COMPARISON OF SENTOSA® SQ DEEP SEQUENCING-BASED HIV-1 GENOTYPING COUPLED TO INTEGRATED WORKFLOW WITH SANGER SEQUENCING METHOD FOR DETECTION OF DRUG RESISTANCE MUTATIONS

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Abstract. Sanger sequencing of viral quasispecies has limited sensitivity in detecting drug resistance mutations (DRMs) at frequencies less than 20%. On the other hand, deep sequencing is effective in detecting such mutations, but the protocol still requires manual and time-consuming working steps. Sentosa® SQ HIV-1 Genotyping Assay based on deep sequencing provides an integrated workflow, a robotic liquid handling system for automatic RNA extraction and library preparation, an Ion-torrent-based deep sequencing system and software for data analysis. Thus, we evaluated the performance of deep sequencing assay and compared the results with those from Sanger sequencing for determining DRMs of 120 previously genotyped clinical samples. Deep sequencing assay took 27.7 hours to complete, including 2.3 hours of manual working steps. DRM analysis revealed a total number of 913 and 789 mutations by deep sequencing assay and Sanger sequencing, respectively. Deep sequencing assay detected 99.4% of all DRMs found by Sanger sequencing and additional 129 DRMs at frequencies below and above 20%. Thus, with an integrated workflow, the deep sequencing assay provides a user-friendly platform and has a relatively short turnover time, requirements suitable for adoption in a routine clinical laboratory.

Keywords: deep sequencing, drug resistance mutation, HIV-1, Sanger dideoxy sequencing

INTRODUCTION

Genotypic HIV-1 drug resistance typing is considered an important tool for management of HIV-1 patients (Günthard *et al*, 2014). The assay plays a vital role in detecting drug resistant mutations (DRMs) in relevant viral genes, thereby assisting clinicians to select and construct an optimal antiretroviral regimen more likely to achieve and maintain viral suppression. Currently, HIV treatment guidelines recommended that drug resistance testing should be performed in treatment-experienced patients with

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virologic failure and in treatment-naïve patients for baseline virus resistance evaluation prior to therapy (Vandamme *et al*, 2011; Günthard *et al*, 2014; Panel on Antiretroviral Guidelines for Adults and Adolescent, 2016).

The gold standard method for genotyping DRMs is based on Sanger sequencing, using in-house protocols or FDA-approved commercial kits such as TRUGENE HIV-1 Genotyping Kit, to sequence protease (PR) and reverse transcriptase (RT) genes. Nevertheless, the Sanger sequencing method has a limited sensitivity in detecting DRMs present at frequencies < 20% (low-frequency DRMs) in viral quasispecies (Palmer *et al*, 2005; Church *et al*, 2006; Halvas *et al*, 2006). Next-generation sequencing or deep sequencing, an ultrasensitive and high-throughput sequencing method, is capable of generating millions of sequence reads, which allow detection of mutations at frequencies <20%. Previous studies evaluating deep sequencing and Sanger methods in detecting HIV-1 DRMs demonstrated that deep sequencing detects all DRMs found by the Sanger sequencing method including additional low-frequency DRMs undetected by the latter (Stelzl *et al*, 2011; Avidor *et al*, 2013; Garcia-Diaz *et al*, 2013; Gibson *et al*, 2014). This feature has allowed studies on the clinical significance of low-frequency DRMs and HIV-1 evolution (Simen *et al*, 2009; Hedskog *et al*, 2010; Li *et al*, 2011). However, the deep sequencing protocol still requires technical expertise in performing a number of manual and time-consuming working steps, especially in library preparation and in analyzing the large amounts of sequencing data, resulting in a long turnover time.

Hence, this study evaluated the performance of the Sentosa® SQ HIV-1 Genotyping Assay equipped with an integrated workflow with that of Sanger sequencing in detecting DRMs. The results should help in deciding the suitability of adopting the deep sequencing method for HIV-1 DRMs screening in a clinical laboratory.

