1   Limited marginal utility of deep sequencing for HIV drug resistance testing in the age of

2   integrase inhibitors

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Abstract

HIV drug resistance genotyping is a critical tool in the clinical management of HIV infections. Although resistance genotyping has traditionally been conducted using Sanger sequencing, next-generation sequencing (NGS) is emerging as a powerful tool due to its ability to detect lower frequency alleles. However, the value added from NGS approaches to antiviral resistance testing remains to be demonstrated. We compared the variant detection capacity of NGS versus Sanger sequencing methods for resistance genotyping of 144 drug resistance tests (105 protease-reverse transcriptase tests and 39 integrase tests) submitted to our clinical virology laboratory over a four-month period in 2016 for Sanger-based HIV drug resistance testing. NGS detected all true high frequency drug resistance mutations (>20% frequency) found by Sanger sequencing, with greater accuracy in one instance of a Sanger-detected false positive. Freely available online NGS variant callers Hydra and PASeq were superior to Sanger methods for interpretations of allele linkage and automated variant calling. NGS additionally detected low frequency mutations (1-20% frequency) associated with higher levels of drug resistance in 30/105 (29%) of protease-reverse transcriptase tests and 4/39 (10%) of integrase tests. Clinical follow-up of 69 individuals for a median of 674 days found no difference in rates of virological failure between individuals with and without low frequency mutations, although rates of virological failure were higher for individuals with drug-relevant low frequency mutations. However, all 27 individuals who experienced virological failure reported poor adherence to their drug regimen during preceding follow-up time, and all 19 who subsequently improved their adherence achieved viral suppression at later time points consistent with a lack of clinical resistance. In conclusion, in a population with low antiviral resistance emergence, NGS methods detected numerous instances of minor alleles that did not result in subsequent bona fide virological failure due to antiviral resistance.

2

51   Importance

52   Genotypic antiviral resistance testing for HIV is an essential component of the clinical

53   microbiology and virology laboratory. Next-generation sequencing (NGS) has emerged as a

54   powerful tool for the detection of low frequency sequence variants (allele frequencies

55   <20%). Whether detecting these low frequency mutations in HIV contributes to improved

56   patient health, however, remains unclear. We compared NGS to conventional Sanger

57   sequencing for detecting resistance mutations for 144 HIV drug resistance tests submitted

58   to our clinical virology laboratory and detected low frequency mutations in 24% of tests.

59   Over approximately two years of follow-up for 69 patients for which we had access to

60   electronic health records, no patients had virological failure due to antiviral resistance.

61   Instead, virological failure was entirely explained by medication non-adherence. While

62   larger studies are required, we suggest that detection of low frequency variants by NGS

63   presents limited marginal clinical utility when compared to standard of care.

64

3

Introduction

66 Antiretroviral drug therapy (ART) for HIV has been tremendously successful in reducing

67 HIV morbidity, mortality, and transmission (1). However, rising rates of HIV infections

68 resistant to antiretroviral drugs pose a major threat to ongoing efforts to control the

69 pandemic (2). Routine HIV drug resistance genotyping improves health outcomes in HIV

70 patients (3, 4) and is a cost-effective tool in the clinical management of HIV infection (5).

71 Genotypic resistance testing assays have traditionally been based on Sanger

72 sequencing (6). While Sanger methods are highly reproducible and validated, minority HIV

73 resistance mutations present in less than 20% of the viral population may escape detection

74 (with a range of approximately 10-30%, depending on the sample context) (6, 7). The

75 capacity of next-generation sequencing (NGS) to provide additional data on low-frequency

76 drug resistant mutations (DRMs) and its potential for lower costs per sample with large

77 batches has led many clinical laboratories to consider transitioning from Sanger sequencing

78 to NGS (8, 9). A commercial NGS HIV drug resistance test has recently received European

79 and Singaporean in vitro diagnostics approval (10). Numerous previous studies have

80 demonstrated NGS methods detect more resistance-associated mutations at low

81 frequencies that Sanger is unable to detect (11-20). However, the predictive value of

82 detectable low frequency mutations for virological failure or other clinical outcomes

83 remains unclear (21-23).

84 Anticipating the potential utility of NGS for HIV drug resistance detection is also

85 complicated by the ever-changing landscape of HIV therapy. A previous pooled analysis of

86 HIV-1 resistance mutations associated with NNRTI resistance found a dose-dependent

87 increased risk of virological failure with first-line ART (24). However, prior studies seeking

88 to associate low frequency DRMs with virological failure provide insufficient evidence of

89 NGS utility because they lacked an appropriate retrospective comparison group (15), were

4

90   conducted within a clinical trial (12) (thus lacking representation of real-world patient

91   samples submitted for genotyping in routine care), or were conducted more than 3 years

92   ago and thus focused on NNRTIs (11, 24). Current ART treatment guidelines for treatment-

93   naïve HIV-1-infected patients include an integrase strand transfer inhibitor (INSTI) (25).

94   Dolutegravir is a particularly promising therapy due to its high barrier to resistance (26-

95   28). Given that recent studies of low frequency resistance mutations during integrase and

96   protease inhibitor-based treatments have generally failed to find an association with

97   virological failure (27, 29-34), the clinical value of NGS sequencing for a standard clinical

98   laboratory requires more investigation.

99   Our reference laboratory provides clinical virology testing for patients in the

100   western Washington region and performed 817 HIV resistance genotyping tests in 2017.

101   Recently, Seattle became one of the first metropolitan areas in the world to attain the 90-90-

102   90 UNAIDS treatment goals, wherein 90% of the patients receiving ART achieve viral

103   suppression (35). Clearly, these goals were reached in the absence of NGS drug resistance

104   testing. Given the concomitant interest in the perceived superiority of NGS for drug

105   resistance in the setting of already successful drug resistance management, we sought 1) to

106   evaluate the technical capacity of Sanger versus NGS sequencing methods for samples

107   submitted to our clinical virology laboratory for HIV resistance genotyping, and 2) to assess

108   the clinical impact of low frequency DRMs detected only by NGS on viral suppression over

109   time.

110

111   Materials and Methods

112   **Test cohort**

113   Serum/plasma for HIV antiviral resistance testing were collected as part of routine care and

114   Sanger sequenced for antiviral resistance in our reference clinical virology laboratory using

5

115   a nested RT-PCR approach described below (Figure 1A)(36). To best compare Sanger

116   versus NGS and to limit PCR cycles prior to NGS library preparation for more accurate allelic

117   representation, we used stored first-round RT-PCR amplicons from prior HIV drug

118   resistance testing: 105 protease-reverse transcriptase (Pr-RT) and 39 integrase (INT)

119   amplicons. To allow clinical follow-up while maintaining drug resistance testing relevance

120   for current HIV regimens, we performed NGS testing on amplicons from tests originally

121   performed approximately two years ago (February to May 2016). Ethical approval for this

122   study was granted by the University of Washington Institutional Review Board.

123

124   **Sanger sequencing, variant calling, and interpretation.** Results of previous genotypic

125   resistance assays performed by our clinical virology laboratory were obtained as the

126   standard of care comparison group. Plasma/serum were previously processed through the

127   laboratory's standardized HIV genotypic resistance assay protocol, which involves HIV RNA

128   separated from plasma via the Boom method silica extraction (37). Extracted RNA was used

129   in a RT-PCR reaction using random hexamers to create cDNA with reagents from GeneAmp

130   RNA PCR kit (Perkin-Elmer, Foster City, CA) according to the manufacturer's protocol.

131   Nested PCR amplification was performed in 50ul volume reactions, consisting of 5ul

132   10X PCR buffer (with 15mM $MgCl_2$), 8 ul dNTP's (1.25mM each), 1ul each forward and

133   reverse primer (20pmol/ul), 0.5 ul Taq (2.5 units per 10μl cDNA sample for first-round

134   PCR; 2.5 units per 5μl PCR product for second round PCR), and nuclease free water. A list of

135   primers is provided in Supplementary Table S-1. A thermal cycler protocol programmed for

136   94°C x 5 minutes for initial denaturation, followed by 35 cycles of 15 seconds at 94°C for

137   denaturation, 30 seconds at 57°C for annealing, 2 min at 72 °C for extension, and final

138   extension at 72 °C for 7 minutes. An annealing temperature of 55 °C was used for second

139   round nested PCR.

