# Technologies for HIV-1 drug resistance testing: inventory and needs

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# Technologies for HIV-1 drug resistance testing: inventory and needs

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**Purpose of review**
HIV-1 drug resistance (HIV DR) testing is routinely performed by genotyping plasma viruses using Sanger population sequencing. Next-generation sequencing (NGS) is increasingly replacing standardized Sanger sequencing. This opens up new opportunities, but also brings challenges.

**Recent findings**
The number of NGS applications and protocols for HIV DR testing is increasing. All of them are noninferior to Sanger sequencing when comparing NGS-derived consensus sequences to Sanger sequencing-derived sequences. In addition, NGS enables high-throughput sequencing of near full-length HIV-1 genomes and detection of low-abundance drug-resistant HIV-1 variants, although their clinical implications need further investigation. Several groups have defined remaining challenges in implementing NGS protocols for HIV-1 resistance testing. Some of them are already being addressed. One of the most important needs is quality management and consequently, if possible, standardization.

**Summary**
The use of NGS technologies on HIV DR testing will allow unprecedented insights into genomic structures of virus populations that may be of immediate relevance to both clinical and research areas such as personalized antiretroviral treatment. Efforts continue to tackle the remaining challenges in NGS-based HIV DR testing.

**Keywords**
HIV-1 drug resistance testing, low-abundance drug-resistant HIV-1 variants, next-generation sequencing, standardization

## INTRODUCTION

Antiretroviral therapy (ART) has fundamentally changed the course of HIV-1 infection. Today, people living with HIV (PLWH) who have access to ART can have a life expectancy approaching that of the general population [1]. However, transmitted or acquired HIV-1 drug resistance (HIV DR) can jeopardize this success [2]. The prevalence of HIV DR is stable or even declining in resource-rich settings, but is increasing alarmingly in resource-limited settings (RLS) [3].

The International Antiviral Society, USA recommends HIV DR testing in newly diagnosed PLWH and in PLWH with virological failure [4]. HIV DR testing can be performed with phenotypic and genotypic assays. This review focuses on genotypic HIV DR testing, although phenotypic assays can provide very valuable data. However, phenotypic assays are laboratory intensive, relatively expensive, not easily standardized, and time-consuming and are therefore rarely performed today [5,6].

Genotypic HIV DR testing is routinely performed by Sanger population sequencing. Several standardized methods are available as well as tools for data analysis. However, Sanger population sequencing is increasingly being replaced by next-generation sequencing (NGS). Sanger population sequencing results in one consensus sequence of the amplified and sequenced region of the HIV-1 genome, meaning that each nucleotide represents the majority at a specific position in the virus

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## KEY POINTS

- Next-generation sequencing will become the new standard for genotypic HIV-1 drug resistance testing.
- Requirements for an optimal genotypic HIV-1 drug resistance assay are defined.
- Standardization and external quality assessment strategies/programs are urgently needed for the implementation of NGS-based genotypic HIV-1 drug resistance testing.
- Meta-analyses of NGS HIV DR and clinical data may allow us to define the clinical impact of low-abundance drug-resistant HIV-1 variants and their clinical relevant thresholds.
- Sequencing virus populations using NGS technologies will provide unprecedented insights into their structures, organization, and evolution.

population. In contrast, NGS technologies result in numerous, up to millions of sequence reads per sample, each ideally representing a single virus variant, ignoring for the moment technical artifacts such as PCR-induced recombination. Consensus sequences can be generated from NGS sequence reads that resemble the results obtained by Sanger population sequencing. NGS offers additional possibilities but is not yet a standardized method for HIV DR testing. This review provides an overview of current NGS technologies and their application to HIV DR testing, as well as challenges and opportunities of these emerging technologies.

## AN OPTIMAL GENOTYPIC HIV-1 DRUG RESISTANCE ASSAY

Figure 1 shows the requirements for an optimal genotypic HIV DR assay. It should cover all HIV-1 subtypes, successfully sequence samples with low viral load and be able to use HIV-1 RNA and/or DNA as genomic input. Different sample sources, e.g. plasma, dried blood spot (DBS), cerebral spinal fluid (CSF), should be suitable. The sequenced region should cover at least the *pol* gene or better yet the near full-length HIV-1 genome to investigate potential mutations related to drug resistance outside the target region. As an additional feature, simultaneous measurement of viral load and/or drug levels would be desirable. From a research and perhaps later clinical practice perspective, it would be desirable to detect low-abundance drug-resistant HIV-1 variants (LA-DRVs) and link drug resistance mutations (DRMs). To reduce or avoid technical artifacts, minimal or no amplification should be required.

