HHS Public Access

Author manuscript

Curr Opin HIV AIDS. Author manuscript; available in PMC 2019 September 11.

Published in final edited form as:

Curr Opin HIV AIDS. 2017 March ; 12(2): 182–189. doi:10.1097/COH.0000000000000344.

Future technologies for monitoring HIV drug resistance and cure

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Abstract

Purpose of review—Sensitive, scalable and affordable assays are critically needed for monitoring the success of interventions for preventing, treating and attempting to cure HIV infection. This review evaluates current and emerging technologies that are applicable for both surveillance of HIV drug resistance (HIVDR) and characterization of HIV reservoirs that persist despite antiretroviral therapy and are obstacles to curing HIV infection.

Recent findings—Next-generation sequencing (NGS) has the potential to be adapted into high-throughput, cost-efficient approaches for HIVDR surveillance and monitoring during continued scale-up of antiretroviral therapy and rollout of preexposure prophylaxis. Similarly, improvements in PCR and NGS are resulting in higher throughput single genome sequencing to detect intact proviruses and to characterize HIV integration sites and clonal expansions of infected cells.

Summary—Current population genotyping methods for resistance monitoring are high cost and low throughput. NGS, combined with simpler sample collection and storage matrices (e.g. dried blood spots), has considerable potential to broaden global surveillance and patient monitoring for HIVDR. Recent adaptions of NGS to identify integration sites of HIV in the human genome and to characterize the integrated HIV proviruses are likely to facilitate investigations of the impact of experimental ‘curative’ interventions on HIV reservoirs.

Keywords

HIV cure; HIV drug resistance; HIV integration; HIV reservoirs; next-generation sequencing

INTRODUCTION

Sensitive, scalable and affordable assays are urgently needed for monitoring the success of interventions for preventing, treating and attempting to cure HIV infection. Eighteen million individuals are currently on antiretroviral therapy (ART), and recent UNAIDS targets aim to increase that number to 90% of all infected individuals [1]. Concurrently, preexposure

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Conflicts of interest

J.W.M. is a consultant to Gilead Sciences and holds share options in Co-Crystal Pharma, Inc. No other conflicts are reported. U.M.P., K.M. and G.V. report no potential conflicts.

prophylaxis (PrEP) roll out with oral tenofovir/emtricitabine, which is also a key component of first-line ART, is planned for thousands of at-risk individuals throughout sub-Saharan Africa, Europe and the United States. The spread of drugesistant HIV remains the greatest threat to undermining the public health benefit of 'Test and Treat' [2] and PrEP rollout [3], yet resistance monitoring is not currently widely available because of high cost and low throughput. This situation is unlikely to change without technological advances that have major effects on cost and capacity.

Although ART and PrEP have the potential to lower HIV incidence, both approaches require drug adherence and continual drug supply, which are resource-intensive. The report of the 'Berlin Patient' in 2009, who was cured of HIV infection [4], galvanized worldwide efforts to achieve an affordable and scalable cure of HIV that would reduce HIV transmission without the need for lifelong ART. A major obstacle to progress toward an HIV cure has been difficulty in quantifying and characterizing the HIV reservoir that leads to viral relapse after ART is stopped. Next-generation sequencing (NGS) is now being adapted to help identify intact (replication competent) HIV proviruses and their integration sites in the human genome. The latter application led to the recognition that clonal expansions of HIV infected cells are common. Further refinements of NGS assays should facilitate the assessment of efficacy of experimental interventions aimed at reducing HIV reservoirs and controlling HIV.

The current review discusses limitations of current assays for drug resistance surveillance and HIV cure research, recent advances in application of NGS as potential solutions and important improvements that are needed to realize the full potential of NGS assays.

LIMITATIONS OF CURRENT HIV DRUG RESISTANCE SURVEILLANCE TECHNOLOGIES

Current assays to identify HIV-1 drug resistance mutations have relied on population sequencing of the HIV-1 protease (*pro)* and reverse transcriptase genes or on identifying specific point mutations in HIV-1 reverse transcriptase associated with resistance (Table 1).

