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The Alphabet Soup of HIV Reservoir Markers

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Abstract
Purpose of Review Despite the success of antiretroviral therapy in suppressing HIV, life-long therapy is required to avoid HIV reactivation from long-lived viral reservoirs. Currently, there is intense interest in searching for therapeutic interventions that can purge the viral reservoir to achieve complete remission in HIV patients off antiretroviral therapy. The evaluation of such interventions relies on our ability to accurately and precisely measure the true size of the viral reservoir. In this review, we assess the most commonly used HIV reservoir assays, as a clear understanding of the strengths and weaknesses of each is vital for the accurate interpretation of results and for the development of improved assays.
Recent Findings The quantification of intracellular or plasma HIV RNA or DNA levels remains the most commonly used tests for the characterization of the viral reservoir. While cost-effective and high-throughput, these assays are not able to differentiate between replication-competent or defective fractions or quantify the number of infected cells. Viral outgrowth assays provide a lower bound for the fraction of cells that can produce infectious virus, but these assays are laborious, expensive and substantially underestimate the potential reservoir of replication-competent provirus. Newer assays are now available that seek to overcome some of these problems, including full-length proviral sequencing, inducible HIV RNA assays, ultrasensitive p24 assays and murine adoptive transfer techniques.
Summary The development and evaluation of strategies for HIV remission rely upon our ability to accurately and precisely quantify the size of the remaining viral reservoir. At this time, all current HIV reservoir assays have drawbacks such that combinations of assays are generally needed to gain a more comprehensive view of the viral reservoir. The development of novel, rapid, high-throughput assays that can sensitively quantify the levels of the replication-competent HIV reservoir is still needed.

Keywords HIV · Reservoir · Assays · QVOA · Replication-competent · Cure

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Introduction

While antiretroviral therapy (ART) has had a dramatic impact on the HIV epidemic, it is not curative and life-long. ART is needed to prevent HIV reactivation from long-lived viral reservoirs [1, 2]. One of the highest priorities for the HIV field is the search for therapeutic interventions that can eliminate or control the HIV reservoir, with the hope of replicating the sustained ART-free HIV remission seen in HIV elite controllers (ECs) or post-treatment controllers (PTCs) [3–5]. However, the optimal method of measuring the HIV reservoir size remains controversial, as all current assays have strengths and weaknesses. This is reflected in the diversity of reservoir assays evaluated as part of previously completed (Table 1) and current clinical trials (Table 2) [3, 6–8]. Overall, there are several categories of HIV reservoir assays. These assays include (1) intracellular HIV DNA, (2) cell-associated HIV RNA, (3) ultrasensitive plasma viremia, (4) viral outgrowth assays, (5) inducible HIV RNA, (6) protein-based assays and (7) murine adoptive transfer assay. In this review, we will summarize the most commonly used HIV reservoir assays,

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Table 1 Effect of latency reversal agents (LRAs) on HIV reservoir measures in previously completed clinical trials

Study	LRA	Total DNA	Integrated DNA	2-LTR circles	CA-RNA	Ultrasensitive plasma RNA	QVOA	TILDA
Gutiérrez, AIDS 2016 [51]	Bryostatin				+	+		
Spivak, CID 2014 [79]	Disulfiram					+	+	
Mothe, JAC 2015 [80]	Disulfiram in combination	+			+			
Rasmussen, Lancet 2014 [8]	Panobinostat	+	+	+	+	+	+	
Søgaard, PLoS Path 2015 [6]	Romidepsin	+		+	+	+	+	+
Leth, Lancet HIV 2016 [60]	Romidepsin in combination	+	+		+	+	+	
Lehrman, Lancet 2005 [41]	Valproic acid		+			+	+	
Siliciano, JID 2007 [81]	Valproic acid						+	
Archin, AIDS 2008 [82]	Valproic acid					+	+	
Sagot-Lerolle, AIDS 2008 [83]	Valproic acid	+	+				+	
Routy, HIV Med 2012 [84]	Valproic acid						+	
Archin, Nature 2012 [3]	Vorinostat	+			+			
Archin, JID 2014 [85]	Vorinostat	+			+	+	+	
Elliott, PLoS Path 2014 [86]	Vorinostat	+	+		+	+		+

QVOA quantitative viral outgrowth assay, *CA-RNA* cell-associated HIV RNA, *TILDA tat*/*rev* inducible limiting dilution assay, *PKC* protein kinase C, *JQ1* a bromodomain inhibitor

providing details on their interpretation, advantages and limitations.

