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Optimization of HIV-1 Next Generation Sequencing genotyping drug resistance testing methods from both RNA and DNA on Ion GeneStudio S5 prime System

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Background

- **Guidance of antiretroviral therapy** (1) for the most commonly used drug classes to treat HIV-1 requires knowledge **of preexisting or emerging mutations in protease (PR), reverse transcriptase (RT), and integrase (INT)** portions of the **HIV-1 *pol* gene** (2–4). Most testing is performed using **population Sanger sequencing** (SS), which has a sensitivity for detecting resistance variants at **~20%** of the total population (4,5).
- Genotypic resistance testing (**GRT**) is widely used to evaluate HIV resistance mutations (DRMs), with SS being the preferred method. Currently, numerous established methodologies and data analysis instruments are available for this purpose. However, there is a **gradual transition occurring in the field from SS to next-generation sequencing (NGS)** (6). Unlike SS, which produces a single consensus sequence for the amplified and sequenced fragment of the HIV-1 genome, **NGS techniques generate a large number of sequence reads, often in the range of millions for a single sample** (7).

Objective

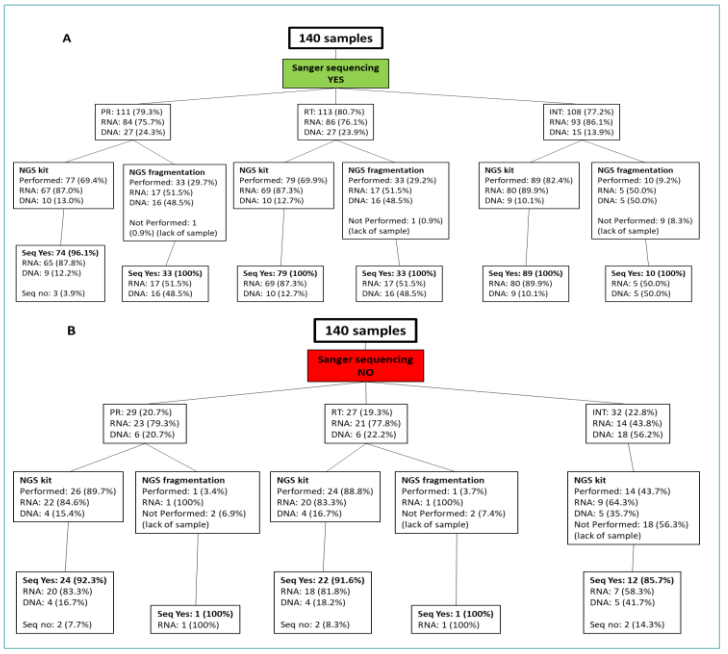
- **Comparison between SS and NGS was evaluated**, considering both DNA and RNA samples from naïve or experienced people living with HIV-1 (PWH).

Materials & Methods

- **One-hundred and forty samples** from PWH were selected, based on viral loads (VL): median (IQR) 4.61(5.51-5.69) log_{10} cp/mL, for NGS sequencing with **Ion GeneStudio S5 prime System by AmpliSeq (AmS) primers pool** (Thermo Fisher), **generating a total of 17 overlapping amplicons** (from PR to INT) or by using **Ion Plus Fragment Library Kit** (Thermo Fisher).
- For samples with positive NGS PR/RT/INT sequencing, **mutations (drug resistance mutations-DRMs, accessory mutations-A, and natural polymorphisms-NPs)**, were compared with those obtained by SS. Sequences were interpreted by **Stanford HIV-db**, both for SS and NGS.
- NGS **minority variants** (MV) were classified with a **frequency between 5-20%**.
- **Phylogeny** was performed to determine **viral subtypes,** and to evaluate the **proper clustering of the SS and NGS** sequences from the same subject.