Standard genotyping

Population genotyping remains the current clinical standard for assessment of HIV drug resistance (HIVDR) mutations in individuals who seroconvert while using PrEP, for individuals starting ART (i.e. pretreatment or transmitted resistance) and for individuals on failing ART regimens. The Abbott Molecular ViroSeq HIV-1 is the only commercially available genotyping system for HIVDR assessment since Siemens discontinued *TruGene* HIV-1 in 2014 [6■■]. Standard genotyping assays have a high cost per sample (>150 USD), require high minimum viral loads (2000 copies/ml), have limited gene coverage (up to codon 335 in HIV-1 reverse transcriptase), are variably successful in genotyping nonsubtype B HIV-1, and only detect resistant variants that comprise more than 20% of the virus population in a sample [5,19–22]. Several in-house assays have reported improved performance for sequencing nonsubtype B HIV-1 and have significantly reduced the cost

over commercial assays (to approximately 50–150 USD), but these methods remain labor-intensive with a high burden of manual data analysis and lack scalability [5,8–10].

Point mutation assays

Several real-time PCR-based point mutation assays (PMA) including allele-specific PCR [13], oligo-ligation assay [23], one-step ligation on RNA amplification [17] and pan-degenerate amplification and adaptation [24] have improved sensitivity (0.01–5%), lower cost per sample (<5 USD) and higher throughput capacity relative to standard genotyping (Table 1). However, large-scale implementation of PMAs has stalled due to issues with primer binding site polymorphisms and mutant codon variants, such as E138E/A/G/K that compromise assay specificity and increase assay complexity and cost [25,26]. Although recent advances in PMAs can accommodate the simultaneous detection of multiple mutations, analysis of mutation combinations, such as thymidine analog mutations for zidovudine resistance, remains challenging [27].

NEXT-GENERATION SEQUENCING FOR PREEXPOSURE PROPHYLAXIS AND ANTIRETROVIRAL THERAPY RESISTANCE MONITORING

NGS has the potential to be adapted into a high-throughput, low-cost HIVDR assay with low frequency mutation detection at 1–5% [5,28■■,29]. For NGS to be implementation-ready for HIVDR surveillance, improvements in nucleic acid preservation and simplification of assay procedures and data analysis are needed.

Current next-generation sequencing assays for sensitive detection of low-frequency resistance

NGS has the capacity to simultaneously obtain reads from millions of copies of HIV genomes per run enabling the potential detection of low-frequency viral quasispecies. The addition of patient identifiers (index sequences) enables multiplexing of samples to increase throughput and reduce cost per genotype relative to standard sequencing [30,31].

Earlier studies using the 454 platform could detect mutants at 1% frequency, but accuracy was compromised by PCRbias and sequencing errors. The newer Illumina platform has increased fidelity and reliability with shorter, more processive reads during NGS and can generate a greater number of reads per run [32]. Two recent advances have increased the sensitivity and accuracy of NGS by correcting for PCR resampling, recombination during PCR and sequencing errors. The addition of unique PrimerIDs composed of degenerate bases during cDNA synthesis can correct for preferential PCR amplification by tagging all the sequences derived from a single RNA template. Using bioinformatics, sequences with identical PrimerIDs are collapsed into one consensus, and sequences with gaps, errors or ambiguous bases indicative of PCR error, recombination or sequencing error are removed [11■,33,34]. Recent improvements of sample processing were made by adding the sequencing adaptors with an oligo-ligation step rather than through PCR amplification, which reduces the potential for recombination and lowers the mutant detection frequency to less than 0.1% [35].

