Future technologies for monitoring HIV drug resistance and cure

Urvi M. Parikh\textsuperscript{a}, Kevin McCormick\textsuperscript{a}, Gert van Zyl\textsuperscript{b}, and John W. Mellors\textsuperscript{a}

**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 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 drug-resistant 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], oligoligation 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...
### Table 1. Technologic approaches for HIV drug resistance testing

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

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.
(index sequences) enables multiplexing of samples to increase throughput and reduce cost per geno-
type relative to standard sequencing [30,31].

Earlier studies using the 454 platform could
detect mutants at 1% frequency, but accuracy was
compromised by PCR bias 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 inno-
vations to improve NGS accuracy and precision, fur-
ther modifications are still needed to simplify sample
processing, NGS library preparation and bioinfor-
matics analysis of sequences. Dried blood spot
(DBS) technology is currently the WHO-recom-
mended 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 Pri-
mestore 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 through-
put could be accomplished by automated sample
extraction (e.g. Abbott m2000sp, Abbott Molecular,
Des Plaines, Illinois, USA) or by replacing laborious
sample preparation steps with liquid-handling equip-
ment. 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 resist-
ance monitoring for epidemiologic surveillance and
patient management.

LIMITATIONS OF CURRENT ASSAYS FOR
HIV CURE

The HIV reservoir consists of HIV-infected cells car-
rying 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 cor-
related 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
good 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 apolipo-
protein B mRNA editing enzyme, catalytic polypep-
tide-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 compet-
ent proviruses [48*]. Use of QVOA for assessing HIV
reservoirs is also limited by large blood volume
requirements, high-cost and low throughput. Sim-
plified, 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].

**Table 2.** Advantages and limitations of newer HIV reservoir assays

<table>
<thead>
<tr>
<th>Assay description</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inducible virus assays: HIV-1 RNA detected in supernatant after cell stimulation</td>
<td>Faster, less costly and more sensitive than QVOA</td>
<td>Does not differentiate inducible virus from replication competent virus</td>
</tr>
<tr>
<td>Limiting dilution and near full-length sequencing of proviruses</td>
<td>More sensitive for likely replication competent virus than QVOA</td>
<td>Limited throughput; costly; apparently intact proviral genome does not prove infectiousness</td>
</tr>
<tr>
<td>Fractional single cell assays by limiting dilution PCR</td>
<td>Allows investigation of the contribution of individual cells in transcription and virus production</td>
<td>Limited throughput. High-throughput single cell assays are in development</td>
</tr>
<tr>
<td>Integration site assays</td>
<td>Investigate the role of clonal proliferation in viral persistence</td>
<td>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</td>
</tr>
</tbody>
</table>

QVOA, quantitative viral outgrowth assay.
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 Biosciences, 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 US-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 allows 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 platforms 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
HIV and diagnostics

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.

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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.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest


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Comprehensive overview of HIVDR monitoring detailing the genetic mechanisms, epidemiology and management of HIVDR as well as the implications of HIVDR for patient management at both individual and population levels across diverse economic and geographic settings.


Study showing that 454 pyrosequencing NGS can be used to genotype from dried blood spot with a high concordance to standard genotyping, from samples with a viral load as low as 1700 copies/ml and an estimated ~50 viral copies per blood spot.


This study describes the proportion of HIV DNA that is intact after acute and chronic infection. It emphasizes the small proportion of intact HIV DNA genomes making up the ‘true’ reservoir.


This article reviews current viral culture and PCR-based methods to characterize and quantify the latent reservoir.


This manuscript describes the tat/rev induced limiting dilution assay, an inducible virus recovery assay that quantifies cells producing multiply spliced mRNA, after stimulation. It is more practicable than the quantitative virus recovery assay but is not specific for replication competent provirus.


This manuscript describes the development of accurate assays to measure total HIV-1 DNA and RNA; the use of a cellular gene quantified in parallel controls for cell input and extraction steps and provides an accurate denominator for the cells assayed.


This study describes the decay rate of residual HIV-1 plasma RNA using an improved single copy assay.


This manuscript describes continuous long read single genome sequencing of HIV genomes with Pacific Biosciences SMRT technology, a method that may allow an accurate study of near full genome HIV haplotypes.

Hong F, Spinder J, Musick A, et al. editor ART Reduces Cellular HIV RNA but Not the Fraction of Proviruses Transcribing RNA. Conference on Retroviruses and Opportunistic Infections (CROI 2016); 2016 February 22–25, 2016; Boston, MA, USA.

This abstract compares the proportion of cells transcribing HIV RNA and the level of HIV RNA expression of infected cells in viremic and successfully treated individuals. It found that the proportion of cells expressing HIV RNA is not significantly different on therapy, but fewer cells on therapy express high levels of HIV RNA.


This investigation describes a method sequencing single HIV DNA genomes of cells and the mRNA that these, which allowed the investigation of clonal expansion and the detection of defective genomes expressing mRNA.


The first description of the HIV genome in single molecules, which is the current standard for HIV RNA.