6

140   A sample of the first-round PCR product was stored in a -20°C sample archive. The

141   second-round PCR product was visualized on a UV light box (254nm wavelength) after gel

142   electrophoresis [1.5 % agarose ethidium bromide gel with 1X Tris Acetate-EDTA buffer

143   (TAE) (Sigma T9650-1L) run at 80mV for 30 minutes. This PCR product was cleaned by

144   adding 2 ul of Exonuclease I and Shrimp Alkaline Phosphatase directly to 10 ul PCR product

145   without changing buffer conditions. Thermal cycler programmed for 37°C x 15 minutes,

146   80°C x 15 minutes, 4° C x infinity. Each sample was diluted with nuclease free water to

147   achieve a final concentration 10-40 ng per reaction. Each sequencing primer (5.0 pmol/uL)

148   was added to 10ul of the pre-cleaned diluted PCR template in a pre-labelled PCR Genemate

149   Skirted 96 well PCR plate (T-3107-1).

150   The samples were sent to a centralized sequencing facility. A Big Dye terminator kit

151   was used for the sequencing reaction. Montage SEQ96 Millipore sequence clean up kit was

152   used for post sequencing. Samples were analyzed using 3730 XI ABI instrument and

153   sequence analysis performed using Sequencher software v5.2.2

154   Criteria for acceptable data included: raw data peak intensities between 1000 and

155   5000, average noise intensity <15%, pure base QV rated high and no abrupt signal changes,

156   elevated base line or spikes. Variant mixes were called when visible in both forward and

157   reverse reads, with and minor peaks visible for at least 25% surface area of the major peak.

158   Results were recorded in a genotypic resistance assay report that was returned to

159   the patient's care provider via the electronic medical record. The report includes a list of

160   mutations associated with drug resistance in the three *pol* gene segments (protease, reverse

161   transcriptase, and integrase) and an interpretation of the resistance profile (what drugs

162   should be avoided) based on the detected mutations. Mutations listed in the genotypic

163   resistance assay report were also interpreted using the Stanford HIV Drug Resistance

164   Database (HIVdb version 8.5) (38). For each mutation, levels of resistance were recorded

7

165   categorically according to the HIVdb standard categories: (0) no evidence of resistance; (1)

166   potential low-level resistance; (2) low level resistance; (3) intermediate resistance; and (4)

167   high level resistance. A sample was considered to have any resistance if it had one or more

168   mutations categorized as a level 1 or higher.

169

170   **NGS sequencing, variant calling, and interpretation.** First-round amplicons for each test

171   were retrieved from -20°C archive and cleaned using 1.0X Ampure beads, quantitated on a

172   Qubit 3.0, and diluted to 1 ng/uL. Libraries were prepped using quarter-reactions of

173   Nextera XT followed by 15 cycles of dual-indexed PCR amplification and sequenced to

174   achieve between 50,000-100,000 reads per sample on an Illumina MiSeq using both 1x192

175   and 2x300 bp runs. Amplicons with less than 10,000x coverage were excluded to ensure

176   sufficient coverage for detection of low frequency mutations. Samples were sequenced in

177   batches of 20-24 samples to minimize the possibility of index cross-talk on the MiSeq

178   platform and each included positive (8E5 cell line) and negative (nuclease-free water)

179   controls to confirm the run. Sequences were preprocessed using cutadapt (39) and

180   uploaded to two online variant callers: PASeq (https://www.paseq.org/) and HyDRA

181   (https://hydra.canada.ca/). These two variant callers were selected because they are

182   highly-developed, free variant callers with user-friendly web interfaces that require

183   minimal bioinformatics skills and provide robust, reproducible, and easy-to-interpret

184   results that could be implemented in a clinical laboratory (40). Both callers use an

185   annotated HXB2 sequence as their reference for variant calling and the well-established

186   Stanford HIV Drug Resistance Database (version at time of use: 8.5) to provide resistance

187   interpretations for DRMs.

188   A "low frequency" DRM was defined as a resistance-associated mutation between

189   1% and 20% allele frequency, based on the default 1% minimum allele frequency needed

8

190   for a mutation to be considered in the drug resistance report produced by both HyDRA and

191   PASeq (40, 41). A DRM was considered "high frequency" if detected at greater than 20%

192   allele frequency. Resistance interpretations were determined by the same method

193   described above for Sanger-detected mutations.

194

195   **Patient characteristics and clinical record abstraction for patient follow-up cohort**.

196   Patients' age, sex, and sample date (baseline) were determined based on patient

197   demographics recorded on the genotypic resistance assay report for all patients included in

198   the test cohort. Electronic medical records were reviewed for clinical outcomes. Patients

199   lacking a recorded plasma HIV RNA test result between the sample date and May 25, 2018

200   were excluded from the patient follow-up cohort due to lack of available follow-up.

201   Laboratory and clinical visit records for patients included in the follow-up cohort were

202   abstracted using a structured data form (information collected is described below), which

203   was designed based on a preliminary in-depth search of five records prior to full record

204   search and abstraction.

205   The following information was recorded for each patient: date of first plasma HIV

206   RNA test in our laboratory information system, plasma HIV RNA load at date of sample (or

207   nearest date), drug regimen prescribed by clinician during clinic visit subsequent to sample

208   date (when both plasma HIV RNA measurement and HIV genotyping results were available

209   to inform clinician actions), and date of most recent office visit where HIV care was

210   received. Adherence (yes/no) was assessed based on clinician visit notes as to whether the

211   patient reported being adherent to the drug regimen prescribed at each clinic visit after

212   baseline. Treatment experience was assessed as (naïve/experienced) based on whether a

213   new diagnosis or initiation of first treatment was noted in the clinical record at baseline

214   clinic date.

215   The primary outcome of interest, virological failure (yes/no), was determined

216   according to the U.S. Health and Human Services definition—failing to achieve or maintain

217   suppression of viral replication to a plasma HIV RNA level ≤200 copies/mL (42)—at a test

218   date more than one month after beginning treatment regimen. Prescribed drug regimens

219   were extracted from the medical record. Low frequency mutation exposure was categorized

220   as present/absent, according to whether the Stanford HIVdb resistance profile determined

221   by NGS was higher in level of resistance compared to the profile reported by Sanger data in

222   the clinical record. In a second calculation of risk, exposure was determined as present

223   when a patient was prescribed a drug regimen which would have been contraindicated

224   based on the resistance profile (including low-frequency mutations) found by NGS.

225

226   **Statistical analyses**

227   Agreement of DRMs called by each NGS caller (HyDRA and PASeq) was analyzed for

228   concordance using percent agreement and Cohen's Kappa (a standard agreement coefficient

229   for binary ratings). The PASeq and HyDRA allele frequency measures for all called

230   mutations were compared by linear regression analysis. Any variants not called by either

231   caller were assumed to be 0% allele frequency and excluded from the concordance analysis

232   due to absence of quantitation. The linear model coefficient (HyDRA's allele frequency

233   measurement as a function of PASeq's) was also reported. Incidence rate of virological

234   failure was determined based on patients' first occurrence of HIV RNA level ≥200

235   copies/mL divided by time at risk (between sample date and censored date). Patients were

236   censored at date of measured virological failure or last available plasma HIV RNA

237   quantification test prior to May 25, 2018. Relative virological failure rate ratios were

238   calculated using the *fmsb* package (43) and all statistical analyses were conducted in R

239   (version 3.4.3) through the RStudio interface (version 1.0.153).

10

240

241 Results

242 **NGS estimated higher levels of drug resistance in test cohort.**

243 Of 144 total tests, 29 patients had both Pr-RT and INT tests ordered for them during the

244 sample period and two patients had a repeat Pr-RT test ordered in the sample period.

245 Therefore, a total of 113 unique patients were ultimately included in the test cohort (Figure

246 1B). Patients were predominantly male (91%) with a median age of 40 (range, 5-67). Most

247 (95%) had HIV subtype B, 5 patients had subtype C (contributing 6 tests), and one had

248 subtype A (1 test). Additional descriptive statistics for the test cohort are listed in Table 1.