The future of next-generation sequencing for resistance monitoring

Although there have been several technical innovations to improve NGS accuracy and precision, further modifications are still needed to simplify sample processing, NGS library preparation and bioinformatics analysis of sequences. Dried blood spot (DBS) technology is currently the WHO-recommended sample collection method in low–middle-income countries (LMIC) for plasma HIV-1 RNA and genotyping assays and has been shown to preserve specimens at ambient temperatures in sufficient quantities for population-based NGS on the 454 plat-form [12,36,37,38■]. There is potential, however, for improvements in DBS; for example, impregnating the filter paper with antioxidants and inhibitors of RNases to preserve HIV RNA templates [39]. Though novel blood storage devices such as HemaSpot (Spot On Sciences, Inc.; Austin, Texas, USA) [40] and Primestore Molecular Transport Media (Longhorn Vaccines and Diagnostics, LLC, Bethesda, Maryland, USA) [41] have improved recovery of HIV nucleic acids over DBS, their cost is too high for widespread use in LMIC. Additional advances in sample throughput could be accomplished by automated sample extraction (e.g. Abbott *m*2000sp, Abbott Molecular, Des Plaines, Illinois, USA) or by replacing laborious sample preparation steps with liquid-handling equipment. Adaptor ligation steps, necessary for sensitive and accurate allele detection by NGS [34,35], could be simplified with commercial adaptor ligation kits and automated liquid handling. Finally, PrimerID bioinformatics scripts that are required for data analysis could be integrated into an automated internet-accessible pipeline processing application. Overall, the future is promising for higher throughput, lower cost and automated NGS plat-forms that will greatly increase accessibility of resistance monitoring for epidemiologic surveillance and patient management.

LIMITATIONS OF CURRENT ASSAYS FOR HIV CURE

The HIV reservoir consists of HIV-infected cells carrying intact (replication-competent) proviruses, which are the source of rebounding virus after ART interruption. HIV reservoir assays attempt to either directly quantify intact proviruses or indirectly measure a biomarker that is strongly correlated with the viral reservoirs. The first major challenge of detecting reservoir cells is that they are very rare in peripheral blood or tissues in patients who initiated therapy early [42–44] or who have been on long-term suppressive therapy.

Although total HIV-1 DNA quantity correlates well with the number of infected cells [45], more than 90% of HIV-1 DNA is defective as a result of deletions, insertions, point mutations or apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)-mediated hypermutation [46■■]. Such defective proviruses accumulate rapidly after acute infection [46■■]. As a consequence, assays of total HIV-1 DNA grossly overestimate the size of the HIV reservoir (Fig. 1). By contrast, the gold standard cell culture-based quantitative viral outgrowth assay (QVOA), which is most specific for the HIV reservoir, underestimates the reservoir relative to intact proviral sequences by as much as 60-fold because not all intact proviruses can be activated with a single round of cell activation [47]. Additional rounds of stimulation increases the yield but still only activate a small proportion of competent proviruses [48■]. Use of QVOA for assessing HIV reservoirs is also limited by large blood volume requirements, high-cost and low throughput. Simplified,

culture–based and inducible virus recovery assays are more practicable and sensitive than QVOA but do not detect replication-competent virus [49,50■]. Similarly, current assays to detect intact provirus that rely on limiting dilution PCR and sequencing have low throughput and limited sensitivity (Table 2).

RECENT DEVELOPMENTS THAT HAVE IMPROVED UPON HIV PERSISTENCE ASSAYS

Improvement in highly sensitive plasma HIV-1 RNA assays requires sufficiently large volume plasma processing, viral concentration and efficient exclusion of PCR inhibitors and detection of inhibition. This has enabled the detection of low-level viral persistence at levels of less than 1 copy per milliliter of plasma [51]. Similarly, for HIV-1 DNA detection, greater sampling through large blood volume draws or leukapheresis improves the chance of detecting rare HIV-infected cells, whereas the background human DNA signal can be reduced by purification of $CD4^+$ cells or resting $CD4^+$ memory cells [52■■].

Analytical improvements in HIV DNA/RNA detection

High HIV diversity contributes to reduced sensitivity by delaying the threshold cycle when primers or probes mismatch a viral template [53■■]. To address this, the choice of conserved genome targets in *integrase* [51], *gag* or the long terminal repeat (LTR) region have improved assay performance and the inclusion of more than one genome target in a multiplex assay has reduced the risk of mismatches to all targets [54,55]. Digitalization of PCR reactions into individual nanoliter or picoliter reactions, followed by detection of the number of positive reactions, has been reported to be more robust to primer mismatches [56] but has limited throughput and is prone to background signal that could be reduced by touchdown PCR [57].