MATERIALS AND METHODS

Study samples

A retrospective study was conducted on 120 EDTA blood samples collected from HIV-1 infected patients whose plasma samples contained HIV-1 viral load >1,000 copies/ml and which were previously genotyped using a TRUGENE HIV-1 Genotyping Kit (Siemens Healthcare Diagnostics, Tarrytown, NY) with consensus sequences submitted to Stanford University HIV drug resistance database for HIV-1 subtyping analysis (https://hivdb.stanford.edu). All plasma samples were obtained from the Virology Unit, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand collected from June to August 2015 and were stripped of data regarding patients' treatment history, antiretroviral regimen history as well as clinical and demographic information.

The study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (MURA 2015/561).

Sentosa® SQ HIV-1 Genotyping Assay with an integrated workflow

Sentosa® SQ HIV-1 Genotyping Assay (Vela Diagnostics, Singapore) can detect DRMs simultaneously in HIV-1 protease (PR), reverse transcriptase (RT) and integrase (IN) genes in 15 clinical samples per run (Fig 1). HIV-1 RNA was extracted from 730 µl each of 15 plasma

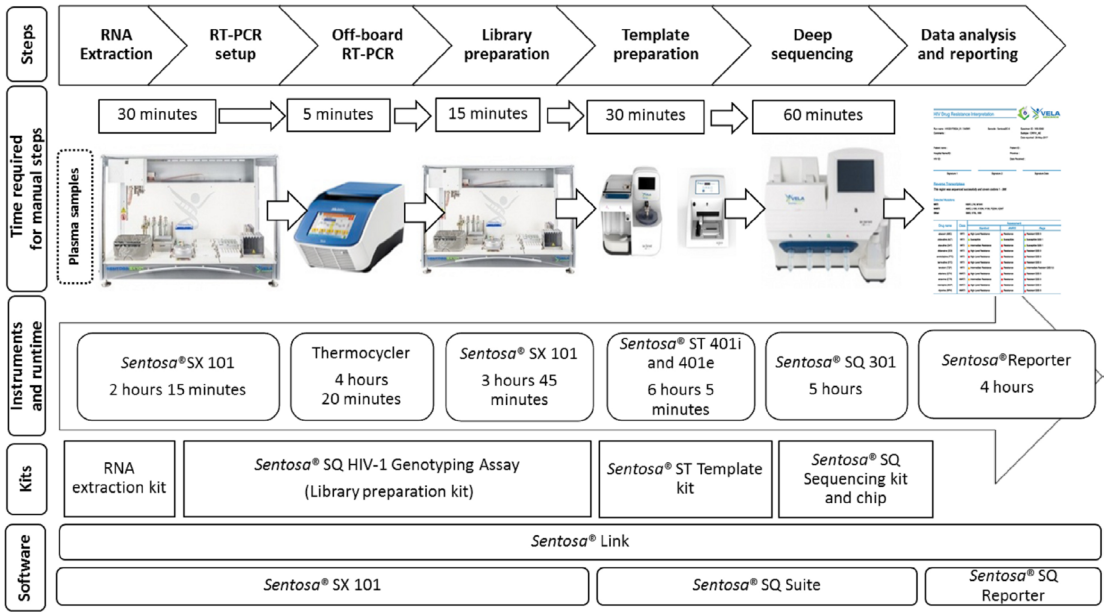

Fig 1–Workflow of Sentosa® SQ HIV-1 Genotyping Assay.

sample, performed using a robotic liquid handling system (Eppendorf Emotion 5075 system, Hamburg, Germany), to obtain 60 µl of RNA eluate. HIV extraction control (HIV-EC) was added to all samples at the beginning of RNA extraction process to normalize both RNA extraction efficiency and library preparation steps. Then a master mix for RT-PCR was mixed with each RNA sample including a HIV-EC blank control in a reaction plate using the robotic liquid handling system. RT-PCR amplification was performed using Veriti™ Dx 96-well Thermal Cycler (Applied Biosystems™, Foster City, CA). Amplicons of HIV-1 PR, RT and IN genes were used for automatic library preparation performed on the same robotic liquid handling system. Pooled DNA libraries were utilized for template preparation and emulsion (em)PCR followed by enrichment of template-positive ion sphere particles (ISPs) by using Sentosa® ST 401i and ST 401e, respectively. The template-positive ISPs were loaded onto a Sentosa® SQ 318 chip and sequenced using Sentosa® SQ 301 sequencer based on a semiconductor sequencing technology. Sentosa® SQ Reporter was employed to analyze the raw sequence readouts to detect DRMs and generate the results.