249 Using the standard of care genotyping test by Sanger sequencing, 40 (28%) tests

250 were positive for resistance to at least one drug. By NGS, 63 (44%) tests were positive for

251 resistance by PASeq and 60 (42%) by HyDRA. NNRTI resistance was most common among

252 both methods; PI and INSTI resistance were least common (Table 1). Of 105 Pr-RT tests,

253 Sanger detected 2 (2%) with PI DRMs, 18 (17%) with NRTI DRMs, and 28 (27%) with

254 NNRTI DRMs. Among 39 INT tests, 1 (3%) had a DRM associated with resistance to INSTI.

255 PASeq detected 9 (9%) more samples with PI resistance; 6 (6%) more samples with NRTI

256 resistance, 11 (10%) more samples with NNRTI resistance, and 3 (8%) more samples with

257 INSTI resistance. HyDRA found similarly higher resistance compared to Sanger: 9 (9%)

258 more samples with PI DRMs, 8 (8%) more with NNRTI DRMs, 7 (9%) more with NRTI

259 DRMs, and 4 more (10%) with INSTI DRMs. Overall, NGS estimated higher levels of drug

260 resistance to one or more antiretroviral drugs for 34 (24%) tests—30/105 (29%) protease-

261 reverse transcriptase tests and 4/39 (10%) of integrase tests.

262

263 **Sanger and NGS variant calls have high concordance with some limitations.**

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264   Sanger and NGS showed almost perfect agreement in their detection of high frequency

265   DRMs (97% of tests). In total, four unexpected differences were observed in a comparison

266   of Sanger versus NGS-detected variant calls (overview in Figure 2 and Figure S-1; detailed in

267   Figure 3A-D). All were the result of position-specific over-interpretation of variants in

268   Sanger. One Pr-RT test was discordant in the mutation called, resulting in a false-positive

269   error by Sanger for antiviral susceptibility. Sanger called a mixed L210CW mutation, which

270   is associated with high resistance to didanosine (ddI), abacavir (ABC) and tenofovir (TDF)

271   (38). NGS, however, did not call this mutation (Figure 3A). Upon manual inspection of the

272   NGS sequence, it became clear that the Sanger read was a false positive for resistance due to

273   the inability of Sanger to interpret linkage between two adjacent nucleotide bases. In this

274   instance, the reference codon was TTG (L - leucine) but the viral population contained a mix

275   of the reference codon and TGT (C - cysteine). Thus, NGS read the variant as L210C and did

276   not call it as a DRM. Sanger, on the other hand, interpreted the codon as having a mix of Gs

277   at both the second and third bases, which led to an interpretation of TGT (C - cysteine) and

278   TGG (W – tryptophan). In three other Pr-RT tests, Sanger reported mutations that were

279   detected at less than 20% allele frequency by NGS: V108I was called by HyDRA (15.9%) and

280   PASeq (17.1%) (Figure 3B), K103N was called by HyDRA (8.9%) and PASeq (7.0%), and

281   H221Y was called by HyDRA (17.9%) and PASeq (17.7%) (Figure 3C). These results

282   demonstrate that DRMs at allele frequencies under 20% can be detected by Sanger

283   sequencing.

284

285   **NGS callers HyDRA and PASeq are essentially equivalent in their DRM calls.**

286   At a 20% allele frequency threshold, HyDRA and PASeq had 100% agreement in

287   terms of which mutations they detected (binary: detected/not; Kappa = 1.0). Concordance

288   of DRM allele frequencies (continuous: 0-100%; Bland-Altman plot in Figure 4A) detected

12

289   by HyDRA versus PASeq was also very high (linear model coefficient = 1.00; R^2 = 1.00). Of

290   note, HyDRA reported many more accessory variants and polymorphisms compared to

291   PASeq (Figure 4B). Of 56 unique DRMs detected among 105 Pr-RT sequences and 3 unique

292   DRMs detected in 39 INT sequences, 40 (71%) and 3 (100%) mutations, respectively, were

293   detected with perfect agreement between the callers (Kappa = 1.0).

294   DRMs on which the two callers disagreed were predominantly very low frequency

295   calls (Figure 4B); the median frequency of mutations detected only by either HyDRA or

296   PASeq (and thus "missed" by the other) was 1.15%. Upon manual inspection of DRMs

297   between 1-2% allele frequency, the discrepancies were attributable to minute differences in

298   the number of reads registered by each caller. For further comparisons of NGS and Sanger

299   results, the callers were assumed to have performed sufficiently similarly and thus the

300   variant and resistance profiles called by HyDRA were used to represent the NGS results for

301   subsequent analyses comparing NGS to Sanger in the patient follow-up cohort.

302

303   **Patient follow-up cohort included 69 patients followed for an average of 674 days.**

304   Of 113 patients included in the test cohort, 69 received care at UW Medicine and had

305   obtainable clinical follow-up data. The median follow-up time was 674 days (IQR: 560-728

306   days). This cohort was a subset of the test cohort, with a similar age and sex distribution

307   (median age = 40; 88% men) and prevalence of low frequency DRMs (32% of patients).

308   Summary statistics for the cohort are provided in Table 2. A higher proportion of patients

309   with low frequency DRMs were female, compared to those without. In the patient cohort, 27

310   patients (39%) experienced virological failure of any plasma HIV RNA ≥200 copies/mL

311   during the follow-up period, 12 (17%) patients had changes to their drug regimen in follow-

312   up and 12 (17%) patients received additional Sanger resistance tests as part of regular care.

13

313   None of these additional Sanger resistance tests detected any additional DRMs compared to

314   the baseline Sanger resistance test.

315

316   **NGS detected more resistance mutations but these did not significantly associate with**

317   **virological failure, which was more likely attributable to drug non-adherence.**

318   Of 69 patients with clinical follow-up, 22 (32%) had low frequency DRMs detected by NGS

319   that were not found by Sanger. Low frequency DRMs were most commonly associated with

320   NRTI resistance (9 patients), followed by NNRTI (7 patients), PI (6 patients), and INSTI (4

321   patients). Five (7%) patients received drugs which would have been contraindicated based

322   on the resistance profiles found by NGS.

323   We were not able to detect a difference in the risk of virologic failure between

324   patients with any low frequency DRMs at baseline compared to those without (RR = 1.61;

325   95% CI: 0.75-3.48). Three patients had only level 1 low frequency DRMs; when we restrict

326   our exposure definition to patients with level 2 or higher resistance (see definition provided

327   in methods section), the virological failure rate in patients with any low frequency DRMs is

328   not significantly higher than in those without (RR = 1.80; 95% CI: 0.82 – 3.92). Among five

329   patients with low frequency DRMs specifically associated with one of their prescribed

330   drugs, four experienced virological failure. Compared to patients without affected regimens,

331   these five patients had a higher rate of virological failure (RR = 3.61; 95% CI: 1.25-10.44).

332   However, all participants who experienced virological failure and subsequently

333   reported improved adherence to their drug regimen were able to achieve viral

334   suppression—regardless of whether or not they had low frequency DRMs—even though

335   most of them (16 of 19 patients) did not change drug regimens in that time period (Figure

336   5A).

14

Discussion

337

338   Our study suggests a limited marginal utility of next-generation sequencing

339 compared to Sanger sequencing for determining HIV antiviral drug resistance in a

340 population with a relatively low prevalence of antiviral resistance. We also demonstrate

341 two free, online variant callers PASeq and HyDRA were marginally superior compared to

342 Sanger sequencing for detection of high frequency DRMs. Both NGS callers were highly

343 concordant with each other in respect to their measured allele frequencies for DRMs.

344 Besides HyDRA's automatic reporting of many more accessory variants and polymorphisms

345 compared to PASeq, the two callers provided nearly interchangeable results.

346   PASeq and HyDRA improve upon Sanger sequencing's manual variant calling

347 procedures by being free and automated, and thus faster, easier, and less expensive. Our

348 study also illustrated two limitations of manual examination procedures for interpreting

349 Sanger chromatograms: missing linkage and reliance on trained laboratory professionals.

350 First, Sanger was unable to impute linkage between two alleles, which manifested as a DRM

351 mixture (L210CW) instead of a polymorphism (L210C). In this example case, the erred call

352 was a false-resistance determination that did not negatively impact patient care because the

353 potentially affected drug types are uncommonly prescribed in this care setting, and the

354 erred variant call is associated with only potential and low levels of associated resistance

355 (zidovudine, stavudine, and didanosine) according to HIVdb. Second, Sanger called three

356 resistance mutations that were below Sanger's expected 20% threshold of sensitivity. This

357 "over-calling" is illustrative of how the procedures rely on a highly-trained lab technician to

358 review DRM regions in a Sanger sequenced resistance test—had the technician not been

359 experienced enough to expect a DRM in that location, it may have been missed. Both

360 manifestations of Sanger "error" resulted from the human element of Sanger variant calling.