An innovative approach combining the principles of quantitative PCR with digitalization is the use of real-time PCR with multiple replicates at the highest dilution. This is less prone to non-specific background and does not rely on the cycle threshold for the quantification of the highest dilutions [54].

Importance of postanalytical standardization of reporting

When reporting cure assay results, it is important to report the denominator of cells actually assayed [52■■]. This could be achieved by parallel quantification of a human reference gene, which controls for all assay steps.

NEW ASSAYS THAT WILL IMPROVE OUR UNDERSTANDING OF VIRAL PERSISTENCE AND IMPROVE MONITORING OF CURATIVE INTERVENTIONS

Advances in NGS, single cell and fractional expression assays, and assays of integrated provirus are providing new tools to characterize HIV reservoirs.

Next-generation single genome sequencing

Improvements in PCR and NGS will result in higher throughput assays for intact provirus. DNA polymerases with improved processivity and proofreading (3′–5′ exonuclease activity) allows amplification of near–full-length amplicons, but when relying on multiple Sanger sequencing reactions, have limited throughput. Recent NGS platforms (e.g. Pacific Bio-sciences, Menlo Park, California, USA) have improved template read length that allows the sequencing of whole HIV genomes [58■■]. This high-throughput approach has the potential to characterize individual full-length viral genomes and determine which are intact. Viral templates must be diluted to one template per PCR reaction to avoid artifacts from recombination between multiple viral genomes, but this endpoint dilution limits throughput.

Single cell and fractional expression assays

Recently, a limiting dilution assay has been developed to quantify cellular RNA levels expressed by individual cells, showing that the reduction of cellular HIV RNA during successful ART is not due to a smaller proportion of infected cells expressing HIV RNA but due to a smaller fraction of cells expressing high levels of RNA [59■■]. New developments in single cell assays will soon allow the simultaneous investigation of different characteristics of a single cell: the cellular phenotype, HIV-1 DNA and mRNA expression and virion production. Sequencing at a single cellular level could also investigate whether individual genomes are intact or defective [60■].

Assays of integrated provirus

Two assays to detect HIV integration sites have been developed. The one HIV integration site loop amplification assay makes use of primers that have a random 3′ decamer tail and an LTR U5-specific 5′ region, which through several steps generates a stem-loop structure with a known HIV-1 LTR sequence in the stem region and unknown human genome sequence in the loop region. Limiting dilution PCR and sequencing of the individual integration sites allow the design of integration-site specific primers, which together with envelope specific primers allow amplification and sequencing of integration sites and HIV 3′ LTR to envelope. Although elegant, this approach is very labor-intensive and requires multiple PCR and Sanger sequencing reactions [61]. The other integration site assay (ISA) approach involves random ultrasonic shearing of HIV-1 DNA, blunt-end ligation of PCR linkers and amplification of the integration sites with and HIV-1 LTR-specific and linker-specific primer followed by a heminested PCR with another internal HIV-1-specific LTR-specific and linker-specific primer that enriches for HIV integration sites. Integration sites are then characterized by high-throughput Illumina sequencing. This provides an efficient approach but the very short HIV-1 sequence does not allow the linkage of the human genome integration site with specific proviral species [62]. Current proviral ISAs are too insensitive to examine the effect of curative interventions on individual clones and therefore need further development. Future assays should also link full HIV genomes to their integration sites. This will likely be facilitated by long fragment PCR and newer NGS platforms that allow single read sequencing of whole HIV genomes [58■■] to investigate the survival and expansion of cells with specific integrated proviruses (Table 2). This capability will accelerate the understanding of whether experimental interventions affect

most cells containing intact proviruses or only a subset as a consequence of variation in host cell and proviral biology.

