Published in final edited form as:

J Virol Methods. 2017 March ; 241: 41–45. doi:10.1016/j.jviromet.2016.12.008.

Performance of Celera RUO integrase resistance assay across multiple HIV-1 subtypes

Carole L Wallis^a, Raquel V Viana^a, Shanmugam Saravanan^b, Carlos Silva de Jesus^c, Clement Zeh^d, Elias K Halvas^e, and John W Mellors^e

^aBARC-SA and Lancet Laboratories, Corner of Napier and Menton, Richmond, 2190, South Africa

^bYRG Centre for AIDS Research and Education, Chennai, India

^cOswaldo Cruz Foundation-FioCruz, Laboratory of AIDS & Molecular Immunology, Brazil

^dKemri-CDC, Kenya

^eUniversity of Pittsburgh, PA, USA

Abstract

Background—HIV-1 sequence variation is a major obstacle to developing molecular based assays for multiple subtypes. This study sought to independently assess performance characteristics of the ViroSeqTM HIV-1 Integrase RUO Genotyping Kit (Celera, US) for samples of multiple different HIV-1 subtypes.

Methods—264 samples were tested in the validation, 106 from integrase inhibitor naïve patients^{*} sent for routine HIV-1 drug resistance testing after failing a 1st- or 2nd-line regimen, and 158 samples from an external virology quality assurance program (VQA). For the latter, 53 unique VQA samples were tested in two to five different laboratories to assess assay reproducibility. For all assays, viral RNA was extracted using the ViroSeq extraction module, reverse transcribed, and amplified in a one-step reaction. Four sequencing primers were used to span codons 1–288 of integrase. The Rega subtyping tool was used for subtype assignment. Integrase polymorphisms and mutations were determined as differences from the HXB2 sequence and by the Stanford database, respectively. Sequences obtained from the different laboratories were aligned and sequence homology determined.

Results—HIV-1 RNA in the 264 samples ranged from 3.15 to 6.74_{log}copies/ml. Successful amplification was obtained for 97% of samples (n=256). The 8 samples that failed to amplify were subtype D (n=3), subtype C (n=1), CRF01_AE (n=1), subtype A1 (n=2), and an unassigned subtype (n=1). Of the 256 that successfully amplified samples, 203 (79%) were successfully sequenced with bidirectional coverage. Of the 53 unsuccessful samples, 13 (5%) failed sequencing

Corresponding Author and reprints request: Dr. Carole Wallis, Molecular Pathology, Lancet Laboratories, Corner of Napier and Menton, Richmond, 2190, South Africa, carole.wallis@lancet.co.za, Telephone: 27-11-358-0816, Fax: 27-11-358-1464.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

and 40 (16%) did not have full bidirectional sequence, as a result of failure of sequencing primers: Primer A (n=1); Primer B (n=18); Primer C (n=1); Primer D (n=7) or short sequences (n=16). For the 135 VQA samples (30 unique samples) that were assayed by different laboratories, homology of the sequences obtained ranged from 92.1% to 100%. However, Laboratory 2 detected more mixtures (74%) compared to the other four laboratories, whereas Laboratory 1 detected the least number of mixtures (35%), likely due to differences between the labs in the methods of sequence analysis. Mutations associated with integrase resistance were observed in seven of the 106 (7%) clinical samples [one sample: Q148K; E138K; G140A; two samples: T97A and four samples: L74I]. Of the four samples with L74I, 3 were subtype G.

Conclusion—Of the total 264 samples tested, 243 (92%) of samples were able to be amplified and sequenced to generate an integrase genotype. Sequencing results were similar between the testing laboratories with the exception of mixture detection. Mutations associated with integrase inhibitor resistance were observed in only 7% of integrase inhibitor naive samples, and some of these mutations are likely to be due to subtype-specific polymorphisms rather than selection by an integrase inhibitor.