361 Given the ease of use and technical capacity of HyDRA and PASeq, the two callers are both

15

362   pragmatic options for the detection of variants and interpretation of NGS results in clinical

363   laboratory HIV resistance testing.

364   In our comparison of overall DRM detection rates, NGS detected more mutations

365   associated with higher levels of drug resistance in 30/105 (29%) of Pr-RT sequences and

366   4/39 (10%) of INT sequences, due to its capacity to detect low frequency DRMs. These

367   findings are consistent with numerous studies in which NGS detected more DRMs compared

368   to Sanger (11-14, 16-20). However, few studies have performed clinical follow up after

369   detection of low frequency DRMs to determine whether the additional DRMs are relevant to

370   patient care or outcomes. Here, we have presented the first study to investigate the effect of

371   low frequency DRMs on viral suppression in patients on multi-drug therapeutic regimens in

372   a pragmatic retrospective cohort of samples submitted for routine HIV antiviral resistance

373   genotyping to a clinical virology lab.

374   We found that the 7% of patients prescribed a drug regimen to which they may have

375   been contraindicated based on low frequency DRMs detected by NGS may be at higher risk

376   of virological failure compared to patients without drug-regimen associated DRMs. With 69

377   patients and a 34% rate of virological failure in those *without* DRMs, we had 80% power to

378   detect increases in the virological failure rate among those *with* DRMs at 75% failure rate or

379   higher. All virological failures in the cohort were associated with medication non-

380   adherence. Low frequency DRMs were most commonly associated with NRTI and NNRTI

381   resistance, even though NNRTI drugs were rarely included in drug regimens of patients in

382   the follow-up cohort (3 patients; 4%). This finding is consistent with previous research (20,

383   44, 45) and suggests that some proportion of DRMs may have originated during prior drug

384   experience or been transmitted upon infection. Low frequency DRMs may also arise de novo

385   as part of natural viral diversification in the body (46). Poor adherence to ART muddles the

386   association between low frequency mutations and virological failure because it can

16

387 contribute to the emergence of resistant viral subpopulations (observed as low frequency

388 DRMs), as well as be a primary cause of a patient's high plasma HIV RNA (Figure 5B). The

389 association may also be confounded by higher viral loads, which could increase the

390 likelihood that a minor variant is present in a sufficient quantity for detection by NGS.

391 However, we think the contribution of this source of confounding is minimal in our study

392 because the laboratory acceptance criteria for the test is 1000 copies/mL and just eight

393 (6%) tests were below this threshold. Furthermore, low frequency variants were detected

394 across a range of viral loads (Figure S2).

395   More than 80% of patients in our cohort were prescribed a regimen that included an

396 integrase inhibitor at baseline. However, despite this high prevalence of INSTI prescriptions

397 and low prevalence of INSTI resistance mutations, 39% of patients in the cohort

398 experienced virological failure (70% of whom were on an INSTI-based regimen), and just

399 four (15%) failures occurred in patients on drug regimens contraindicated by their NGS

400 resistance profile. Furthermore, the majority of patients never changed drug regimens

401 following their "virological failure," and patients who continued to report poor adherence

402 experienced uncontrolled viral loads well over 200 copies/mL for the duration of follow-up

403 while those with improved adherence achieved suppression (below the limit of detection;

404 <40 copies/mL). This suggests that poor adherence may be a primary factor affecting rates

405 of viral suppression in this cohort, irrespective of the prevalence of low frequency DRMs.

406   By evaluating the utility of NGS in the context of a pragmatic patient population, we

407 provide a real-world example of its potential to inform clinical care and elucidate factors

408 that could affect its implementation. Our focus on a single high-resource setting is a

409 potential limitation of the study. Seattle is unique in already having achieved UNAIDS goals

410 of 90% of patients on ARV with suppressed virological loads, indicating a smaller potential

411 return on investment for NGS antiviral testing and a low overall prevalence of antiviral

17

412   resistance. In a high-resource clinical setting, any indication of resistance on a Sanger

413   resistance assay report—even at low levels—is sufficient to rule out that drug for an ARV

414   regimen. DRMs causing any degree of change to the level of resistance interpreted by HIVdb

415   were therefore considered in this analysis. In less resource-rich settings, however,

416   "potential" (level 1) and "low-level" (level 2) resistance may need to be excluded from the

417   analysis because their clinical significance is not well understood and may be overly

418   conservative for prescribing practices in resource-limited settings.

419   Another limitation of our study is its low power. Despite significant efforts exerted

420   to obtain a sufficiently large cohort size for the analysis, more than a third of patients in the

421   test cohort were excluded from the sample cohort due to lack of follow-up (or inability to

422   obtain medical records). The exclusion of these individuals is not a major concern as a

423   source of selection bias because loss to follow-up is a regular occurrence in the course of

424   clinical management and remains representative of a pragmatic population receiving

425   testing in a clinical lab. Given the clinical and potential economic importance of this study's

426   objectives, a long-term cohort study involving many more patients receiving HIV care and

427   repeat sequencing is needed. However, we also caution that such a study may have limited

428   generalizability in the current age of fast-changing HIV clinical pharmacology. As has been

429   the case for efforts to interpret prior similar studies conducted in populations and time

430   periods without integrase inhibitors (11, 24), evolving treatment regimens make it difficult

431   to develop informative pragmatic cohort studies to validate the clinical relevance of low

432   frequency variants.

433   Regardless, our findings demonstrate two online, free, and easy-to-use NGS variant

434   callers have high concordance with Sanger in a pragmatic clinical setting and thus may be

435   good candidates for implementation as part of a clinical laboratory analysis pipeline for HIV

436   drug resistance. However, given growing prevalence of integrase inhibitor regimens with

18

437   high barriers to resistance and high prevalence of low frequency DRMs (predominantly

438   NNRTI and NRTI) in patients with poor adherence, we suggest clinical laboratories use

439   caution in overvaluing the low-frequency variant detection afforded by NGS for clinical

440   antiretroviral resistance genotyping as it may not provide much improvement over

441   standardized Sanger methods for detection of clinically relevant resistance in a real-world

442   context.

443

444

445   **Acknowledgements**

19

449 **Table and Figures**

450 **Table 1.** Demographic and clinical characteristics of patients included in the test cohort.

Patient characteristics[1]	(n = 113)	
Patient age, median (IQR)	40 (33-49)	
Men, n (%)[1]	103 (91)	
Plasma HIV RNA at baseline [log10(copies/mL)] – median (IQR)	4.2 (3.7-4.8)	
Missing plasma HIV RNA measure at baseline	27 (24)	
B subtype, n (%)	107 (95)	
C subtype	5	
A subtype	1	
Test characteristics	**(n = 144)**	
Any (≥1) DRM reported by clinical genotyping (Sanger sequencing), n (% of tests)	40 (28)	
PI	2 (2)	
NRTI	18 (17)	
NNRTI	28 (27)	
INSTI	1 (3)	
	HyDRA[2]	PASeq
Any (≥1) DRM detected by NGS, n (% of tests)	60 (42)	63 (44)
PI	11 (11)	11 (10)
NRTI	27 (26)	24 (23)
NNRTI	36 (34)	39 (37)
INSTI	5 (13)	4 (10)

451 [1] Percentages calculated based on 113 unique patients in test cohort: 29 patients had both

452 Pr-RT and INT tests and 2 patients had a repeat Pr-RT test during the sample period.

453 (*Protease-reverse transcriptase test*) PI: protease inhibitors; NRTI:

454 nucleoside/nucleotide reverse transcriptase inhibitors; NNRTI: non-nucleoside

455 reverse transcriptase inhibitors

456 (*Integrase test*) INSTI: integrase inhibitors

20

457

458   2 HyDRA DRMs were used as the "NGS" value for subsequent analyses in the patient follow-

459   up cohort that compared Sanger to NGS resistance profiles.

460   **Table 2.** Demographic and clinical characteristics of patients included in the patient follow-

461   up cohort.

