 3 runs at the 3 test sites. Reproducibility of sample detection, mutation detection and HIV-1 PC performance were considered. For mutation detection, a 10% mutant frequency threshold was also applied to account for the assay's LOD3. However, for completeness, reproducibility data for <10% mutation frequencies are also included. Percent agreement was used to evaluate similarity of data generated by the 3 test sites.

Clinical Reproducibility

Reproducibility Test	Total	Agreement between sites				All others	Overall Percent Agreement
		S1(3/3) S2(3/3) S3(3/3)	S1(2/3) S2(2/3) S3(2/3)	S1(1/3) S2(1/3) S3(1/3)	S1(0/3) S2(0/3) S3(0/3)		
Sample detection	20	19	0	0	0	1	95.0% (19/20)
Mutant Variant ≥ 20% VF	161	149	0	0	3	9	94.41% (152/161)
Mutant Variant 10-20% VF	7	7	0	0	0	0	100% (7/7)
Mutant Variant 5-10% VF	22	6	1	2	0	13	40.91% (9/22)
Mutant Variant <5% VF	46	0	0	1	0	45	2.17% (1/46)

Reproducibility Test	Total	Complete agreement		All others	Percent Agreement
		S1(9/9) S2(9/9) S3(9/9)	*S1(x/9) S2(x/9) S3(x/9)		
PC detection	1	1	0	0	100% (1/1)
PC mutant detection	10	9	0	1	90.0% (9/10)

*Total counts of complete agreement for x=8, 7, 6, 5, 4, 3, 2, 1 and 0

Clinical Sensitivity and Specificity

The clinical sensitivity and specificity of the *Sentosa*® SQ HIV-1 Genotyping Assay were validated in correlation with Sanger sequencing, using 20 HIV-1 positive clinical plasma samples. To account for LOD of Sanger sequencing and for a fair comparison, only mutations with >20% variant frequency are presented.

Clinical sensitivity and specificity

Overall		Sanger Sequencing-based methods (Reference)		
		>20% VF		
		Mutant	Non-mutant	Total
Sentosa® SQ HIV-1 Genotyping Assay (Test)	Mutant	1376	36	1412
	Non-mutant	37	60111	60148
	Total	1413	60147	61560
	Sensitivity:	97.38% (1376/1413) 95%CI: (96.41%, 98.09%)		
	Specificity:	99.94% (60111/60147) 95% CI: (99.92%, 99.97%)		

Population Sensitivity and Specificity

The population sensitivity and specificity of the *Sentosa*® SQ HIV-1 Genotyping Assay were validated in correlation with Sanger sequencing, using 107 HIV-1 positive clinical plasma samples. To account for LOD of Sanger sequencing and for a fair comparison, data with >20% variant frequency cutoff only presented.

Population sensitivity and specificity

Overall		Sanger Sequencing-based methods (Reference)		
		>20% VF		
		Mutant	Non-mutant	Total
Sentosa® SQ HIV-1 Genotyping Assay (Test)	Mutant	819	32	851
	Non-mutant	33	35514	35547
	Total	852	35546	36398
	Sensitivity:	96.13% (819/852) 95%CI: (94.61%, 97.23%)		
	Specificity:	99.91% (35514/35546) 95% CI: (99.87%, 99.94%)		

Sentosa® *SQ HIV-1 Genotyping Assay DRM target list*

The *Sentosa*® SQ HIV-1 Genotyping Assay has a target list of 342 DRMs reportable as a consequence of NGS technology. These 342 DRMs includes DRMs listed in CBER200222, FDA Guidance for Industry Class II Special Controls Guidance Document- *In Vitro* HIV-1 Drug Resistance Genotype Assay (henceforth referred to as Guidance Document). The DRM target list can be classified into 4 categories:

i) 121 Verified and Validated DRMs (Table i)
ii) 53 Verified only DRMs (Table ii)
iii) 36 Validated only DRMs (Table iii)
iv) 132 Not verified and not validated DRMs (Table iv)

Verified DRMs are defined as mutations detected with at least 10 replicates with >90% accuracy during the *Sentosa*® SQ HIV-1 Genotyping Assay verification studies.

Validated DRMs are defined as mutations detected by the *Sentosa*® SQ HIV-1 Genotyping Assay in 127 samples and confirmed with Sanger sequencing during clinical validation.

Not verified and not validated DRMs are defined as mutations that did not meet the criteria of both verified DRMs and validated DRMs.

DRMs listed in Tables A-E of Guidance Document are as indicated in superscript where applicable in Tables i to iv.

Table i. Verified and Validated DRMs

Protease		RT		Integrase
L10I[C]	D60E	M41L[A,D]	E138Q	T66I
L10V[C]	I62V	E44D[D]	V179D	L74I
V11I	L63P	A62V[A,D]	V179I	L74M
I15V	I64L	K65R[A,D]	Y181C[A,E]	E92Q
G16E	I64M	D67N[A,D]	Y181I[A,E]	T97A
K20I	I64V	S68G[A]	M184I[A,D]	E138K
K20M[C]	H69K	T69D[A,D]	M184V[A,D]	G140S
K20R[C]	H69Q	T69N	Y188L[A,E]	S147G
K20T	H69Y	K70E	V189I	Q148H
V32I[C]	A71T[C]	K70R[A,D]	G190A[E]	Q148R
L33F[C]	A71V[C]	L74I	L210W[A,D]	V151I
L33V	G73C	V75M	T215D	N155H
E34Q	G73S[C]	V90I	T215E	K156N
E35N	G73T	A98G	T215F[A,D]	E157Q
M36I[C]	T74S	A98S	T215H	G163E
M36L	V77I[C]	L100I[A,E]	T215Y[A,D]	G163K
K43R	V82A[B,C]	K101E	K219E[A]	G163Q
K43T	V82I	K101Q	K219N	G193E
M46I[B,C]	I85V	K101R	K219Q[A,D]	T206S
M46L[C]	N88D[B,C]	K103N[A,E]	K219R	S230N
I47A	N88S	K103R	H221Y	R263K
I50L	L89I	K103S	P225H[E]	
I50V[B,C]	L89M	V106M	K238T	
F53L	L90M[B,C]	V108I[A,E]		
I54L[C]	I93L	Y115F[A,D]		
I54V[B,C]		V118I[D]		