CONCLUSION

Population-based Sanger sequencing of HIV provided essential, initial insights into HIVDR and has been used for patient monitoring in well resourced settings, but recent advances in sample collection, automated sample processing and sequencing technologies are poised to greatly expand availability of resistance monitoring. Because low-frequency mutations have recently been shown to affect treatment outcome, NGS plat-forms offer the additional advantage of greater sensitivity than population sequencing for detection of minor viral variants.

Higher-throughput quantitative assays of proviral competence are a high priority to assess the effects of interventions on the HIV reservoir size. Recent developments in full-length sequencing show promise and could identify intact proviruses, but have limited throughput. Current assays for HIV integration sites have increased our understanding of clonal expansion as a key mechanism of HIV persistence but further assay refinements are needed to assess the impact of curative interventions on individual clonal populations of infected cells. Such assay refinements and other advances will undoubtedly occur and provide much greater insight into HIV reservoirs and the impact of interventions designed to achieve an HIV cure.

Acknowledgements

We acknowledge Lauren Berner and Lorraine Pollini for assistance in preparing this article and Kerri Penrose for helpful discussions.

Financial support and sponsorship

This article is made possible by generous support of the American people through the United States Agency for International Development (USAID) and the U.S. President's Emergency Plan for AIDS Relief (PEPFAR). The contents are the responsibility of the University of Pittsburgh and do not necessarily reflect the views of USAID, PEPFAR or the United States Government. Cooperative agreement AID-OAA-A-15–00031. Support from the AIDS Clinical Trials Group Network (ACTG) to the University of Pittsburgh Virology Specialty Laboratory funded by National Institutes of Health (NIH)/National Institute of Allergy and Infectious Diseases (NIAID) (UM1 AI106701). Support from the National Cancer Institute, National Institutes of Health, under Contract number HHSN261200800001E. The content of this publication does not necessarily reflect the views of policies of the Department of Health and Human Services, nor does mention of trade name, commercial products or organizations imply endorsement by the U.S. Government.

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KEY POINTS

- The shortcomings of current methods for HIV drug resistance testing limit global access to resistance monitoring.
- Next-generation sequencing technology has the potential to be adapted into a high-throughput, low-cost assay that will expand resistance testing to that needed for ‘Test and Treat’ and PrEP rollout programs.
- Advances in DNA polymerase enzymes and next-generation sequencing technologies are providing new tools to characterize HIV reservoirs.
- Improvements in the interpretation and throughput of sequencing assays for intact proviruses and clonal expansions of infected cells are needed before they can be applied to assess the impact of experimental interventions on HIV reservoirs.