Patient characteristics	No low frequency DRMs (n=47)	Low frequency DRMs detected (n=22)	p-value*	(n=69)
Patient age – median (IQR)	40 (32 – 48)	40 (36 -51)	0.34	40 (33-48)
Men (%)	43 (91)	18 (82)	0.44	61 (88)
Total follow-up [days] – median (IQR)	656 (557-705)	713 (638-750)	0.46	674 (560-728)
Plasma HIV RNA, baseline [log10(copies/mL)] – median (IQR)	4.2 (3.6-4.9)	4.2 (3.9-4.6)	0.41	4.2 (3.8-4.8)
- Missing	4	1		5
Virological failure (%)	16 (34)	11 (50)	0.32	27 (39)
- Rate (per 10,000 person-days)	6.4	10.3		7.5
Changed drug regimen from baseline (%)	10 (21)	2 (9)	0.37	12 (17)
Received repeat Sanger resistance genotyping in follow-up (%)	9 (19)	3 (14)	0.82	12 (17)
Treatment naïve (%)	10 (25)	1 (7)	0.26	11 (25)
- Missing	7	7		14
Drugs included in prescribed regimen (% of patients):				
PI	15 (32)	6 (27)	0.91	21 (30)
NRTI	47 (100)	22 (100)	-	69 (100)
NNRTI	3 (6)	0 (0)	0.20	3 (4)
INSTI	39 (83)	17 (77)	0.81	56 (81)
Reported as resistant to any drug by clinical genotyping, n (% of tests)	16 (34)	9 (41)	0.78	25 (36)
Low frequency DRM detected (% of patients):				22 (32)

21

PI	0	6 (27)	-	6
NRTI	0	9 (41)	-	9
NNRTI	0	7 (32)	-	7
INSTI	0	4 (18)	-	4

462   *Student t-test (means) or proportion test, as applicable: comparing patients with no low

463   frequency DRMs to those with low frequency DRMs.

464

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647

648   **Figure 1. Sequencing protocol comparison.**

649   A) First-round amplicons from clinical resistance genotyping by Sanger sequencing were

650   also processed by next-generation sequencing and the resultant resistance profiles

651   compared.

652   B) Test cohort included drug resistance genotyping tests originally performed as part of

653   routine HIV care at UW Medicine between February and May 2016, and patient cohort

654   included those patients who provided tests in the test cohort and had available follow-up

655   records between sample date and May 25, 2018.

656

657   **Figure 2. Histogram of DRM frequency by method of sequencing.**

658   Number of DRMs detected at each allele frequency, by NGS only (red), by both Sanger and

659   NGS (blue), and by Sanger only (yellow). A-D letters highlight disagreements found between

30

660   NGS and Sanger variant calls made at the 20% allele frequency threshold expected for

661   Sanger sequencing. Each instance is detailed in the corresponding letter of Figure 3.

662

663   **Figure 3. Discrepancies between Sanger and NGS variant calls at 20% allele frequency**

664   **threshold.**

665   A) Sanger called a mixed L210CW variant, which is associated with resistance to three NRTI

666   drugs. However, neither NGS caller called the variant as a DRM because they recognized the

667   linkage between two adjacent nucleotide bases, which clarified the variant as TGT (C –

668   cysteine; polymorphism), rather than a mixture that included TGG (W – tryptophan; DRM).

669   Three different DRMs were called by Sanger sequencing that were detected at less than

670   20% allele frequency by NGS, including V108I (16-17%, B), K103N (7-9%, C), and H221Y

671   (18%, D).

672

673   **Figure 4. Concordance of allele frequency calls by HyDRA and PASeq variant callers**.

674   A) Bland-Altman plot comparing allele frequency measurements for variants called by

675   PASeq and HyDRA.

676   B-1) Correlation plot of allele frequency measurements for variants called by PASeq (x-axis)

677   versus HyDRA (y-axis). Accessory mutations are in blue and DRMs are labeled in red. The

678   default setting of HyDRA includes calls of many more accessory variants than PASeq, which

679   led to the large number of accessory mutations called by HyDRA but not by PASeq. The

680   accessory mutation S168G (blue) was called by both callers at 90% allele frequency. B-2)

681   Zoomed in view of 1-2% frequency range (cluster of observations in bottom left corner of

682   Figure 4B-1). *For figure illustration, all true zero values were assigned a random uniform

683   value between 0-1% in (A) and 0-0.1% in (B).

684

31

685   **Figure 5. Results of clinical follow-up for patients and theoretical model.**

686   A) Low frequency DRMs are correlated with ongoing medication adherence, as a result of

687   prior drug experience and current selective drug pressure, which is thus associated with

688   virological failure, regardless of drug resistance. B) Stratification of clinical outcomes in

689   patient cohort, given the presence or absence of low frequency DRMs associated with

690   reduced susceptibility to their drug regimen. Adherence was defined as documentation of

691   poor medication adherence in clinical chart. Virological failure was defined as plasma HIV

692   RNA level >200 copies/mL at a test date more than one month after baseline.

693   [a] Lamivudine/zidothymidine was replaced with emtricitabine/tenofovir following poor

694   adherence in patient's regimen, potentially contributing to subsequent suppression.

695   [b] Darunavir was replaced with dolutegravir in both patients' regimens, potentially

696   contributing to subsequent suppression.

697   Days-at-risk refers to time between sample date and censor date. Patients were censored at

698   date of measured virological failure or last available plasma HIV RNA quantification test.

699   Days follow-up includes all days between sample date and last plasma HIV RNA

700   quantification test.

701

702   **Supplemental Material**

703

704   **Figure S-1**. **Agreement of Sanger and NGS variant resistance interpretations at 20%**

705   **and 1% cutoffs.**

706   Low frequency DRMs refer to those between 1-20% frequency and were only called by NGS.

707   Tests with agreement between Sanger and NGS (green); disagreement (blue); Sanger false-

708   positive due to inability to detect linkage (orange).

709

32

710 **Figure S-2. Low frequency variants detected in patients with a range of viral loads.**

711 A) Boxplot distribution of patients' viral load [plasma HIV RNA log(copies/mL)] at sample

712 date for samples with and without detected low frequency DRMs.

713 B) Viral load of patient in which a low frequency DRM was detected and the allele frequency

714 of the detected DRM(s).

715

33

Figure 1. Sequencing protocol comparison.

A) First-round amplicons from clinical resistance genotyping by Sanger sequencing were also processed by next-generation sequencing and the resultant resistance profiles compared.

