Highly Automated Deep Sequencing-based HIV-1 Drug Resistance Monitoring System

Dr. Elian Rakhmanaliev
Senior Scientist
Vela Research Pte. Ltd.

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Background

**Facts that encouraged us to develop HIV-1 Drug Resistance Monitoring System:**

• Resistance of HIV to antiretroviral drugs is the most common cause for therapeutic failure in people infected with HIV;

• Timely detection and reporting of drug resistant mutations (DRMs) is critical for drug regimen;

• All HIV strains contain DRMs;

• Limited choice of HIV-1 drug resistance monitoring systems.
HIVDR Monitoring Systems Available on the Market

HIV-1 drug resistance monitoring systems approved for *in vitro* diagnostics (IVD) by FDA:

1) Sanger sequencing-based ViroSeq™ HIV-1 genotyping system (Applied Biosystems);

2) CLIP-based TruGene HIV-1 Genotyping Kit (SIEMENS) – discontinued in 2016.

Several Sanger sequencing and NGS-based Research Use Only (RUO) assays and Laboratory Developed Tests (LDT).
Technology Choice

Sanger sequencing. Open questions:
What is correct mutant sequence – CTT or TGA or CTA or TTT or CGA or CGT or TGT?
How many strains? Two or more than two?

Deep or Next Generation Sequencing (NGS)

Strain 1: A>C, A>G, G>A, T>C
Strain 2: A>T, G>A

Major advantages:
1) Ability to distinguish between different strains;
2) Sensitive detection of low-frequency variants (up to 5%).
HIV Genome

- Targets: Protease, Reverse Transcriptase and Integrase regions of the *pol* gene.
- Number of amplicons: 2 (~1500 bp (Protease and RT) and ~1000 bp (Integrase)).
- The amplicons cover 272 target variants in 103 codons across three target genes.
Sentosa® SQ HIV NGS Workflow
TAT is ~27 hours with hands-on time ~3.5 hours

1. RNA Extraction
   - Time: 2 hrs. 45 min
   - Instruments: PX1 Plate Sealer, Veriti Dx Cycler
   - Reagents: Sentosa® SX Virus Total Nucleic Acid Plus II (4x16) Kit
   - Software: Sentosa® SX101 Software

2. Library Preparation
   - Time: 8.5 hrs.
   - Instruments: ST401i, ST401e
   - Reagents: Sentosa® SQ HIV Genotyping Assay (4x16)
   - Software: Sentosa® ST Template Kit

3. Template Preparation
   - Time: 7 hrs.
   - Instruments: ST401i, ST401e
   - Reagents: Sentosa® ST Template Kit
   - Software: Sentosa® SQ Suite

4. Sequencing
   - Time: 5.5 hrs.
   - Instruments: Sentosa® SQ301
   - Reagents: Sentosa® SQ Sequencing Kit
   - Software: Sentosa® Link

5. Data Analysis and Reporting
   - Time: 3.5 hrs.
   - Instruments: Sentosa® SQ Reporter Server
   - Reagents: Sentosa® SQ 318 Chip Kit
   - Software: Sentosa® SQ Reporter

Notes:
* PX1 PCR Plate Sealer, Bio-Rad (Mat. No: 181-4000). Not available from Vela Diagnostics
** Veriti Dx 96-well Cycler, Life Technologies (Mat. No: 4452300). Not available from Vela Diagnostics
Sentosa® SQ Reporter

Reporting System – Pathology Report

Pathology Report

Patient Information:
- Name of Patient: [Name]
- Date of Collection: [Date]
- Name of Lab: [Lab Name]
- Age: [Age]
- Gender: [Gender]
- Specimen Type: [Type]
- DNA Quality/Yield: [Value]
- Tumor Cellularity: [Cellularity]

Pathology Comments:
- Pathologist's signature: [Signature]
- Name printed: [Name]
- Date: [Date]

Genotypes:

<table>
<thead>
<tr>
<th>No.</th>
<th>Subtype</th>
<th>Number of Contigs</th>
<th>Total Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PROTEASE</td>
<td>1</td>
<td>67556</td>
</tr>
<tr>
<td>2</td>
<td>INTEGRASE</td>
<td>1</td>
<td>5891</td>
</tr>
</tbody>
</table>

Interpretation:
1 subtype detected: G

Methodology:
Targeted next-generation sequencing was performed on this sample. HIV RNA was extracted from sample and was used for one-step RT-PCR. The amplicons were enzymatically fragmented and ligated with barcoded adaptor and subjected to next-generation sequencing on the Sentosa® SQ201 sequencing instrument. Subsequently, Sentosa® SQ Suite software performs primary analysis (signal processing and base-calling) on the raw sequencing data generated by Sentosa® SQ201. After primary analysis, the data was transferred to Sentosa® SQ Reporter Server for secondary analysis and report generation.