In general, this test should be easy to use, rapid and cost-effective so that it can be used worldwide. NGS usually offers a high sample throughput and is therefore often very expensive per sample when only a few samples are sequenced. This means that HIV DR testing must be performed in central laboratories, which is often time-consuming due to the need to transport samples to the central laboratory.

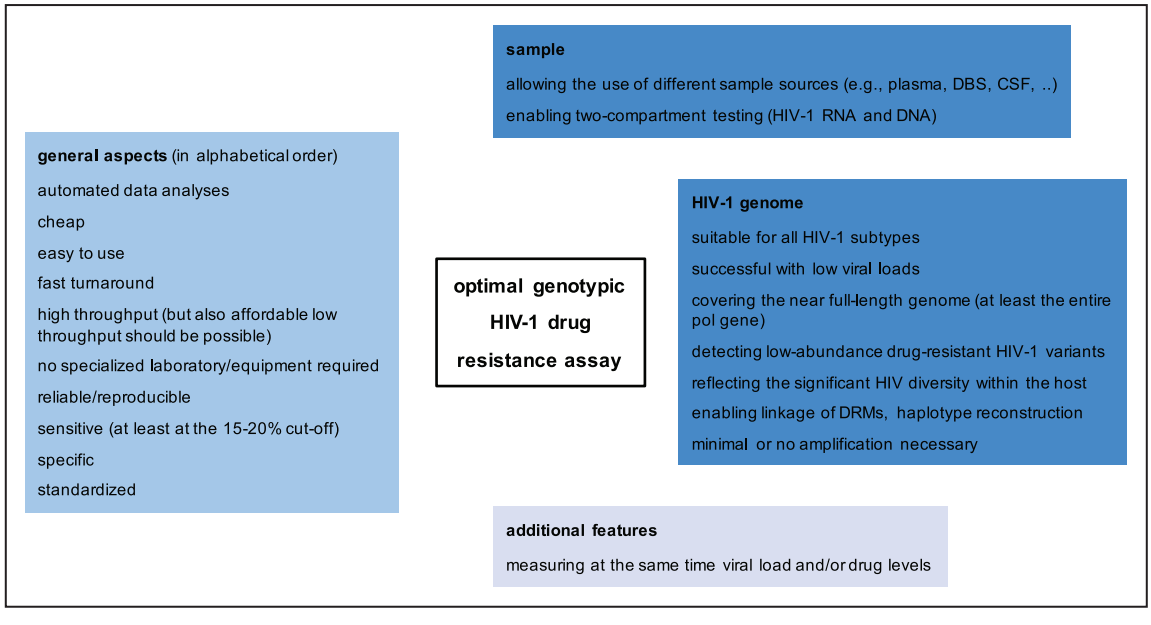


**FIGURE 1.** Requirements on an optimal genotypic HIV-1 drug resistance assay. CSF, cerebrospinal fluid; DBS, dried blood spot; DRM, drug resistant mutation.

**Table 1.** Current next-generation sequencing technologies.

| Next-generation sequencing supplier | NGS technology | Maximum read lengths (bases) | Substitution error rate (%) | Insertion/deletion errors | Sequencing run time (hours) | Sequencing run data volume (Gb) | Remarks |
|---|---|---|---|---|---|---|---|
| Illumina | Sequencing-by-synthesis | 2 × 300 | 0.1 | Very rarely (<0.1%) | 4-56 | 1.2–3,000 | Numerous devices available |
| Ion Torrent | Sequencing-by-synthesis | 600 | <0.1 | Common in homopolymeric regions | 2.5–31 | 1–24 | |
| Oxford Nanopore | Single molecule sequencing | $10^6$ | 2–13 | 7–9% in homopolymeric regions | 1 min to 72 hrs | 1–300 | Portable device available; high input RNA/DNA concentration required |
| Pacific Biosciences | Single molecule sequencing | 60,000 | <0.2–14[a] | Very common[a] | up to 30 | 75–600 | High input RNA/DNA concentration required |

[a]Single molecule circular consensus sequences reduce error rates.