B) Test cohort included drug resistance genotyping tests originally performed as part of routine HIV care at UW Medicine between February and May 2016, and patient cohort included those patients who provided tests in the test cohort and had available follow-up records between sample date and May 25, 2018.
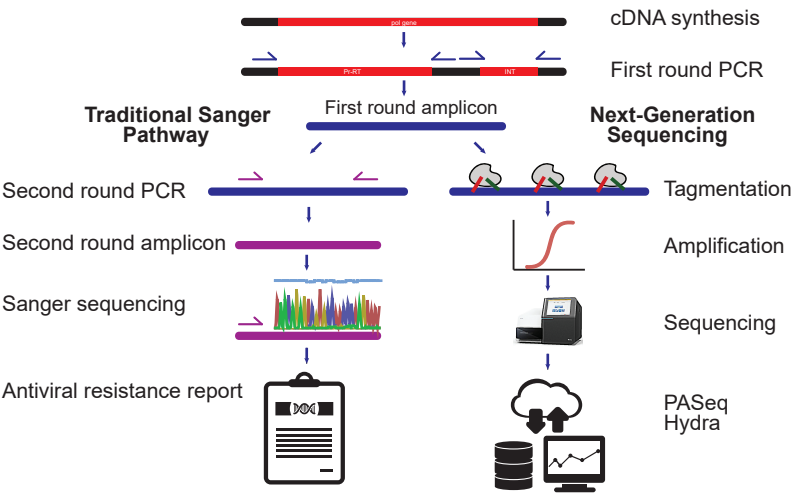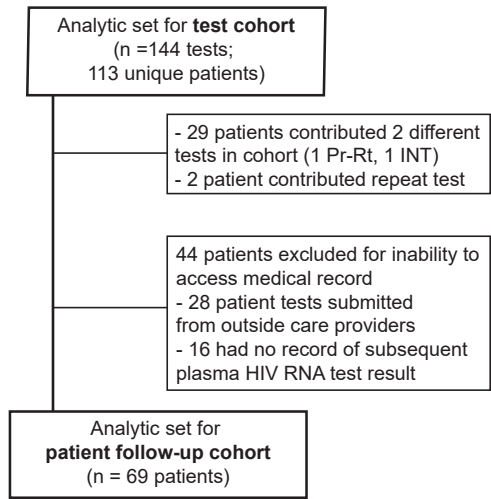
Figure 2. Histogram of DRM frequency by method of sequencing.
Number of DRMs detected at each allele frequency, by NGS only (red) by both Sanger and NGS (blue), and by Sanger only (yellow). . A-D letters highlight disagreements found between NGS and Sanger variant calls made at the 20% allele frequency threshold expected for Sanger sequencing. Each instance is detailed in the corresponding letter of Figure 3.
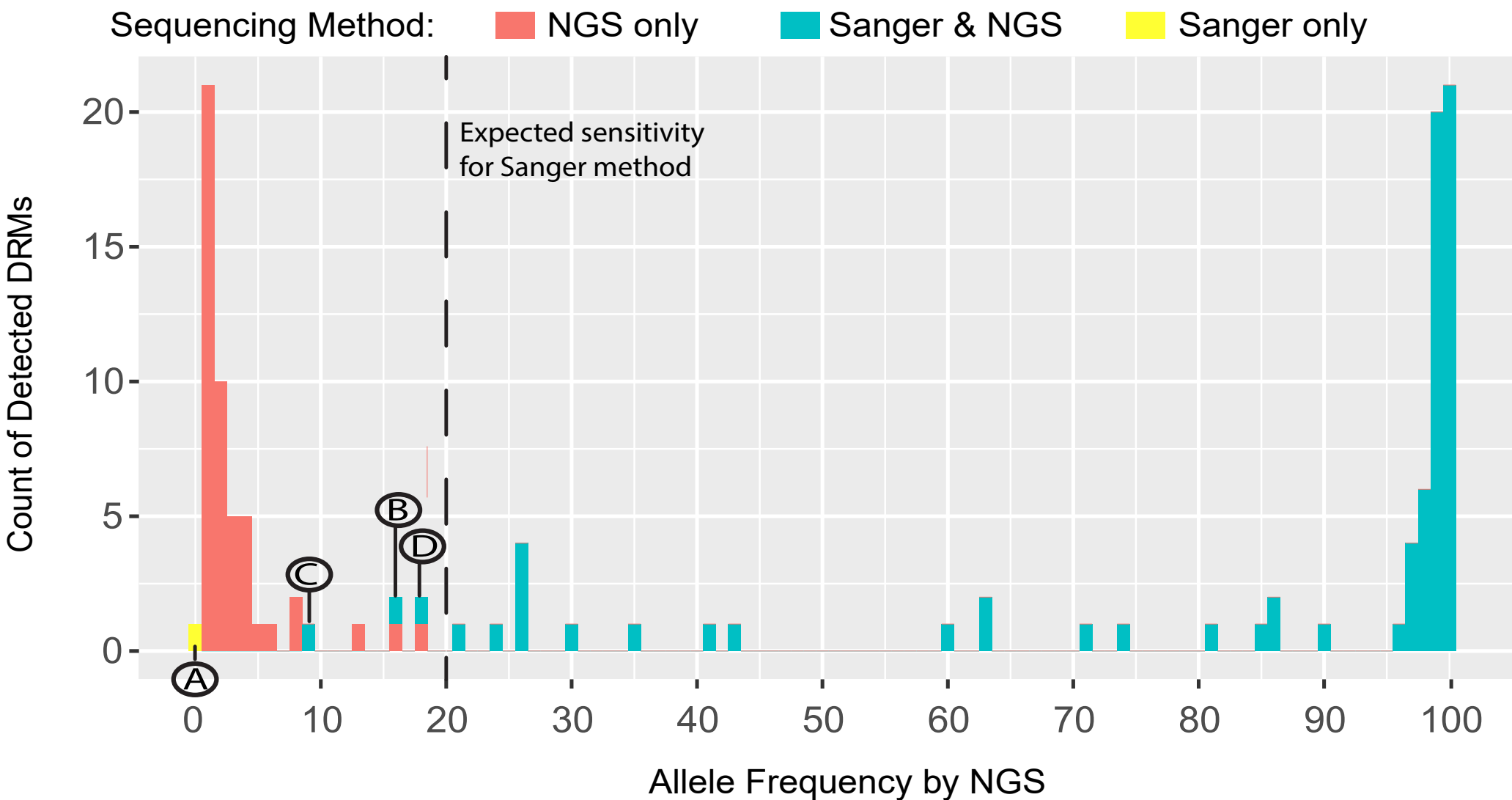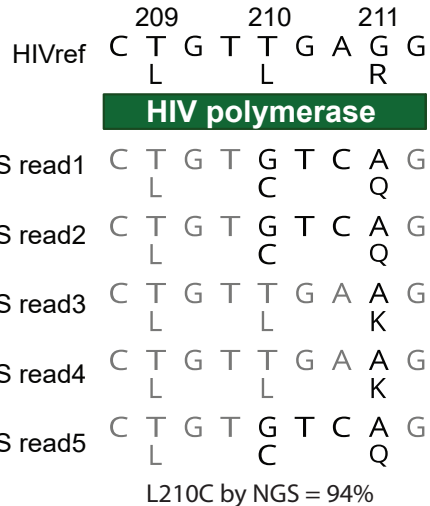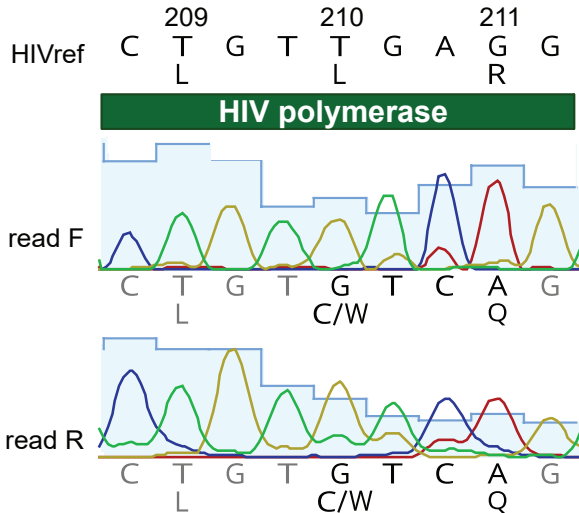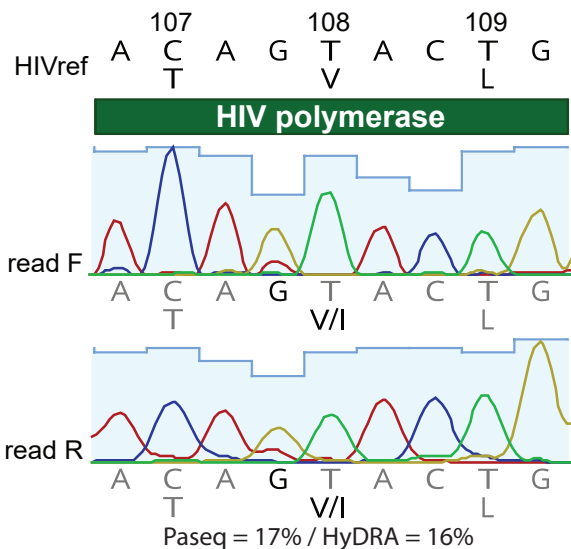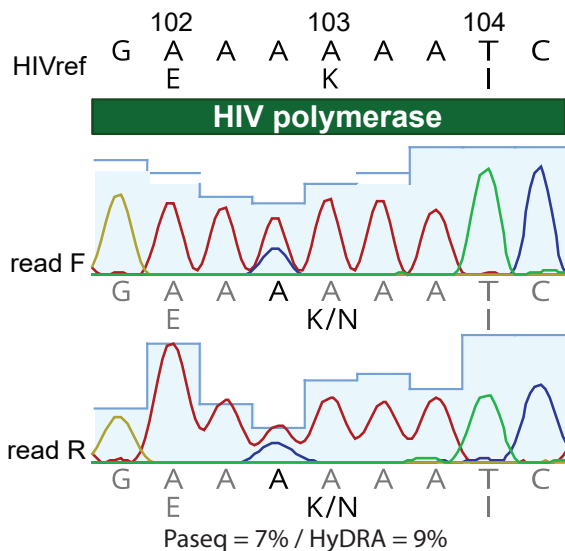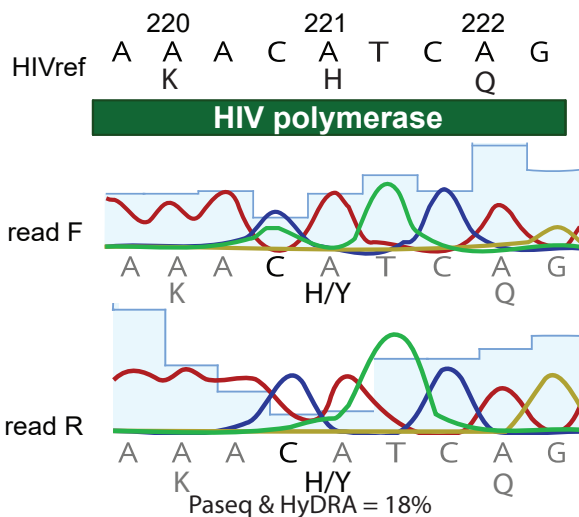
Figure 3. Discrepancies between Sanger and NGS variant calls at 20% allele frequency threshold.