HIV DNA Assays

Total HIV-1 DNA includes all forms of HIV-1 DNA: circular unintegrated, linear unintegrated and linear integrated. The relative abundance of each species has been reported to be in the following descending order: non-integrated linear DNA > integrated proviral DNA > non-integrated circular DNA [9, 10]. Specifically in non-suppressed patients, non-integrated forms make up the vast majority of HIV DNA in the nucleus, which is approximately 100-fold more frequent than the integrated proviral DNA form [11]. Separate assays have been developed to quantify each species.

Quantitative real-time PCR (qPCR) or droplet digital PCR (ddPCR) for conserved viral regions can be performed to determine levels of HIV nucleic acid, as will be discussed below. qPCR monitors the progression of amplification in each cycle through the use of fluorescent probes, and quantification is performed by measuring the threshold cycle (C_t) at which

Table 2 A selection of current clinical trials for HIV remission and their primary outcome measures

ClinicalTrials.gov identifier	Trial name	Primary outcomes measured
NCT02092116	REDUC	QVOA, total HIV DNA, integrated DNA
NCT02707900	VOR VAX	QVOA
NCT02707692	Perturbing of HIV reservoir with immune stimulation	CA-RNA
NCT02336074	RIVER	Total HIV DNA
NCT02628340	RESEACHRON	Total HIV DNA
NCT02191098	ALT-803	TILDA
NCT01933594	A5315	SCA and CA-RNA
NCT02028403	A5326	Gag-specific CD8+ T cells and changes in SCA
NCT02411539	A5342	CA-RNA/DNA ratio

QVOA quantitative viral outgrowth assay, *CA-RNA* cell-associated HIV RNA, *TILDA tat*/*rev* inducible limiting dilution assay, *SCA* single-copy assay

fluorescence is higher than a certain threshold. While a standard curve is necessary for quantification of copy number by qPCR, it provides a wide dynamic range. Other caveats include susceptibility to primer/probe sequence mismatches that lead to inaccurate quantification and sensitivity challenges in detecting low copy numbers. The former can be tackled using patient-matched primers [12] or calculating the patient-specific mismatch-related quantification errors (MRQE) by comparing the amplification of a patient sample with that of a control template without mismatches [13].

Another option is using the more recently described ddPCR, which measures only endpoint fluorescence after all thermal cycles are completed to provide absolute quantification for HIV DNA. Through a microfluidic system, the aqueous PCR reaction mixture is emulsified in a thermostable oil to form droplets, allowing a large number of microscopic reactions to be performed at the same time, at a level where some droplets will have no template and others may have one or more template copies [14, 15]. After the reaction is complete, the samples are loaded into a droplet reader and each droplet is streamed in a single file past an optical detector to detect the amplitude of fluorescence from each droplet. The readout is a count of the number of positive droplets formed per sample well. Based on Poisson distribution, the data can be used to calculate the starting copy number of the target DNA template in the original sample. The dynamic range of ddPCR is determined by the number of replicates (droplets) formed in the reaction.

A recognized advantage of ddPCR over qPCR is avoiding the need for standard curves since ddPCR gives an absolute quantification. Furthermore, ddPCR may result in improved precision [14, 16] and is more robust towards target sequence variation, which is especially important when working with genetically diverse clinical samples [14]. On the other hand, ddPCR has a more limited dynamic range, demonstrates a higher risk of false-positive wells and is more time-consuming due to the additional time needed for droplet generation and droplet reading after the PCR is complete [14, 16]. In addition, the ddPCR system and reagents are generally more costly than those for qPCR.

The two most commonly used ddPCR platforms include systems from Bio-Rad and RainDance. The Bio-Rad platform represented the first commercially available ddPCR system and is the most widely adopted. The RainDance platform was more recently introduced into the field [17], but may be able to yield a significantly greater number of droplets. A rigorous comparison of the two ddPCR platforms for HIV detection is still needed.

Total HIV DNA

To determine levels of total HIV DNA, qPCR or ddPCR for conserved viral regions can be performed [18]. In both of these platforms, probes for HIV DNA have targeted HIV-1 *gag* [18, 19], *pol* [14] or the LTR region [20–22]. When the three different targets were compared side-to-side, targeting the LTR region was shown to be superior and resulted in some cases in a $\log_{10}$-higher number of HIV copies [23]. To determine the copy number of HIV-1 DNA per cell, a parallel measurement of a control gene (such as CCR5 [24], β-globin [25] or albumin [18]) is frequently conducted to quantify the number of cells assayed during the HIV DNA measurement. Alternatively, a parallel blood sample, drawn simultaneously, can be stained for CD4 and run for flow cytometric analysis [19].