An optimal genotypic HIV DR assay should not require a specialized laboratory and/or equipment and should also allow the processing of a small number of samples at affordable prices. Like all diagnostic tests, HIV DR tests must be sensitive, i.e., a DRM should be reliably detected if it is present at a frequency of ≥15–20%; and HIV DR tests should be specific, i.e., not detect DRMs that are not present. An optimal genotypic HIV DR assay should be standardized and data analysis automated. To date, such an optimal genotypic HIV DR assay is not available.

## CURRENT NEXT-GENERATION SEQUENCING TECHNOLOGIES: PROS AND CONS

Several next-generation technologies are currently available (Table 1). The sequencing-by-synthesis technology from Illumina is by far the most widely used technology. It produces short reads of a maximum 2 × 300 bp with high accuracy (error rate ~0.1%) and good coverage. However, the sequencing run time is quite long and cost efficiency can only be achieved by high-throughput analysis. The US Food and Drug Administration recently approved the first and so far only commercial NGS HIV DR assay released from Vela Diagnostics (Vela Sentosa SQ HIV-1 Genotyping). This assay is based on the sequencing-by-synthesis technology from Ion Torrent, which provides short reads of a maximum 600 bp with low substitution error rate (<0.1%) and good coverage. However, insertions/deletions in homopolymeric regions are common, and the cost per sample is high (US $400) when using the assay from Vela Diagnostics.

The single-molecule sequencing technology from Oxford Nanopore results in very long reads (1,000–100,000 bp on average), is fast (can be stopped any time when real-time analysis has reached the desired amount of reads), and has the unique feature of providing a portable device, i.e., it can be used in any location without the need for a specialized laboratory and/or equipment. However, error rates are currently high (2–13%), it is relatively expensive if only a small number of samples are sequenced, and a high concentration of RNA or DNA is required as input material (up to several hundred nanograms), so in case of HIV-1, nested PCR is usually required to obtain the desired amount of input material. The same is true for the single-molecule sequencing technology from Pacific Biosciences, which requires up to 1 microgram of DNA. This technology also results in very long reads (up to 60,000 bp), and its high error rate of ~14% can be significantly reduced to <0.2% when a single molecule is sequenced multiple times to generate a single molecule circular consensus sequence. However, the Pacific Bioscience's technology is currently laboratory intensive, time-consuming, and expensive.

## RECENT ACHIEVEMENTS AND FUTURE NEEDS FOR GENOTYPIC HIV-1 DRUG RESISTANCE TESTING

As summarized above, none of the current NGS technologies can meet all requirements for an optimal genotypic HIV DR assay. Nevertheless, recent achievements and future requirements for an optimal genotypic HIV-1 resistance test (Fig. 1) are discussed below. This review does not address recent achievements in data interpretation of NGS-based genotypic HIV DR testing, as this is covered in another chapter in this issue.

HIV DR testing begins with the collection of samples from the compartment being tested. Primarily, plasma is used because it is relatively easy to access and the virus population tested reflects the circulating virus population at that time. However,

if storage and transport of liquid plasma are required, it must be frozen and kept frozen to ensure sample integrity, and this is not readily available everywhere. As an alternative, dried plasma spots (DPS) are used or DBS, if no centrifuge is available. DPS/DBS cards can be stored safely at room temperature even for several days to weeks. DBS have been used successfully in samples from Cameroon [7] and other studies [8]. It should be noted that the sensitivity is usually lower when using DBS/DPS, mainly due to the lower blood or plasma volume stored on DBS/DPS cards. A very recent meta-analysis of HIV-1 viral load testing using DPS showed that DPS are suitable samples for the measurement of viral loads [9▪]. In certain PLWH, it may be useful to test HIV DR in CSF, as it has recently been shown that not only the composition but also the frequency of DRMs can differ in the two compartments, blood and CSF [10].

An optimal genotypic HIV DR assay should allow for a two-compartment test, i.e., sequencing both HIV-1 RNA and HIV-1 DNA. This could be particularly advantageous in PLWH with low viral loads <1,000 HIV-1 RNA copies/ml plasma, where genotypic HIV DR testing of plasma virus is not always successful, as a substantial number of treatment-experienced PLWH with low viral loads already carry DRMs [11▪]. A recent study showed that 11/16 treated PLWH with low viral loads harbored DRMs in HIV-1 DNA [12]. Moderate to high concordances were observed when comparing DRMs in HIV-1 DNA in PLWH on suppressive ART with DRMs in historical HIV-1 RNA samples using Sanger population sequencing [13–16]. This is where the use of NGS can be beneficial, as tools are available to exclude APOBEC-related hypermutated NGS sequence reads, as it has been shown that the majority of DRMs in HIV-1 DNA are found in hypermutated sequences [17▪]. Another noteworthy point is that there can be high variability within a sample in the detection of DRMs in HIV-1 DNA even at a cut-off of 20% [18]. In conclusion, HIV-DR using HIV-1 DNA may be beneficial in certain situations, but interpretation of the results remains difficult.