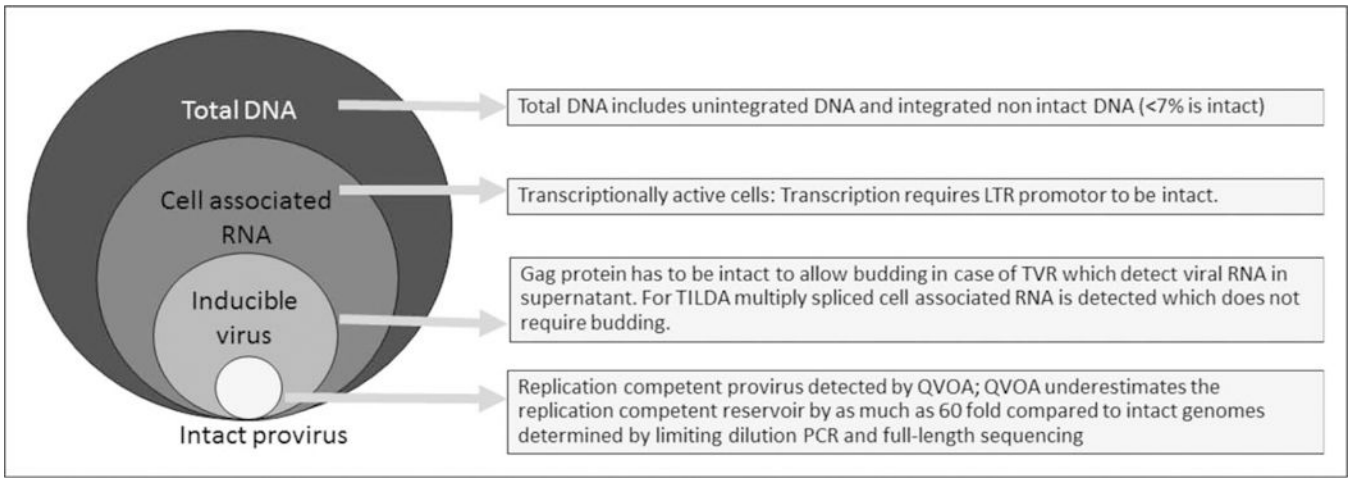

FIGURE 1.

What do reservoir assays measure? QVOA, quantitative viral outgrowth assay; TILDA, tat/rev induced limiting dilution assay; TVR, total virus recovery assay [49].

Table 1.

Technologic approaches for HIV drug resistance testing

Category	Type	Assay	Application	Unique features	Status	References
Population-based	Sanger 'Standard' sequencing	Viroseq	Centralized	Genotyping HIV-1 protease gene from codons 1–99 and *RT* gene from codons 1–335	Commercially available	[5,6■■]
				Cost 120 USD per sample, VL threshold =2000cpm, Sensitivity >20%		
	Sanger 'Standard' sequencing	TruGene	Centralized	Genotyping HIV-1 protease gene from codons 4–99 and RT codons 38–248	Discontinued	[7]
				Cost 150 USD per sample, VL threshold = 200cpm		
	Sanger 'Standard' sequencing	In-house	Centralized	Genotyping for non-B subtypes and flexible amplification of resistance codons	In development	[5,8–10]
				Cost 50–150 USD per sample		
Sensitive	NGS	Illumina	Centralized	Detection of minor variants using unique tagging of individual virion genomes	In development	[11■]
				High number of reads per run, but the read lengths are shorter than 454		
	NGS	454 Pyrosequencing	Centralized	Longer read lengths, but limited to 1 million reads, high error rates with polybases >6	In development	[12]
	Point mutation assay PCR primer amplification	ASPCR	Point-of-care	Selective amplification of PCR product by match or mismatch of 3' end of primer	In development	[13]
				Cost <5 USD per sample, low VL threshold, problems with specificity (polymorphisms)		
	Point mutation assay PCR primer ligation	OLA	Point-of-care	Selective ligation of tagged-oligonucleotides on HIV PCR product by match or mismatch of 3' end of primer, ligated-oligonucleotides can be identified with ELISA, plate or paper capture detection methods	Currently field testing in Kenya: Clinical trial: NCT01898754	[14–16]
	Point mutation assay PCR primer ligation	LRA	Point-of-care	Simplified ligation amplification assay using a one-step single-buffer method and sequence-specific dual-labeled probe for detection	In development	[17,18]

ASPCR, allele-specific PCR; LRA, one-step ligation on RNA amplification; NGS, next-generation sequencing; OLA, oligonucleotide ligation assay; RT, reverse transcriptase; VL, viral load.

Table 2.

Advantages and limitations of newer HIV reservoir assays

Assay description	Advantages	Limitations
Inducible virus assays: HIV-1 RNA detected in supernatant after cell stimulation	Faster, less costly and more sensitive than QVOA	Does not differentiate inducible virus from replication competent virus
Limiting dilution and near full-length sequencing of proviruses	More sensitive for likely replication competent virus than QVOA	Limited throughput; costly; apparently intact proviral genome does not prove infectiousness
Fractional single cell assays by limiting dilution PCR	Allows investigation of the contribution of individual cells in transcription and virus production	Limited throughput. High-throughput single cell assays are in development
Integration site assays	Investigate the role of clonal proliferation in viral persistence	Current assays have a low throughput and cannot link proviral sequences to integration sites. Not suitable to study the effect of interventions on population size and survival of particular clones

QVOA, quantitative viral outgrowth assay.