Results

Sequencing performance



Flowchart showing how, of the **140 total samples analyzed**, those with **good SS methodology** (A) and **those for which an interpretable sequence was not obtained with SS methodology** (B) (divided into the three regions PR, RT, INT), were processed with **NGS methodology**, using the **two different commercial kits ("Ion AmpliSeq™ Library 2.0" kit and "Ion Xpress™ Plus Fragment Library" kit)** depending on the type of sample and its availability. Specifically, first the **success rates of SS for the three gene portions analyzed are shown** (A), then how many of these samples and which **NGS protocol was used** and, finally, **the success rates of the two protocols divided by the same three portions analyzed are shown**. In addition, the percentages of **RNA and DNA samples are shown**.

Description of the 41 DRMs/NPs found in the NGS of the 77 samples with complete sequencing both in SS and NGS at the 5% threshold

Mutation (N)	Type of mutation	NGS %	Resistance drug	Drug class	Naïve	RNA/DNA
Protease						
L10H (1)	P	5.9	-	-	No	RNA
L10I (1)	P	9.3	-	-	Yes	RNA
L10R (1)	P	6.0	-	-	No	RNA
V11I (1)	**A**	**8.1**	**DRV**	**PI**	**Yes**	**RNA**
K20I (1)	P	8.8	-	-	No	RNA
L33V (1)	P	17	-	-	No	RNA
M36I (3)	P	6.5-10.0	-	-	Yes (3)	RNA (3)
L63A (1)	P	18	-	-	No	RNA
L63P (3)	P	5.2-18.0	-	-	No (3)	RNA (3)
L63Q (1)	P	12.0	-	-	Yes	RNA
L63T (1)	P	5.8	-	-	No	RNA
G73C (1)	**A**	**8.5**	**ATV**	**PI**	**No**	**DNA**
V77I (1)	P	6.1	-	-	Yes	RNA
V82I (1)	P	18.0	-	-	Yes	RNA
I93L (1)	P	18.0	-	-	No	RNA
I93T (1)	P	5.1	-	-	Yes	RNA
Reverse transcriptase						
M41I (1)	**R**	**6.5**	**AZT**	**NRTI**	**Yes**	**RNA**
K65E (1)	**R**	**6.6**	**ABC, TDF**	**NRTI**	**No**	**RNA**
K65R (1)	**R**	**18**	**ABC, FTC, 3TC, TDF**	**NRTI**	**No**	**RNA**
D67E (1)	**R**	**5.3**	**AZT**	**NRTI**	**Yes**	**RNA**
V75M (1)	**A**	**19.0**	**AZT**	**NRTI**	**No**	**RNA**
L100I (1)	**R**	**11.0**	**DOR, EFV, ETR, NVP, RPV**	**NNRTI**	**No**	**RNA**
K101P (1)	**R**	**15.0**	**DOR, EFV, ETR, NVP, RPV**	**NNRTI**	**No**	**DNA**
K103N (1)	**R**	**11.0**	**EFV, NVP**	**NNRTI**	**No**	**DNA**
E138K (1)	**R**	**6.9**	**EFV, ETR, NVP, LPV**	**NNRTI**	**Yes**	**RNA**
K219N (1)	**R**	**5.2**	**AZT**	**NRTI**	**Yes**	**RNA**
Integrase						
E138K (1)	**R**	**11.0**	**BIC, CAB, DTG, EVG, RAL**	**INSTI**	**Yes**	**RNA**
G140A (6)	**R**	**5.3-16.0**	**BIC, CAB, DTG, EVG, RAL**	**INSTI**	**Yes (3), No (3)**	**RNA (6)**
G140S (1)	**R**	**16.0**	**BIC, CAB, DTG, EVG, RAL**	**INSTI**	**No**	**DNA**
Y143H (1)	**R**	**5.4**	**CAB, EVG, RAL**	**INSTI**	**No**	**RNA**
G163R (1)	**R**	**17.0**	**EVG, RAL**	**INSTI**	**No**	**DNA**
G163S (1)	**R**	**5.0**	**EVG, RAL**	**INSTI**	**Yes**	**RNA**