DRM analysis

The DRMs were analyzed using the 2015 edition of the IAS-USA drug resistance mutation list (Wensing *et al*, 2015). DRMs were divided into two main groups, which were composed of mutations in PR gene associated with resistance to protease inhibitors (PI mutations) and mutations in RT gene associated with resistance to RT inhibitors (RTI mutations) comprising mutations associated with nucleoside and nucleotide analogs (NRTI

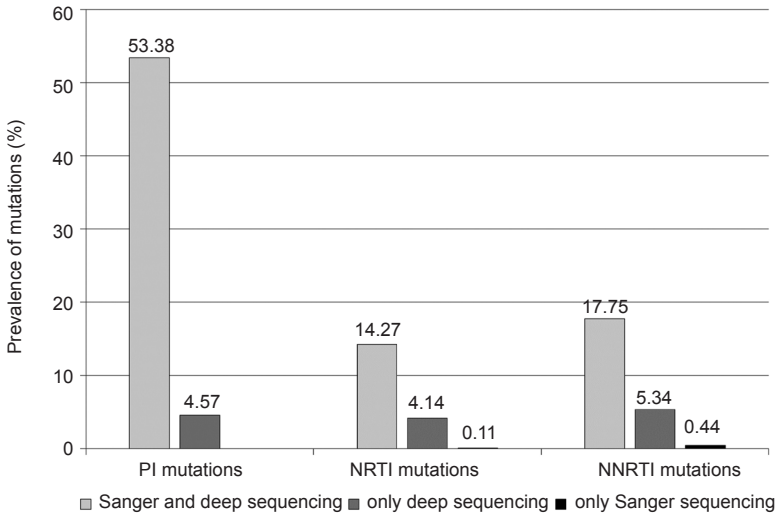

Fig 2–Prevalence of mutations associated with resistance to antiretrovirals against HIV-1 of 120 blood plasma samples from the Virology Unit, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University. PI, protease inhibitor; NRTI, nucleoside and nucleotide analog reverse transcriptase inhibitor; NNRTI, non-nucleoside analogue reverse transcriptase inhibitor.

mutations) and those associated with non-nucleoside analogue (NNRTI mutations).

RESULTS

Performance of Sentosa® SQ HIV-1 Genotyping equipped with an integrated workflow compared to Sanger sequencing

The 120 plasma samples contained a median viral load of 15,600 copies/ml (range 1,080 - >10,000,000 copies/ml). Deep sequencing assay equipped with an integrated workflow took 27.7 hours to process the samples, which included 2.3, 21.4 and 4 hours for the manual steps of operating the instruments, data analysis and generating results, respectively.

One hundred and nine (91%) samples had PR and RT genes successfully genotyped by both Sanger and deep sequencing methods, comprising 100 (92%) samples with HIV-1 subtype CRE01_AE, 8 (7%) subtype B and 1 (1%) subtype C. Of the 11 samples untyped by both methods, 2, 8 and 1 samples were genotyped by the Sanger method only, deep sequencing method only and neither method, respectively.

Comparison of HIV-1 DRMs detection by Sentosa® SQ HIV-1 Genotyping and Sanger sequencing methods

In order to compare the performance in detecting DRMs between sequencing deep sequencing and Sanger sequencing methods, mutations detected in 109 samples by both assays were analyzed for PI and RTI mutations using IAS-USA DRM list (Wensing *et al*, 2015). PI mutations were detected in 108 (99%) samples and none in one sample, while RTI mutations were present in 81 (74%) samples and none in 24 (22%). Deep sequencing assay detected ≥1 RTI mutations present at frequencies <20% of viral quasispecies in

4 (4%) samples, and these RTI mutations had sequence reads >1,000 per nucleotide position of variant nucleotides.