Keywords

HIV-1 drug resistance; non-subtype B; Integrase

1. Introduction

Global access to antiretroviral therapy (ART) has increased rapidly over the last ten years in developing countries, with over 15.8 million infected individuals receiving ART by June 2015 (UNAIDS, 2015). ART can fail to suppress HIV-1 as a result of transmitted drug resistance, medication toxicity, incomplete medication adherence, suboptimal pharmacokinetics or inadequate regimen potency, resulting in the emergence of drug-resistant HIV-1 (Boulle et al., 2007; Carr and Cooper, 2000; Haas et al., 2004; Haas et al., 2003; Rhee et al., 2015). When HIV drug resistance develops, ideally, a new regimen containing at least three active drugs should be administered. Optimal third-line options are still being investigated in studies such as the AIDS Clinical Trials Group (ACTG) Protocol A5288: “Management Using the Latest Technologies in Resource-limited Settings to Optimize Combination Therapy After Viral Failure (MULTI-OCTAVE)” (ClinicalTrials.gov Identifier: NCT01641367). This study includes the drug raltegravir from the integrase class of inhibitors.

Raltegravir was the first FDA-approved integrase inhibitor that blocks viral replication by inhibiting integration of HIV-1 DNA into the host genome (Cahn and Sued, 2007; Colquitt and Pham, 2007; Grinsztejn et al., 2007). Decreased susceptibility to raltegravir develops as a result of several different mutations in the integrase gene. To date, the majority of work in detecting integrase resistance has centred on HIV-1 subtype B. However, the subtypes circulating in developing countries, where the need for treatment is greatest, are generally non-subtype B. The subtypes that predominate in these developing countries include sub-subtype A, subtypes C, D, F and several recombinant forms. Sequence variations in the binding sites for PCR amplification primers and sequencing primers are a major obstacle to developing molecular based assays for this range of subtypes. One commercially available

test for integrase resistance is available (ViroSeq™ HIV-1 Integrase RUO Genotyping Kit [Celera, US]). In the current study, we sought to assess the performance characteristics of this HIV-1 Integrase RUO Genotyping Kit with patient samples across a broad range of subtypes and across different testing laboratories.

2. Materials and Methods

2.1. Samples

Two hundred and sixty four plasma samples were tested in the assessment. One hundred and six of the 264 (40%) plasma samples were from integrase inhibitor naïve patients that were sent for routine HIV-1 drug resistance testing between 2013–2014, after failing either a first-line NNRTI based regimen or a second-line PI based regimen. All samples selected had plasma HIV-1 RNA > 1000 copies/ml.

One hundred and fifty eight well characterised plasma samples with a variety of HIV-1 subtypes were obtained from the National Institutes of Health (NIH) Division of AIDS (DAIDS) Virology Quality Assessment Program (VQA) Laboratory, Rush Institute, USA. Of the 158 samples from the VQA, 53 were unique with the following subtypes: B (n=26); C (n=15); CRF-1_AE (n=8); D (n=2); F (n=2). Moreover, the six samples had minor and major integrase mutations. Thirty of these 53 were identical samples (panels 23–28), which were tested in at least two and up to five different laboratories to assess the reproducibility of the ViroSeq™ HIV-1 Integrase RUO Genotyping Kit. Twenty three samples (panels 20–22 and NED [NIH-ENVA-DOD]) of which seven were subtype AE were tested in one laboratory.