NGS-based genotypic HIV DR assays can be used to generate a consensus sequence of all individual NGS sequence reads and report DRMs with a cut-off of ~15–20%, such that the results are comparable to standard Sanger population sequencing and are shown to be highly concordant [19▪▪]. In addition, NGS-based genotypic HIV DR assays provide the ability to detect low-abundance LA-DRVs. In this context, the use of primer identifiers (primer ID) can be advantageous as it minimizes the impact of technical errors on the interpretation of NGS data [20–23]. However, two important limitations are the high number of sequence reads required, making this method costly, and the difficulty in applying it to near full-length HIV-1-genome sequencing.

In a recent publication by the WHO HIVResNet working group, we systematically reviewed 103 studies reporting LA-DRVs in ART-naïve PLWH. We demonstrated that the clinical impact of LA-DRVs on first-line ART remains challenging, and we defined open questions about LA-DRVs [24▪▪]. For example, we have yet to define clinically relevant thresholds of LA-DRVs that are associated with increased risk of virological failure that may be dependent on ART regimens and/or DRMs. Could any linkages of low-abundance DRMs have any effects? Further research is needed, and the increasing use of NGS-based genotypic HIV DRV testing will be of great benefit in this regard.

The assessment of HIV DR is not the only critical parameter in the care of PLWH treated with ART. Therefore, it would be of great advantage if a genotypic HIV DR assay could simultaneously measure the viral load. The virus-enrichment method to generate whole-genome HIV sequences (veSEQ-HIV) combines genotypic HIV DR testing with quantification of HIV-1 viral load, while being a low-cost, high-throughput assay [25▪,26]. In addition, monitoring antiretroviral drug levels in PLWH who are failing ART can support clinical decision-making [27]. A recent study has shown that lopinavir levels can be measured in plasma and DBS that correlate with the presence of lopinavir resistance [28].

Most NGS technologies require specialized laboratories and equipment and are therefore not suitable for the increasingly desired point-of-care diagnostics. Oxford Nanopore is currently the only provider offering a portable device, MinION, for NGS. Last year, an initial proof-of-concept study demonstrated the utility of MinION sequencing for HIV DR detection [29▪]. NGS or Sanger sequencing is often not feasible and/or affordable in RLS. Therefore, point mutations assays (PMAs) have recently been evaluated and may be of clinical value in the prevention of virological failure [30,31]. However, PMAs, although less costly, can only cover a few DRMs and face the problem of HIV-1 diversity using DRM-specific primers.

## QUALITY ASSESSMENT AND STANDARDIZATION OF NEXT-GENERATION SEQUENCING-BASED GENOTYPIC HIV-1 DRUG RESISTANCE TESTING

There are numerous technical and analytical challenges to the use of NGS-based HIV DR testing that can arise in literally any of the individual steps (Fig. 2) [19▪▪,32,33▪▪]. Errors in sample collection,

 