Summary of DRMs/NPs agreement between SS and NGS at 20% and 5% variant frequency threshold of the 77 samples with complete PR/RT/INT sequencing

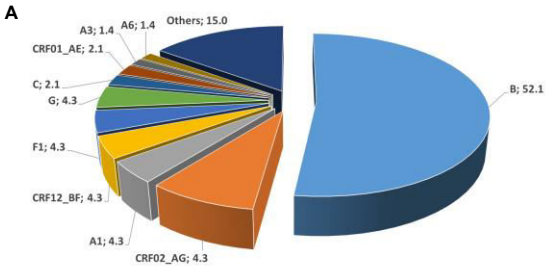
Gene	≥20% cut-off (n, %)				≥5% cut-off (n, %)			
	Sanger and NGS variants agree	Sanger negative & NGS positive	Sanger positive & NGS negative	Total	Sanger and NGS variants agree	Sanger negative & NGS positive	Sanger positive & NGS negative	Total
PR	246 (93.2)	13 (4.9)	5 (1.9)	264 (100)	246 (86.6)	33 (11.6)	5 (1.8)	284 (100)
RT	46 (79.3)	8 (13.8)	4 (6.9)	58 (100)	46 (68.7)	18 (26.9)	4 (5.9)	68 (100)
INT	13 (100)	0 (0.0)	0 (0.0)	13 (100)	13 (54.2)	11 (45.8)	0 (0.0)	24 (100)
Overall	305 (91.0)	21 (6.3)	9 (2.7)	335 (100)	305 (81.3)	62 (16.5)	9 (2.4)	376 (100)

DRMs/NPs divided into PR, RT, and INT, are listed in order of amino-acid position with the specification of how many samples they were detected in; the type of mutation (P = polymorphism; A= accessory resistance mutation; R= resistance-associated mutation), the frequency in percentage, the drugs, and the class of drugs resistance are described. Acronyms: DRV: darunavir; ATV: atazanavir; AZT: zidovudine; ABC: abacavir; FTC: emtricitabine; 3TC: lamivudine; TDF: tenofovir; DOR: doravirin; EFV: efavirenz; ETR: etravirine; NVP: nevirapine; RPV: rilpivirine; BIC: bictegravir; CAB: cabotegravir; DTG: dolutegravir; EVG: elvitegravir; RAL: raltegravir; PI: protease inhibitors; NRTIs: nucleoside reverse transcriptase inhibitors; NNRTIs: non-nucleoside reverse transcriptase inhibitors; INstI: integrase inhibitors.

Of the total samples analyzed, **77** showed **complete sequencing of the three regions PR, RT and INT** in both SS and NGS. In particular, a total of 335 DRMs/NPs were identified at the 20% threshold. As expected, the majority (91.0%) were present in both SS and NGS-generated sequences; a small percentage of these (2.7%) were present only in those obtained with SS, while 6.3% only in those obtained with NGS.
Decreasing the threshold to 5%, **41 additional mutations were detected**, reaching a total of 376 DRMs/NPs. In this case, there was a clear increase (16.5%) at this threshold of mutations present only in sequences obtained with NGS, as expected.

Phylogenetic analysis

Subtypes prevalence



Phylogenetic three

Phylogenetic analysis was performed to determine **viral subtypes** (A), and to evaluate the **proper clustering of the SS and NGS sequences** from the **same subject** of the 77 samples with complete PR/RT/INT sequences (B).

Conclusions

Overall, our **results show that NGS by Ion Torrent S5 assays performance was comparable to SS**, **both using RNA and DNA** at several **viral loads and subtypes**. As expected, **setting cut-off to ≥5% NGS detected MV that could not be detected by SS**, which could improve treatment selection and clinical outcomes. However, the real weight of these MV is yet to be determined, also implementing studies with a larger sample size.

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