2.2. Population Genotype Analysis

Population based genotyping was performed using the ViroSeq™ HIV-1 Integrase RUO Genotyping Kit (Celera, US) according to the manufacturer's instructions. Viral RNA was extracted from plasma using the ViroSeq extraction module. Briefly, 500µl of plasma was centrifuged at 23000xg for 1 hour at 4°C and the viral pellet resuspended in 600µl of lysis buffer supplied in the extraction module. Viral RNA was then precipitated with 100% isopropanol and washed with cold 70% ethanol. The extracted viral RNA was resuspended in 50µl kit provided buffer. The extracted viral RNA was used to generate a 1.1 kilobase amplicon in a one-step reverse transcribed (RT)-initiated polymerase chain reaction encompassing the entire integrase region. After column or enzymatic purification, the amplicon was used as a template to sequence 0.9kb of the integrase region using four sequencing primers. Sequencing was performed with an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, USA). If sequencing primers failed they were repeated. Sequences were analysed using the ViroSeq™ Integrase Software V1.0.0 to give the drug resistance profile across codons 1–288 of integrase (Figure 1).

2.3. Data Analysis

The Rega HIV-1 subtyping tool (<http://dbpartners.stanford.edu/RegaSubtyping/>) was used to identify the HIV-1 subtype of each sample. Integrase polymorphisms and mutations were determined using both the ViroSeq™ Integrase V1.0.0 algorithm and the Stanford database

to determine the level of predicted susceptibility to raltegravir, elvitegravir and dolutegravir. A phylogenetic tree analysis using MEGA 6.0¹¹ was performed on the sequences generated from the two different testing laboratories to ensure all duplicate samples clustered together.

To access reproducibility, the paired samples from the two to five different laboratories had the nucleotide sequences aligned and the level of disagreements compared. The level of disagreement was compared after a consensus sequence from the different laboratories was generated as follows: a) number of sequencing differences (complete difference of nucleotide at the same position); b) the number of partial-mismatches (for example R vs. G). Sequence similarity was calculated for each of the parameters evaluated and a greater than 98% for complete mismatches was considered acceptable. Lastly, the mutation lists obtained through the Stanford database were compared for sequences derived from all the laboratories.

A Fisher's exact test was used to determine the difference in sequencing primers that failed. As the sample number was small, subtype C was compared to all other subtypes.

3. Results

3.1. Amplification

The ViroSeqTM HIV-1 Integrase RUO Genotyping Kit indicates that plasma HIV-1 RNA of 1000 copies/ml is the lower limit of detection. No samples with HIV-1 RNA less than the reported limit of detection were tested. Of the 264 samples tested, the HIV-1 RNA ranged from 3.15 to 6.74_{log} copies/ml with a mean viral load of 4.30_{log} copies/ml. Successful amplification was obtained for 97% (256 of 264) of samples. The HIV-1 RNA of the samples that failed to amplify ranged across HIV-1 RNA levels, indicating that HIV-1 RNA copy number was not the main determinant of amplification success. Of the eight samples that failed to amplify, the subtypes, determined by independent sequencing of the reverse transcriptase and protease region, were subtype D (n=3), subtype C (n=1), CRF01_AE (n=1), subtype A1 (n=2), and an unassigned subtype (n=1) (Table 1). The highest frequency of amplification failures was observed in subtype D (15%), followed by A1 (7%) and recombinants (7%), although the distributed of failures was similar across subtypes.

3.2. Sequencing

Of the 256 samples that successfully amplified, sequence could not be obtained for 13 (5%) samples because all four sequencing primers failed. Of the 13 samples that failed to sequence, the subtypes were subtype D (n=1), subtype A1 (n=4), CRF01_AE (n=1) and subtype C (n=7; Table 1). Sequencing failure was most frequent in subtype A1 (14%), followed by subtype C (7%). Sequence was therefore obtained for 243 of samples; however, complete bidirectional sequencing was only obtained for 203 (84%) samples. Of the 40 samples (16%) that did not have full bidirectional sequence, Table 2 showed the sequencing primers that failed and the HIV-1 subtypes. Subtype C samples had the highest number of primer failures, resulting in incomplete bidirectional sequencing of the integrase region in 21 of 94 (22%) samples which is significantly different compared to the other subtypes combined (14%, p=0.015).