Table ii. Verified only DRMs

Protease		RT		Integrase
L10F[C]	Q58E	D67E	Q151M[A,D]	T66K
V11L	T74P	D67G	I167V	G140A
L23I	L76V	T69S	V179F	Y143R
L24IC	V82C	T69del	V179T	Q148K
M46V	V82L	L74V[A,D]	Y181F	
I47V[C]	V82M	V75I[A,D]	Y188C[A,E]	
G48M	V82S[B,C]	V75L	G190E	
G48Q	V82T[B,C]	K101H	G190S[E]	
G48V[B,C]	N83D	K101T	G190T	
F53Y	I84V[B,C]	K103H	L210S	
I54A		V106A[A,E]	M230L	
I54M		F116Y[A,D]	K238R	
I54S		I132L	N348I	

Table iii. Validated only DRMs

Protease	RT		Integrase
K20V	T69A	V179L	T66A
D30N[B,C]	K70N	Y181V	L68V
L33I	K70Q	Y188H[E]	A128T
E35G	K70T	L210F	E138D
I66F	K101P	T215A	Y143C
A71I	V106I	T215C	Q146K
T74A	E138A	T215L	G193R
L89V	E138G	T215N	S230R
I93M	E138K	T215S	
	V179E		

Table iv. Not Verified and Not Validated DRMs

Protease		RT		Integrase	
L10C	A71L	E40F	I132M	A49G	S153F
L10M	G73A[C]	M41I	E138R	H51Y	S153Y
L10R[C]	G73D	E44A	E138S	V54I	N155S
L10Y	G73F	K65E	Q151L	E92G	N155T
I15A	G73V	K65N	V179M	E92V	G163R
K20N	T74E	D67H	Y181G	Q95K	G163S
L24F	V77A	D67Q	Y181S	H114Y	S230G
L24M	V77T	D67S	Y188F	G118R	
V32L	V82F[B,C]	D67T	G190C	F121Y	
L33M	V82G	D67del	G190Q	E138A	
E34V	N83S	T69E	G190V	E138T	
M36V	I84A	T69G	T215G	G140C	
L38W	I84C	T69I	T215I	Y143A	
R41I	N88G	T69K	T215V	Y143G	
R41T	N88T	T69ins[A,D]	K219H	Y143H	
K45I	L89R	K70G	K219W	Y143K	
M46T	L89T	K70S	F227C	Y143S	
G48A	C95F	V75A	F227L	P145S	
G48L		V75S	M230I	Q146I	
G48S		V75T[D]	M230V	Q146L	
G48T		F77L[A,D]	Y232H	Q146P	
F53W		L100V	Y232N	Q146R	
I54T		K101I	L234I	Q148E	
H69I		K101N	P236L[E]	Q148G	
H69N		K103E	K238N	Q148N	
H69R		K103Q	Y318F	V151A	
A71G		K103T		V151L	

HIV-1 gag mutations

The *Sentosa*® SQ HIV-1 Genotyping Assay does not interrogate the HIV-1 *gag* gene. However, mutations in *gag* cleavage sites may confer resistance to all protease inhibitors (PI) and may emerge before mutations in protease [10]. A large proportion of virus samples from patients with confirmed virologic failure on a PI-containing regimen are not found to have PI resistance–associated mutations. Preliminary data from recent studies suggest that several mutations in the Gag protein may be responsible for reduced PI susceptibility in a subset of these patients [11].

Instrument maintenance

After every run, discard used sample tubes, plates, reagents and tips according to the local safety regulations. All samples and waste should be considered potentially infectious.

A reservoir collects liquid waste generated during the nucleic acid extraction procedure. Dispose of the liquid waste according to the local safety and environmental regulations. Dispose of the biohazard bags after each run.

Perform regular cleaning of the *Sentosa*® SX101, the Veriti® Dx 96-Well Thermal Cycler, 0.2 mL and the *Sentosa*® SQ301 instruments after each run. Please refer to the respective instrument user manuals for detailed procedures.

Ensure that maintenance is performed regularly to minimize the risk of error. Always wear the appropriate personal protective equipment (PPE: laboratory coat, gloves, goggles) during cleaning / maintenance procedures.

Resources

The latest edition of the MSDS and user manuals of the *Sentosa*® SQ HIV-1 Genotyping Assay, the *Sentosa*® SX Virus Total Nucleic Acid Plus Kit and the *Sentosa*® SX IA Template Prep Kit are available for download at www.veladx.com by logging in as an authorized user, or requesting them via email.

Symbols used

Symbol	Description
	Contains reagents sufficient for <*n*> tests
	Use-by date
	Do not reuse
	Catalog number
	Component
	Number
	Content
	Lot number
	Control
	Document / label identification number
	Temperature limit
	Manufacturer
	Consult instructions for use
	Cut here

Assay name: *Sentosa*® SQ HIV-1 Genotyping Reagents

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