Of the 918 DRMs detected, 913 were by the deep sequencing assay and 789 by Sanger sequencing procedure, with 784 (99%) of the latter obtained by the deep sequencing assay, while 773 (99%) DRMs were detected by both assays and 11 (1%) by deep sequencing assay at frequencies of 5-20%. There were 5 DRMs identified only by Sanger sequencing method. Deep sequencing assay detected an additional 129 DRMs unidentified by Sanger's method, consisting of 102 low-frequency DRMs and 27 high-frequency DRMs at frequencies of 21-85%. Based on the IAS-USA DRM list, the 918 DRMs comprised 532 (57.9%), 170 (18.5%) and 216 (23.5%) PI, NRTI and NNRTI mutations, respectively. Deep sequencing assay detected all PI mutations and 99.2% of NRTI and 97.6% NNRTI mutations of DRMs revealed by Sanger's method (Fig 2). The most frequent codons associated with PI resistance were M36I, H69K and L89M, that of NRTI resistance was M184V and that of NNRTI resistance was K103N (Fig 3).

Low-frequency DRMs

Low-frequency DRM was defined as that with frequency <20% of the viral quasispecies. One hundred and two low-frequency DRMs in 29 samples were detected by deep sequencing assay. The most common low-frequency PI mutations were minor types but two major mutations (M46I and L90M) also were found. There were 27 (26%) low-frequency NRTI mutations found in 22 samples, the most frequent being the thymidine analog mutations (TAMs), namely, D67N (30%), K70R (7%), T215F (7%), and K219Q/E (7%), and M41L (4%); while there were 38 (37%) low-frequency NNRTI mutations from 28 subjects, the most frequent being V106I (16%), G190A (13%), A98G (10%) K103N (10%), and V108I (8%).

DISCUSSION

Deep sequencing has revolutionized genotypic HIV-1 drug resistance screening allowing detection of DRMs at frequencies <20% of viral quasispecies (Stelzl *et al*, 2011; Avidor *et al*, 2013; Garcia-Diaz *et al*, 2013; Gibson *et al*, 2014). However, the original deep sequencing protocol required several manual and time-consuming working steps, particularly in library preparation resulting in human errors and a long total turnover time. Integration of a workflow has solved this problem (Manee *et al*, 2017). Although both Sanger sequencing and deep sequencing assay genotyped PR and RT genes in 91% of the samples, the remaining samples could only be genotyped by either method or not at all. The failure of Sanger's method might be due to inadequate extracted RNA required for this technique compared to that of the deep sequencing assay. Failure in both assays was probably stemmed from insufficient amounts of viral RNA.

As anticipated, deep sequencing assay was able to detect nearly all DRMs found by Sanger sequencing and, in addition, detected low-frequency DRMs, 10% of which could only be reliably identify by Sanger's method. Deep sequencing was unable to find five DRMs indicated by the Sanger's method, but detected four wild-type viruses and a non-identical DRM. A manual review of the electropherograms and BAM of these five DRMs demonstrated that the four wild-type viruses, sorted from BAM files, had only wild-type nucleotides, while electropherograms showed peaks that included a