3.3. Reproducibility

Reproducibility of the ViroSeq™ HIV-1 Integrase RUO Genotyping assay was determined by sending the same blinded sample panels (supplied by the Virology Quality Assurance [VQA] program from Rush University, Chicago) to five different laboratories for multiple testing runs. Twenty samples were run in triplicate by three different laboratories (Lab 1–3); twenty five samples were run in duplicate by two different laboratories (lab 4–5); and 5 samples were run at least once by all five laboratories. Of the 135 samples, one laboratory could not amplify one sample. Of the 101939 nucleotide sequences obtained (ave. 864 nucleotides per sequence) there were 105 complete mismatches, resulting in a sequence similarity of 99.9%. When comparing each sequence from each laboratory to the other sequences obtained from other laboratories for the same sample, sequence homology ranged from 92.1% to 100%. Complete mismatches were mainly attributed to one laboratory (Lab 1; 104 of the 105). Differences in amino acid and mutations were also observed as a consequence of the nucleotide differences. Seventy two amino acid differences were observed across the 33979 amino acids compared (99.8% homology). Differences in mutations were observed at three codons in integrase associated with integrase drug resistance (A128T, T97A and V151I). Different levels of mutant mixtures were detected by each laboratory, with laboratory 2 reporting the most mixtures (74%) and laboratory 1 reporting the least (35%) with the mean number of mismatches being 55%. The mutational differences that were observed were not localized to any specific region or codon of integrase.

3.4. Integrase Mutations and Polymorphisms

All the 106 patient samples tested were raltegravir naïve; however, seven (7%) had mutations linked to reduced susceptibility to an integrase inhibitor as determined by the Stanford Database. The following mutations were observed: Q148K; E138K; G140A in one sample; T97A in two samples; and L74I in four samples. Of the four samples with L74I, three were subtype G. Polymorphisms not linked to integrase resistance that occurred at greater than 10% overall are shown in Figure 2 and by sub-subtype A, subtypes B, C and D in Figure 3. The majority of the polymorphisms were observed in HIV-1 subtype C.

4. Discussion

The ViroSeq™ HIV-1 Integrase RUO Genotyping assay obtained amplification and full bidirectional sequences in 77% of samples across all subtypes tested. Sequencing results were similar between the five testing laboratories with the exception of mixture detection. Mutations associated with integrase inhibitor resistance determined by the Stanford database were observed in only 7% of integrase inhibitor naïve samples, and most of these mutations are likely to be due to subtype-specific polymorphisms rather than selection by an integrase inhibitor, although further analysis of samples from before and after integrase therapy is needed.

Although the ViroSeq™ HIV-1 Integrase RUO Genotyping assay successfully obtained sequences in 243 of samples across all subtypes tested, only 84% had complete bidirectional sequences. This level of success is similar to what has been observed in other regions of

HIV-1 with subtype independent assays. A 15% no amplification rate was observed with subtype D samples and a 7% with subtype A1 and recombinant samples (CRF01_AE and unassigned). Failed amplification for subtype D was significantly different ($p=0.001$) when compared to all other failed amplification subtype samples. Although the assay has limitations in non-subtype B samples for obtaining complete bidirectional sequences, the overall high success rate in obtaining integrase sequences and the ease of the one step RT-PCR assay make it a feasible and useful assay for roll-out in areas where subtypes other than subtype B are most frequent.

The five laboratories that tested the same samples showed the ViroSeq integrase assay is reproducible for major base peaks; however, detection of mixtures (minor peaks) varied widely across the laboratories. The differences in mixture detection are likely due to variation in manual analysis of electropherograms, suggesting that automated sequence analysis could improve consistency.

Seven percent of the routine samples tested contained integrase resistance mutations. This is unlike what has been observed in the US where no major integrase mutations have been observed in samples from integrase inhibitor naïve individuals in a large multisite study that sequenced 1090 samples (Volpe et al., 2015). This difference could be a result of subtype specific polymorphism as L74I was noted in three subtype G samples. Alternatively, it could reflect undisclosed raltegravir use, given that the drug was being registered in some African counties starting in 2013. Nevertheless, testing of samples is needed to monitor integrase resistance as use of integrase inhibitors increases in Africa.