References Used:
- Human immunodeficiency virus type 1 (HIV-1)

Assay Information:
The Sentosa® SQ HIV Genotyping Assay is a next generation sequencing based in vitro diagnostic test intended for viral genotyping in patients diagnosed with HIV infection from human plasma or serum. This test is intended to be used on the Sentosa® SX101 with the Sentosa® SX-Total Nucleic Acid Plus Kit in conjunction with the Sentosa® ST401 and Sentosa® SQ201 instruments.

Gene List:
- Selective mutation analysis of Protease (codons 1-99), NRTI (codons 1-250), NNRTI (codons 1-335) and Integrate (codons 1-289) was performed.

Disclaimer:
This test was developed and its performance determined by this laboratory. It has not been cleared or approved by U.S. Food and Drug Administration. Since FDA is not required for clinical use of this test, this laboratory has established and validated the test’s accuracy and precision, pursuant to the requirements of CLIA 88. This laboratory is licensed and accredited under CLIA and CAP.

Vela is a trademark of Vela Holding Pte Ltd. Sentosa® is a registered trademark of Vela Holding Pte Ltd in several markets including the US and the European Union.
**Sentosa® SQ HIV Genotyping Assay**

**Reporting System – Drug Resistance Interpretation Report**

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### HIV Drug Resistance Interpretation

#### Protease

*This region was sequenced successfully and covers codons 1 - 99*

**Detected Mutations**

<table>
<thead>
<tr>
<th>Major</th>
<th>L90M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accessory</td>
<td>None</td>
</tr>
<tr>
<td>Other</td>
<td>K20R, M36I, I62V, L63P, I64V, V771, V82I, I93L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Class</th>
<th>Assessment</th>
<th>Stanford</th>
<th>ANRS</th>
<th>Rega</th>
</tr>
</thead>
<tbody>
<tr>
<td>atazanavir/r (ATV/r)</td>
<td>PI</td>
<td>Low-Level Resistance</td>
<td></td>
<td>Susceptible</td>
<td>Susceptible GSS 1.5</td>
</tr>
<tr>
<td>darunavir/r (DRV/r)</td>
<td>PI</td>
<td>Susceptible</td>
<td></td>
<td>Susceptible</td>
<td>Susceptible GSS 1.5</td>
</tr>
<tr>
<td>fosamprenavir/r (FPV/r)</td>
<td>PI</td>
<td>Low-Level Resistance</td>
<td></td>
<td>Susceptible</td>
<td>Susceptible GSS 1.5</td>
</tr>
<tr>
<td>Indinavir/r (IDV/r)</td>
<td>PI</td>
<td>Intermediate Resistance</td>
<td>Resistance</td>
<td>Susceptible GSS 1.5</td>
<td></td>
</tr>
<tr>
<td>lopinavir/r (LPV/r)</td>
<td>PI</td>
<td>Low-Level Resistance</td>
<td></td>
<td>Possible resistance</td>
<td>Susceptible GSS 1.5</td>
</tr>
<tr>
<td>nelfinavir (NFV)</td>
<td>PI</td>
<td>High-Level Resistance</td>
<td>Resistance</td>
<td></td>
<td>Resistant GSS 0</td>
</tr>
<tr>
<td>saquinavir/r (SQV/r)</td>
<td>PI</td>
<td>Intermediate Resistance</td>
<td>Resistance</td>
<td></td>
<td>Intermediate Resistant GSS 0.75</td>
</tr>
<tr>
<td>tipranavir/r (TPV/r)</td>
<td>PI</td>
<td>Susceptible</td>
<td></td>
<td>Susceptible</td>
<td>Susceptible GSS 1.5</td>
</tr>
</tbody>
</table>
Clinical Validation

Study Synopsis

Objective:
To evaluate the performance of the Sentosa® SQ HIV Genotyping Assay (4x16) and define the following characteristics of the assay:

Clinical sensitivity – ability of the Sentosa® SQ HIV Genotyping Assay to detect HIV-1 Group M, successfully amplify and sequence the target regions.

Variant detection correctness – ability of the Sentosa® SQ HIV Genotyping workflow to correctly detect and report target variants.

Testing sites:
1) Institute of Virology (Cologne, Germany) – 150 prospective EDTA plasma clinical samples;

2) Vela Research (Singapore) in collaboration with Stanford University (CA, USA) – 50 retrospective EDTA plasma clinical samples.