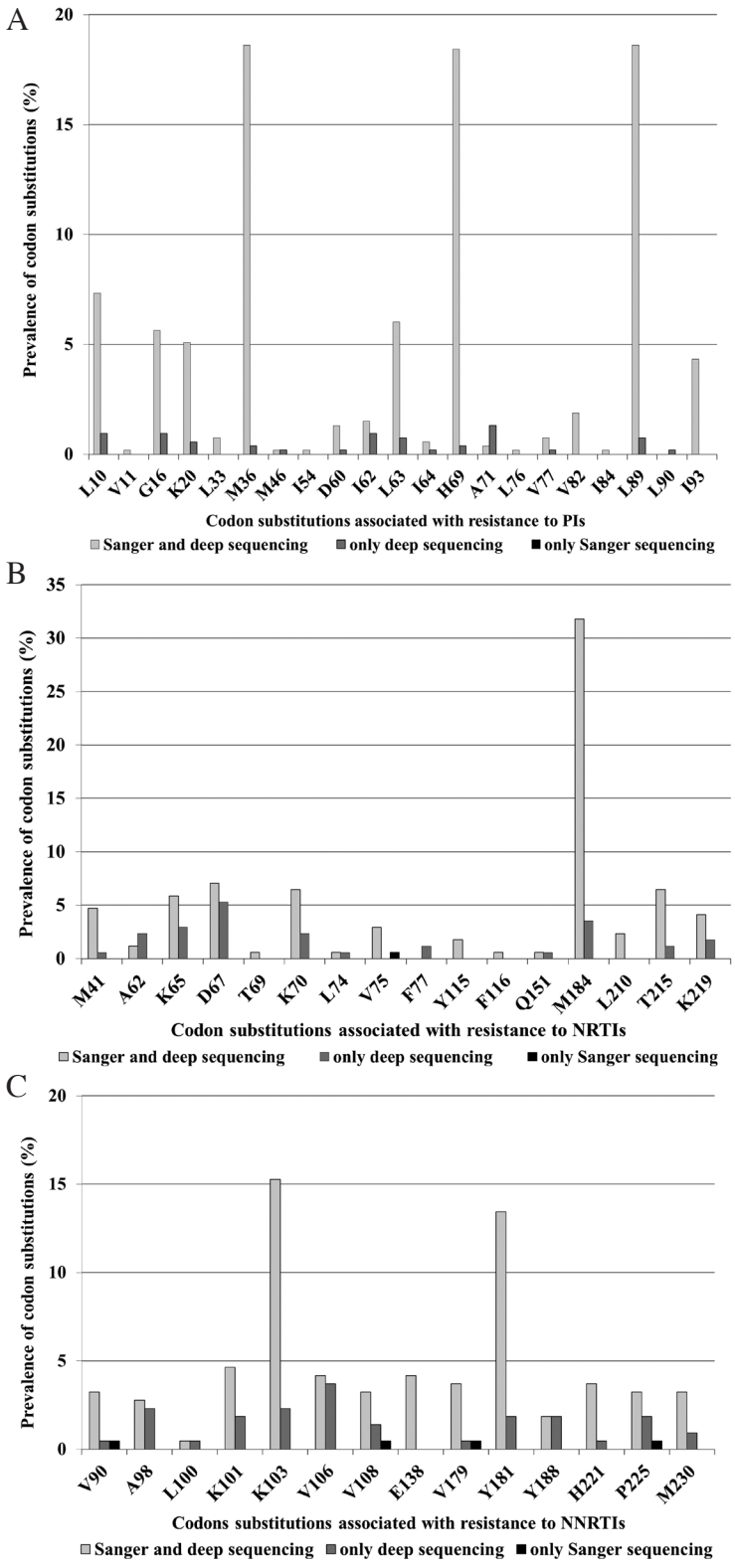

Fig 3–Prevalence of codon substitutions associated with resistance to antiretrovirals against HIV-1 of 120 blood plasma samples from the Virology Unit, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University. A. Protease inhibitors. B. Nucleoside and nucleotide analogue reverse transcriptase inhibitors. C. Non-nucleoside analogue reverse transcriptase inhibitors.

mixture of a major (wild type) and minor nucleotide. As for the non-identical DRM, each assay detected a different nucleotide. Stelzl *et al* (2011), testing the accuracy of deep sequencing using samples from QCMD ENVA HIV drug resistance typing EQA program, reported one DRM codon (AAG) detected by deep sequencing is not identical to the expected result (ARR). In addition, Ram *et al* (2015), employing other NGS platforms, showed these NGS platforms are unable to detect a number of mutations found only by TRUGENE HIV-1 Genotyping kit.