In summary, of the total 264 samples tested, 77% ($n=203$) were successfully amplified and a complete bi-directional sequence obtained using the ViroSeq™ HIV-1 Integrase RUO Genotyping assay, and a unidirectional sequence was obtained in 92% ($n = 243$). Sequencing results were similar between the five testing laboratories with the exception of mixture detection. The latter could be improved by automated analysis of electropherograms. Overall, the ViroSeq™ HIV-1 Integrase RUO Genotyping assay is a useful tool for integrase genotyping across different HIV-1 subtypes.

Acknowledgments

We would like to thank Celera Diagnostics for providing the ViroSeq™ HIV-1 Integrase RUO Genotyping Kits and the NIAID Virology Quality Assurance Program (VQA) program at Rush University (HHSN272201200023C) for samples and the ACTG Laboratory Center (UM1-AI-106701) for support.

References

- Boulle A, Orrel C, Kaplan R, Van Cutsem G, McNally M, Hilderbrand K, Myer L, Egger M, Coetzee D, Maartens G, Wood R. Substitutions due to antiretroviral toxicity or contraindication in the first 3 years of antiretroviral therapy in a large South African cohort. *Antiviral therapy*. 2007; 12:753–60. [PubMed: 17713158]
- Cahn P, Sued O. Raltegravir: a new antiretroviral class for salvage therapy. *Lancet*. 2007; 369:1235–6. [PubMed: 17434380]
- Carr A, Cooper DA. Adverse effects of antiretroviral therapy. *Lancet*. 2000; 356:1423–30. [PubMed: 11052597]

- Colquitt, AR., Pham, PA. The Hopkins HIV report: a bimonthly newsletter for healthcare providers. Vol. 19. Johns Hopkins University AIDS Service; 2007. Expanded access drug profile: raltegravir (RAL, MK-0518); p. 11-2.
- Grinsztejn B, Nguyen BY, Katlama C, Gatell JM, Lazzarin A, Vittecoq D, Gonzalez CJ, Chen J, Harvey CM, Isaacs RD, Protocol T. Safety and efficacy of the HIV-1 integrase inhibitor raltegravir (MK-0518) in treatment-experienced patients with multidrug-resistant virus: a phase II randomised controlled trial. *Lancet*. 2007; 369:1261–9. [PubMed: 17434401]
- Haas DW, Ribaud HJ, Kim RB, Tierney C, Wilkinson GR, Gulick RM, Clifford DB, Hulgand T, Marzolini C, Acosta EP. Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. *AIDS (London, England)*. 2004; 18:2391–400.
- Haas DW, Wu H, Li H, Bosch RJ, Lederman MM, Kuritzkes D, Landay A, Connick E, Benson C, Wilkinson GR, Kessler H, Kim RB. MDR1 gene polymorphisms and phase 1 viral decay during HIV-1 infection: an adult AIDS Clinical Trials Group study. *Journal of acquired immune deficiency syndromes (1999)*. 2003; 34:295–8. [PubMed: 14600574]
- Rhee SY, Blanco JL, Jordan MR, Taylor J, Lemey P, Varghese V, Hamers RL, Bertagnolio S, Rinke de Wit TF, Aghokeng AF, Albert J, Avi R, Avila-Rios S, Bessong PO, Brooks JI, Boucher CA, Brumme ZL, Busch MP, Bussmann H, Chaix ML, Chin BS, D'Aquin TT, De Gascun CF, Derache A, Descamps D, Deshpande AK, Djoko CF, Eshleman SH, Fleury H, Frange P, Fujisaki S, Harrigan PR, Hattori J, Holguin A, Hunt GM, Ichimura H, Kaleebu P, Katzenstein D, Kiertiburanakul S, Kim JH, Kim SS, Li Y, Lutsar I, Morris L, Ndembu N, Ng KP, Paranjape RS, Peeters M, Poljak M, Price MA, Ragonnet-Cronin ML, Reyes-Teran G, Rolland M, Sirivichayakul S, Smith DM, Soares MA, Soriano VV, Ssemwanga D, Stanojevic M, Stefani MA, Sugiura W, Sungkanuparph S, Tanuri A, Tee KK, Truong HH, van de Vijver DA, Vidal N, Yang C, Yang R, Yebra G, Ioannidis JP, Vandamme AM, Shafer RW. Geographic and temporal trends in the molecular epidemiology and genetic mechanisms of transmitted HIV-1 drug resistance: an individual-patient- and sequence-level meta-analysis. *PLoS medicine*. 2015; 12:e1001810. [PubMed: 25849352]
- UNAIDS. AIDS by the numbers 2015. 2015.
- Volpe JM, Ward DJ, Napolitano L, Phung P, Toma J, Solberg O, Petropoulos CJ, Walworth CM. Five Antiretroviral Drug Class-Resistant HIV-1 in a Treatment-Naive Patient Successfully Suppressed with Optimized Antiretroviral Drug Selection. *Journal of the International Association of Providers of AIDS Care*. 2015; 14:398–401. [PubMed: 26188010]