A) Sanger called a mixed L210CW variant, which is associated with resistance to three NRTI drugs. However, neither NGS caller called the variant as a DRM because they recognized the linkage between two adjacent nucleotide bases, which clarified the variant as TGT (C – cysteine; polymorphism), rather than a mixture that included TGG (W – tryptophan; DRM). Three different DRMs were called by Sanger sequencing that were detected at less than 20% allele frequency by NGS, including V108I (16-17%, B), K103N (7-9%, C), and H221Y (18%, D).
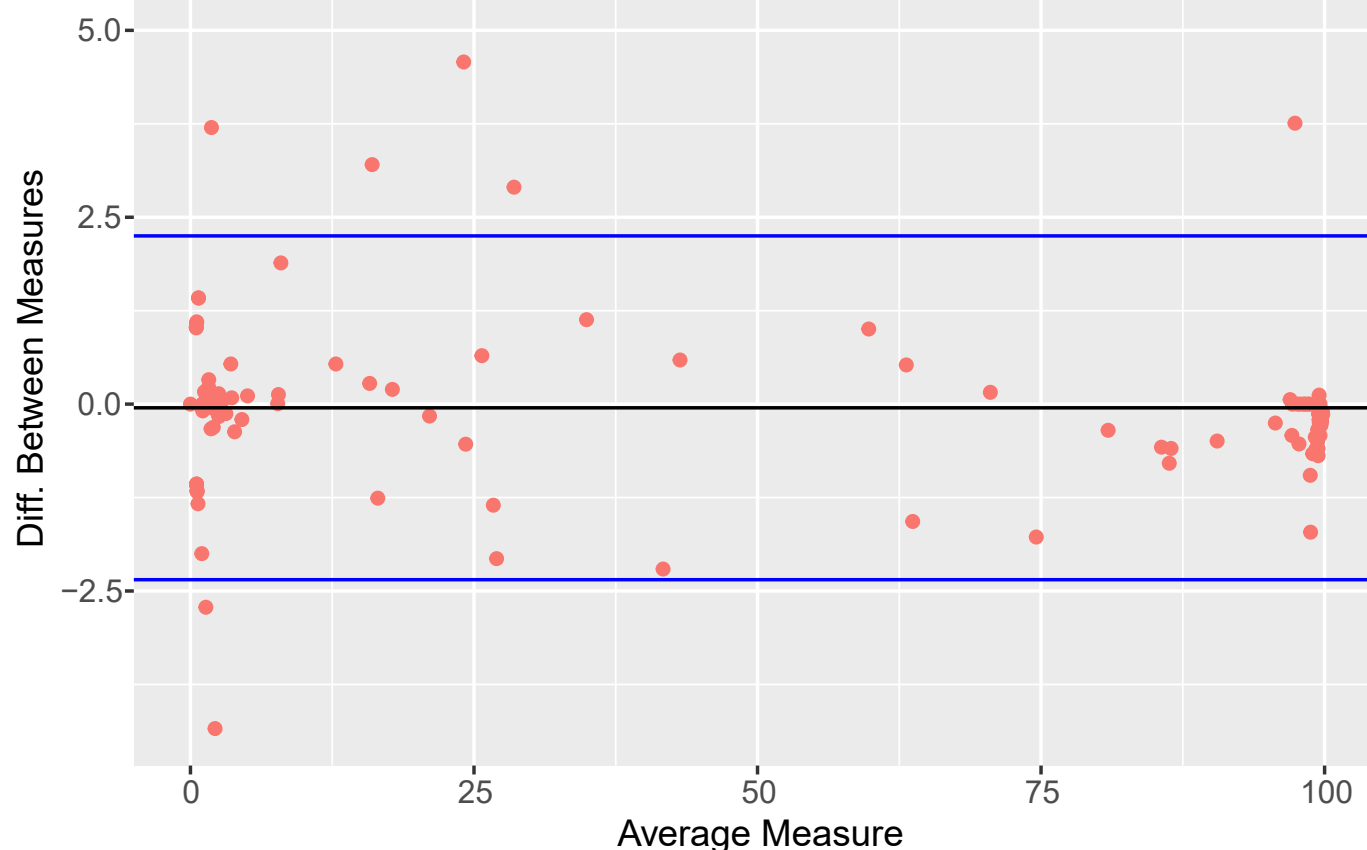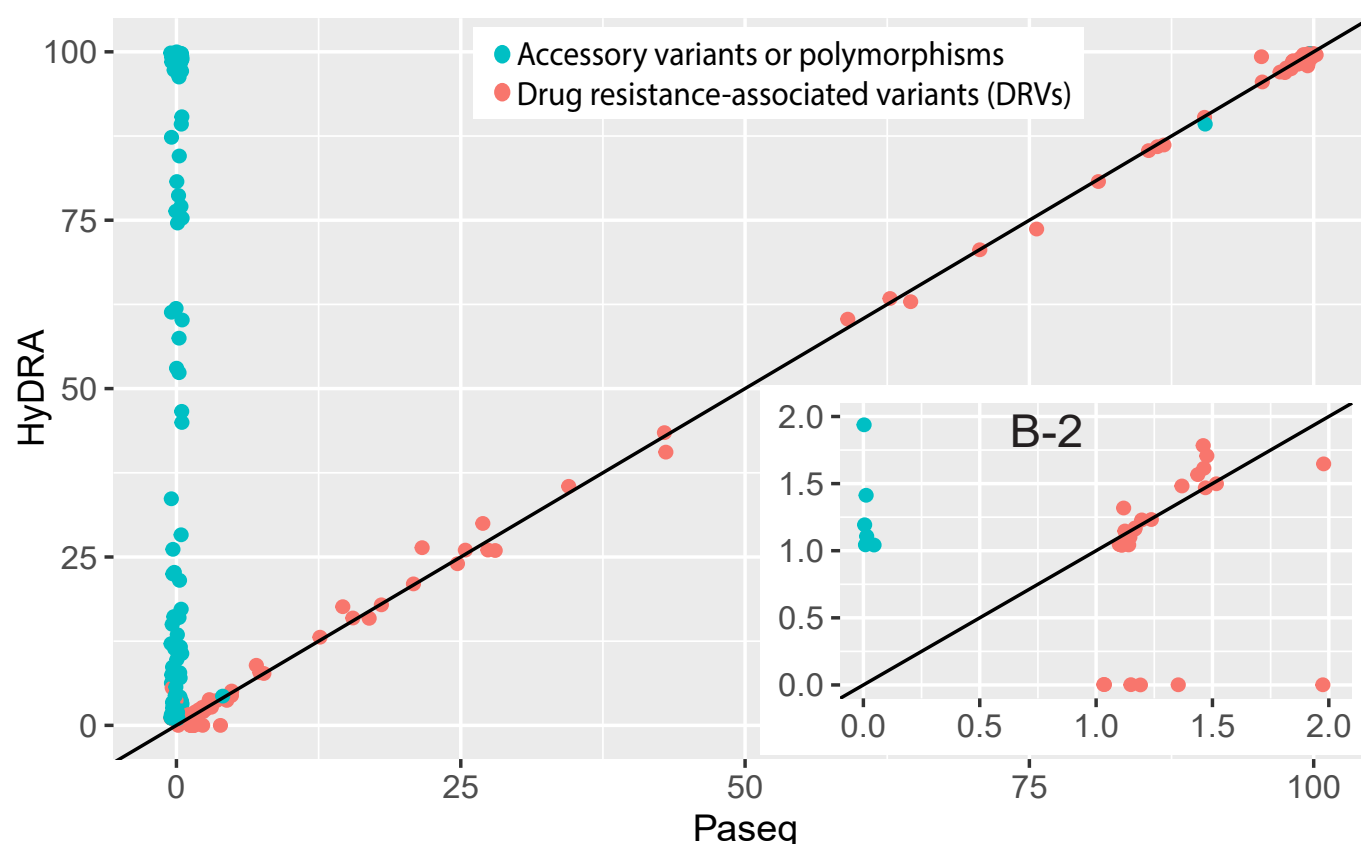
Figure 4. Concordance of allele frequency calls by HyDRA and PASeq variant callers.
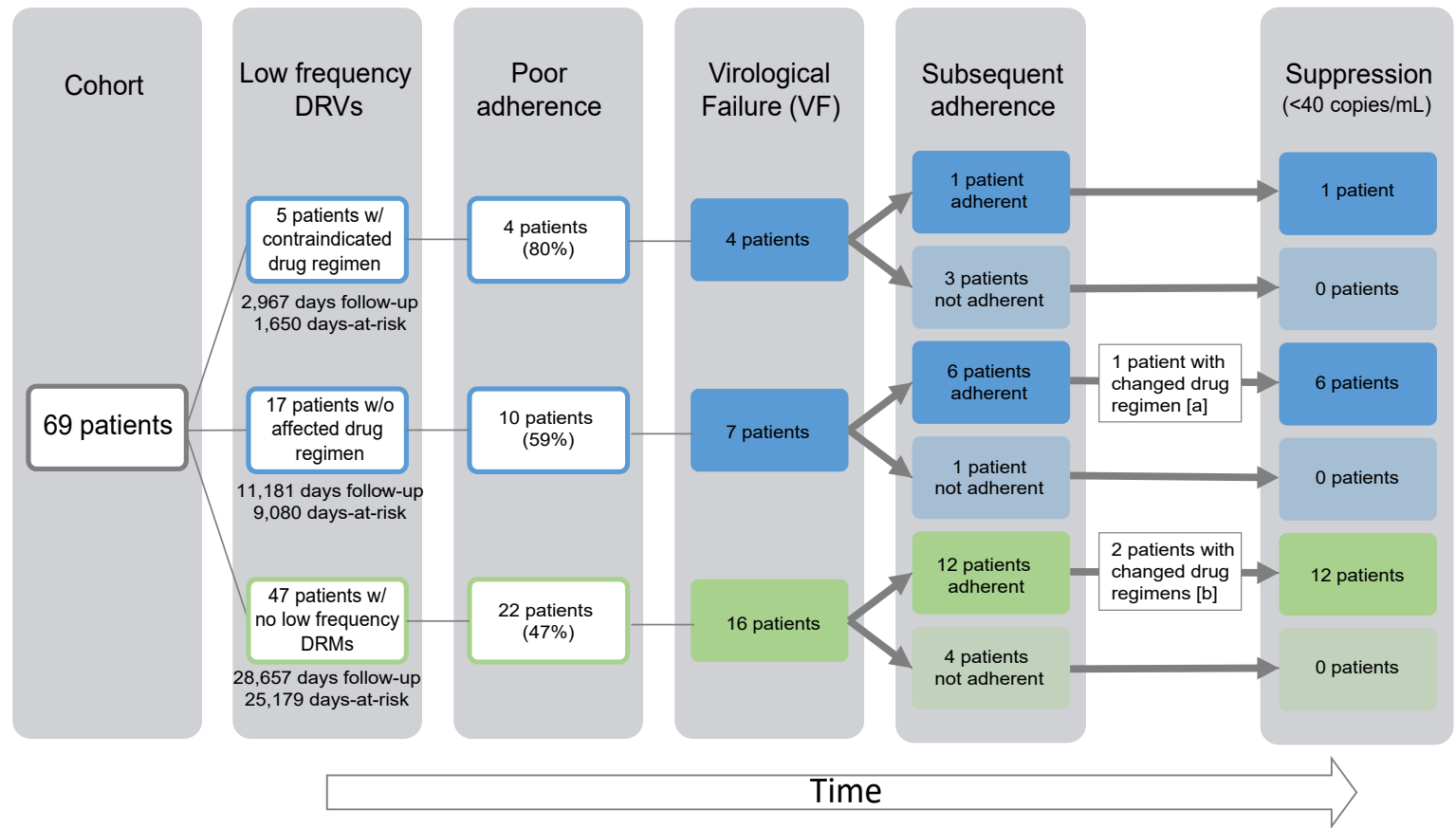A) Bland-Altman plot comparing allele frequency measurements for variants called by PASeq and HyDRA.
B-1) Correlation plot of allele frequency measurements for variants called by PASeq (x-axis) versus HyDRA (y-axis). Accessory mutations are in blue and DRMs are labeled in red. The default setting of HyDRA includes calls of many more accessory variants than PASeq, which led to the large number of accessory mutations called by HyDRA but not by PASeq. The accessory mutation S168G (blue) was called by both callers at 90% allele frequency. B-2) Zoomed in view of 1-2% frequency range (cluster of observations in bottom left corner of Figure 4B-1). *For figure illustration, all true zero values were assigned a random uniform value between 0-1% in (A) and 0-0.1% in (B).