Sanger sequencing method missed 27 high-frequency DRMs detected by deep sequencing assay. This finding is similar to previous studies reporting certain high-frequency DRMs are detected only by deep sequencing (Le *et al*, 2009; Fisher *et al*, 2012; Mohamed *et al*, 2014). A manual review of the electropherograms of these 27 DRMs that (i) Sanger sequencing detected only wild-type nucleotides but could not identify any mutant nucleotide (data not shown but will be supplied upon request), (ii) sequences were edited from a mixture of nucleotides from both wild-type and variant nucleotides, and (iii) the software picked out only major (wild-type) nucleotides even though there existed minor peaks underneath (data not shown but will be supplied upon request) (Ram *et al*, 2015). Furthermore, deep sequencing assay detected a few high-frequency DRMs that had been found in previous resistance testing while Sanger sequencing missed these DRMs. This finding is probably not due to deep sequencing false positives, as according to EQA program reports, a codon containing a mixture of DRMs can be missed by laboratories owing to differences in performance of capability of each laboratory and in both the quality of sequence data analysis and editing (Schuurman *et al*, 2002; Pandit *et al*, 2008).

Results of previous resistance testing form part of the important information used for constructing optimal antiretroviral (ARV) regimens in treatment-experienced patients (Günthard *et al*, 2014). Being an ultrasensitive method, deep sequencing may be able to detect DRMs responsible for the results of the previous resistance tests. Thus, this study investigated whether deep sequencing assay could detect HIV-1 DRMs found in a previous genotypic resistance testing using Sanger sequencing protocol. The results showed that deep sequencing assay was not only capable of detecting DRMs indicated by the Sanger's method, which were mostly mutations in the RT gene but also revealed the presence of wild-type viruses and other additional amino acid substitutions (data not shown). The DRMs had been found in samples of previous resistance testing conducted during the previous 1 to 7 years before the tests were carried out by the deep sequencing assay. The previous study employed ultra-deep pyrosequencing to detect resistant viruses after treatment interruption and showed that resistant viruses rapidly disappeared after treatment interruption and was undetectable as early as after 3 months (Hedskog *et al*, 2010). This study had no ARV regimen history, so these samples were assumed not to be exposed to drug-selective pressure, which results in reduced replication fitness of DRMs such as M184V mutation (Paredes *et al*, 2009). Consequently, such DRMs in plasma may have either decreased rapidly to frequencies below the detection limit or were cleared from the plasma compartment to be replaced by wild-type viruses. The finding suggests that despite using deep

sequencing as an ultrasensitive detection method, it is difficult to detect DRMs found in previous resistance tests if they are not exposed to a long period of drug-selective pressure.

The total time to obtain from results of 120 samples using the Sentosa® SQ HIV-1 Genotyping assay was shorter than that of other HIV-1 deep sequencing protocols (>29 hours) (Stelzl *et al*, 2011; Mohamed *et al*, 2014). As a result of the integrated workflow, the robotic liquid handling system reduced manual working procedures and errors, particularly in library preparation; in addition the semiconductor sequencer performed deep sequencing in less than that of other NGS platforms (Quiñones-Mateu *et al*, 2014). The software automatically analyzed NGS data and generated results. Moreover, the cost effective per sample of deep sequencing assay will tend to be competitive to that of Sanger sequencing method because deep sequencing assay detects DRMs in HIV-1 PR, RT and IN genes concurrently, whereas Sanger sequencing, such as FDA-approved commercial kits, generally detects DRMs in PR and RT genes.

In conclusion, the Sentosa® SQ HIV-1 Genotyping Assay equipped with an integrated workflow shows performance at least as well as Sanger sequencing method in detecting HIV-1 DRMs and has the advantage of detecting DRMs at frequencies below 20% and some DRMs at frequencies above 20% unidentified by the Sanger's method. With an integrated workflow, the deep sequencing assay was user-friendly and had a relatively shorter turnaround time. Furthermore, the cost per sample of deep sequencing will tend to decrease in the coming future. Therefore, deep sequencing is a technology for HIV-1 drug resistance genotyping suitable for adoption in a routine clinical laboratory setting.

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CONFLICTS OF INTEREST

The authors hereby declare no personal or professional conflicts of interest regarding any aspect of this study. Vela Diagnostics Pte, Ltd, has no role in the design, conduction or publication of this study.

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