Reference method: Sanger sequencing
Clinical Validation
Clinical sensitivity – 98.5%

<table>
<thead>
<tr>
<th>Testing site</th>
<th>Number of targets tested*</th>
<th>Number of targets detected*</th>
<th>Clinical Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cologne site</td>
<td>300</td>
<td>296</td>
<td>98.67% (95% CI: 96.62% - 99.48%)</td>
</tr>
<tr>
<td>Singapore site</td>
<td>100</td>
<td>98</td>
<td>98.00% (95% CI: 93.00% - 99.45%)</td>
</tr>
<tr>
<td>Overall</td>
<td>400</td>
<td>394</td>
<td>98.50% (95% CI: 96.77% - 99.31%)</td>
</tr>
</tbody>
</table>

*Individual target regions (Protease/RT and Integrase) are taken as independent data points.

A total of 200 samples were tested. Both, PR/RT and Integrase target regions, were amplified in 196 out of 200 samples. In two samples neither PR/RT nor Integrase regions were amplified, in one sample PR/RT was not detected and in one sample Integrase region was not amplified. Overall clinical sensitivity for the Sentosa® SQ HIV Genotyping Assay was defined at **98.50% (95% CI: 96.77% - 99.31%)**
Clinical Validation

272 target variants were validated

Only variants covered by both methods (NGS and Sanger sequencing) were considered in this analysis.

A total of 42,170 target variants in 164 samples were covered by both systems.

207 variants reported by the Sentosa® SQ Reporter with frequency <20% (below limit of detection of Sanger sequencing method) were excluded from the comparative analysis.

Agreement between Sanger sequencing and the Sentosa® SQ HIV Genotyping NGS system:

<table>
<thead>
<tr>
<th></th>
<th>Mutant variants</th>
<th>Wild type variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanger sequencing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant variants</td>
<td>1085</td>
<td>21</td>
</tr>
<tr>
<td>Wild type variants</td>
<td>53</td>
<td>40,804*</td>
</tr>
</tbody>
</table>

*Excluding 207 variants reported by the Sentosa® SQ Reporter with frequency <20%.
Clinical Validation

Variant detection correctness – 99.82%

<table>
<thead>
<tr>
<th></th>
<th>Number of concordant variants</th>
<th>Number of discordant variants</th>
<th>Reference (Sanger Sequencing)</th>
<th>Variant Detection Correctness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cologne site</td>
<td>31,367</td>
<td>51</td>
<td>31,418</td>
<td>99.84% (95% CI: 99.79% - 99.88%)</td>
</tr>
<tr>
<td>Singapore site</td>
<td>10,522</td>
<td>23</td>
<td>10,545</td>
<td>99.78% (95% CI: 99.69% - 99.84%)</td>
</tr>
<tr>
<td>Overall</td>
<td>41,889</td>
<td>74</td>
<td>41,963</td>
<td>99.82% (95% CI: 99.78% - 99.86%)</td>
</tr>
</tbody>
</table>

Variant detection rate for the *Sentosa*® SQ HIV Genotyping Assay was defined at 99.82% (95% CI: 99.78% - 99.86%)
Sentosa® SQ HIV NGS Workflow

Major Characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core Technology</td>
<td>NGS, Ion Torrent PGM (Dx) platform</td>
</tr>
<tr>
<td>RNA Extraction and Library Prep</td>
<td>Automated</td>
</tr>
<tr>
<td>Turn around time / hands-on time</td>
<td>~27 hours / ~3.5 hours</td>
</tr>
<tr>
<td>Specimen type</td>
<td>EDTA Plasma</td>
</tr>
<tr>
<td>Specimen pre-treatment</td>
<td>Not required</td>
</tr>
<tr>
<td>Number of tests per kit</td>
<td>Up to 60 clinical samples</td>
</tr>
<tr>
<td>Kit format</td>
<td>4x16 (4 runs, up to 15 samples + 1 system control in each run)</td>
</tr>
<tr>
<td>Analytical sensitivity</td>
<td>1000 copies/mL</td>
</tr>
<tr>
<td>Analytical reactivity</td>
<td>HIV-1 Group M (subtypes A to K and CRFs)</td>
</tr>
<tr>
<td>Variant detection</td>
<td>up to 5%</td>
</tr>
<tr>
<td>Carryover contamination prevention</td>
<td>Uracil-DNA glycosylase system</td>
</tr>
<tr>
<td>Sequence data analysis</td>
<td>Fully automatic, linked to Stanford HIVdb.</td>
</tr>
</tbody>
</table>
Conclusion

Sentosa® HIV NGS workflow appears as a highly reliable tool for clinical diagnostics. More sensitive detection of low-frequency variants (up to 5%) resulting a higher predicted level of drug resistance, which offers improvements in HIV-1 drug resistance monitoring.
Acknowledgments

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