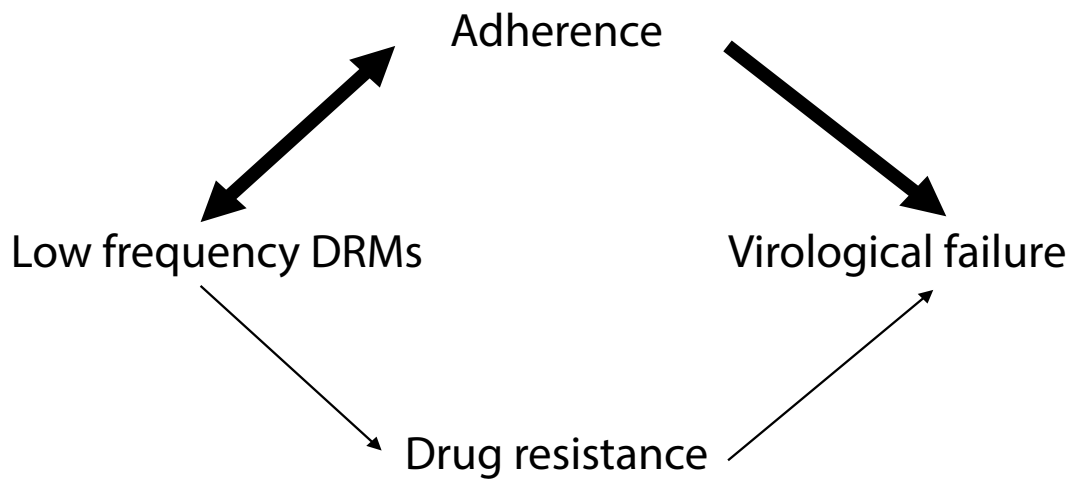
Figure 5. Results of clinical follow-up for patients and theoretical model.
A) Low frequency DRMs are correlated with ongoing medication adherence, as a result of prior drug experience and current selective drug pressure, which is thus associated with virological failure, regardless of drug resistance. B) Stratification of clinical outcomes in patient cohort, given the presence or absence of low frequency DRMs associated with reduced susceptibility to their drug regimen. Adherence was defined as documentation of poor medication adherence in clinical chart. Virological failure was defined as plasma HIV RNA level >200 copies/mL at a test date more than one month after baseline. [a] Lamivudine/zidothymidine was replaced with emtricitabine/tenofovir following poor adherence in patient's regimen, potentially contributing to subsequent suppression. [b] Darunavir was replaced with dolutegravir in both patients' regimens; potentially contributed to subsequent suppression. Days-at-risk refers to time between sample date and censor date. Patients were censored at date of measured virological failure or last available plasma HIV RNA quantification test. Days follow-up includes all days between sample date and last plasma HIV RNA quantification test date.