Highlights

- ViroSeq™ HIV-1 Integrase RUO Genotyping assay validation across multiple subtypes;
- Of the 264 samples tested, 256 successfully amplified and of those 203 (79%) were successfully sequenced with full bidirectional coverage.
- However, at least unidirectional integrase sequences were obtained for 92% of all the samples tested (243 of 264).

Figure 1. Diagrammatic representation of *pol* gene of HIV with the position of the amplicon and sequencing primers of the ViroSeq™ HIV-1 Integrase RUO Genotyping Kit.

Figure 2.

Integrase Polymorphisms in Integrase Naïve subjects. To date, none of these polymorphisms have been linked to reducing integrase activity.

Figure 3.

Integrase Polymorphisms in Integrase Naïve subjects, by subtype (subtype D in purple; subtype C in green; subtype B in red and subtype A1 in blue). Subtype C had the highest number of polymorphisms compared to the other subtypes.

Table 1

Overall performance of the ViroSeq™ HIV-1 Integrase RUO Genotyping, by subtype.

Subtype	No Amplification *	No Sequence Obtained *	Bidirectional Sequence Not Obtained	Full Sequence Obtained (%)	Total
A1	2 (7%)	4 (14%)	9 (33%)	12 (44%)	27
D	3 (15%)	1 (5%)	1 (5%)	15 (75%)	20
Recombinant	2 (7%)	1 (3%)	4 (14%)	22 (76%)	29
C	1 (1%)	7 (7%)	21 (22%)	65 (69%)	94
B			4 (5%)	79 (95%)	83
F1			1 (11%)	8 (89%)	9
G				2 (100%)	2
Total	8 (3%)	13 (5%)	40 (15%)	203 (77%)	264

* Subtype determined by sequencing of protease and reverse transcriptase.

Table 2

Sequencing primers that failed, by subtype.

Subtype (n=40)	Primer A	Primer B	Primer C	Primer D*	Short Sequence
A1 (n=9)				1	8
B (n=4 ^{**})		4		1	
C (n=21 ^{**})	1	13	1	5	3
Recombinant (n=3)					3
F1 (n=1)		1			
D (n=2)					2
Total	1	18	1	7	16

* Failure of sequencing primer D resulted in no sequence for the first 40 codons of integrase.

** The n is the number of samples that a sequencing primer failed. For subtype B there were four samples, one of which had two primers that did not work. Similarly, for subtype C there are 21 samples, two of which had more than one sequencing primer that did not work or generated only a short sequence.