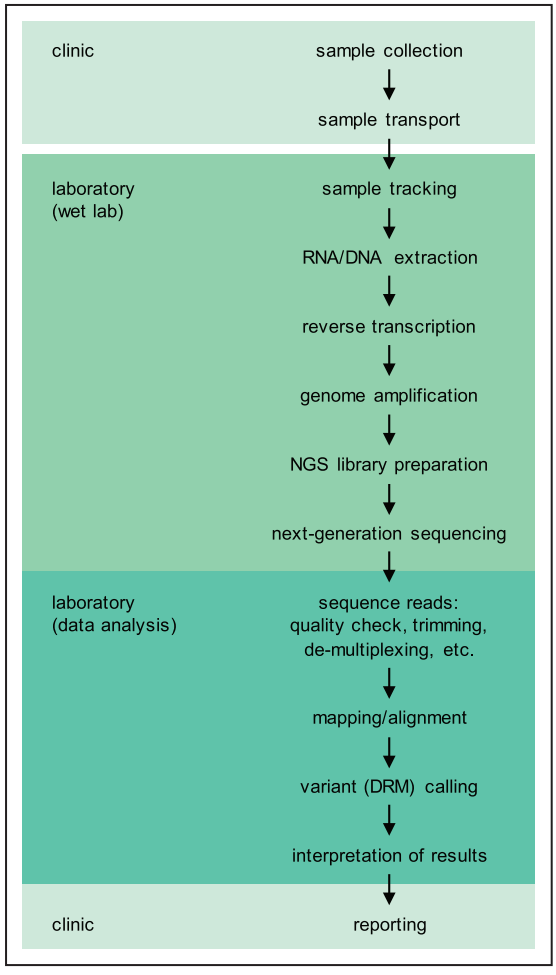


**FIGURE 2.** Flow chart of NGS-based genotypic HIV-1 drug resistance testing, from sample collection to reporting. DRM, drug resistance mutation; NGS, next-generation sequencing.

viral genome extraction and amplification(s), NGS data analysis, and interpretation can result in artificially reduced or increased diversity of virus populations, possibly missing or falsely detecting DRMs. Therefore, standardization and external quality assessment strategies for the implementation of NGS-based HIV DR testing in routine practice are urgently needed, as recently summarized at the second 'Winnipeg Consensus' symposium on NGS-based HIV DR testing [34▪▪].

Quality management starts with intra-laboratory quality control. This is where laboratory information management systems are useful, i.e., software packages for effective monitoring and control of each step in the NGS-based HIV DR assay [35▪].

External quality assessment through inter-laboratory comparisons based on distributed virus samples, so-called wet panels, are very useful to validate NGS applications for genotypic HIV DR testing. However, they require a large amount of individual virus samples and are time and personnel consuming. Therefore, very few laboratory comparisons of NGS-based HIV DR assays have been carried out to date. Two inter-laboratory comparisons of a prototype HIV DR assay based on 454 pyrosequencing, which is no longer available, were performed [36,37]. Two recent inter-laboratory comparisons, in which each laboratory applied its own NGS protocol and analysis tool, were performed based on sequencing-by-synthesis technology from Illumina or Ion Torrent [38,39]. In terms of sensitivity and specificity, good to excellent agreement was observed, with the expected lower agreement at low frequencies of DRMs and/or low viral load [36–39].

At the first 'Winnipeg Consensus' symposium, held in 2018 in Winnipeg, Canada, and attended by leading experts in bioinformatic strategies for processing NGS HIV DR data, the authors defined key functional topics in NGS HIV DR data analysis: ' (1) NGS read quality control and quality assurance, (2) NGS read alignment and reference mapping, (3) HIV variant calling and variant quality control, (4) HIV DR interpretation and reporting; and (5) analysis data management' [40]. Indeed, the biggest challenges in NGS today are no longer in the sequencing procedures in the laboratory, but in the data analysis and their interpretations. Therefore, external quality assessment through inter-laboratory comparisons can also be performed by distributing well-characterized NGS datasets, so called dry panels. Noguera-Julian *et al.* have recently proposed a comprehensive list of the criteria for such panels [41▪▪].

The external quality assessment of NGS-based HIV DR testing is very demanding and not all requirements have been conclusively defined yet [19▪▪,33▪▪,34▪▪,40,41▪▪,42▪▪]. As a transition strategy for the external quality assessment of NGS-based HIV DR assays, it may be useful to use external quality assessment strategies originally implemented for the external quality assessment of HIV DR assays based on Sanger population sequencing [43].

## CONCLUSION

NGS-based HIV DR testing is becoming the new standard not only in research but also in clinical monitoring. An optimal genotypic HIV DR assay should meet a long list of requirements. Such an assay is currently not available and its development is very difficult. Nevertheless, many efforts are currently underway to address the remaining challenges and key needs in NGS-based HIV DR testing, with a particular focus on implementing external quality assessment programs.

NGS-based HIV DR testing enables the detection and quantification of low-abundance LA-DRVs. They have long been studied, but their clinical relevance has yet to be determined. NGS data combined with clinical data from PLWH will hopefully allow us in the near future to perform useful meta-analyses to resolve this important question and define clinically relevant thresholds, which will benefit personalized antiretroviral treatment of PLWH. Furthermore, NGS data from virus populations will be a treasure to study not only low-abundance drug-resistant HIV-1 variants, but also virus evolution and the dynamics of the pandemic, to name a few examples.

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## Conflicts of interest

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