In patients on suppressive antiretroviral therapy, total HIV DNA level is reflective of the total HIV proviral reservoir size and may predict viral rebound timing after treatment interruption [26, 27]. However, a PCR-based approach for measuring the viral reservoir leads to an overestimation of the size of the replication-competent reservoir, as the vast majority of viral genomes quantified are not replication-competent. It has been estimated that measuring total HIV DNA may overestimate the intact HIV reservoir by greater than 100-fold in those treated during chronic infection and by more than tenfold in those treated during acute infection [28••].

It is important to note that unintegrated viral DNA contributes to the total HIV DNA signal, while unintegrated DNA contributes very little to the viral reservoir due to its limited transcription potential [25, 29]. This is an especially important distinction when monitoring reservoir size in viremic non-suppressed patients who have an excess of unintegrated HIV DNA that confounds the interpretation of total DNA levels [30, 31].

Circular Unintegrated HIV DNA

Circular unintegrated forms include 1-LTR and 2-LTR circular HIV DNA. In non-suppressed patients, non-integrated DNA makes up the majority of total DNA [30]. However, in virologically suppressed patients, circular HIV DNA forms represent a minor population and do not give rise to infectious virus. The 2-LTR circles, for example, represent approximately 0.03–5% of total viral DNA [32]. Quantification of 1-LTR circles by qPCR has been technically challenging and unreliable because they lack unique sequence segments to distinguish them from proviral or linear unintegrated DNA [33]. The presence of 2-LTR circles can be assayed by using primers specific for the junction between the two LTR ends and performing either qPCR or ddPCR [14]. Historically, 2-LTR circles have been measured as a reflection of active viral replication, but this has been called into question [34].

Integrated HIV Proviral DNA

Alu-gag PCR

There are situations where measurement of only integrated proviral HIV DNA is indicated. This is especially relevant

for participants who are not virologically suppressed, where linear and circular non-integrated forms may dominate the total DNA measurement. To quantify integrated DNA alone, a nested PCR, known as Alu-*gag* PCR, can be performed [35, 36]. The first PCR utilizes a forward primer that is virus-specific, recognizing the U5 region of the LTR, while the reverse primer binds to Alu repeats. This results in specific amplification of integrated HIV DNA. Alu elements are abundant ~300 bp-interspersed repeat sequences, distributed at a frequency of one Alu element in every 2.5 kb of the human genome [37]. Since the integration of HIV is random within the human genome, Alu-*gag* pre-amplification generates a population of cellular-HIV junction DNA sequences of various lengths. After the initial amplification round, qPCR is used to quantify total copies of integrated provirus [38]. The caveats here are that only integrated forms of the virus that reside close to Alu repeats will be reliably amplified [39] and the high variability between sample replicates. To address this, repetitive sampling has been introduced [36, 39, 40].

Gel Separation

Running DNA samples on a gel can be used to separate genomic (approximately 20 kb) high-molecular weight (HMW) DNA from episomal DNA. HMW DNA is then recovered from the gel and subjected to HIV-specific qPCR. This has been implemented in some studies [41], and authors have reported that this fractionation procedure gets rid of 97–99% of linear HIV-1 DNA and 99% of 2-LTR circles [30]. This assay is not commonly employed but can also determine levels of all non-integrated HIV species, where unintegrated HIV DNA equals HIV DNA from total DNA minus integrated HIV DNA from HMW DNA.

Fluorescence In Situ Hybridization (FISH)

FISH can be used to quantify the number of integrated proviral DNA copies [42] and was successfully used on splenocytes from splenectomized HIV patients [43]. That study demonstrated that the majority of infected cells in HIV patients harbor more than one proviral copy, with a mean of three to four proviruses per infected cell. DNAscope, an optimized in situ hybridization platform that relies on the use of probes spanning the entire length of the viral DNA (vDNA), was demonstrated to detect latently infected cells in lymphoid tissue sections from macaques [44••]. This might represent a sensitive tool to identify sites of latent viral reservoir at the tissue level.

Full-length Single Genome Sequencing (SGS)

Defective proviruses constitute the vast majority (93–98%) of proviral HIV DNA [28••]. To better estimate the true size of intact proviral reservoir, methods to perform full-length single-genome proviral sequencing are becoming increasingly popular. Genomic DNA is extracted from patient cells and subjected to a limiting dilution PCR protocol using primers that span the two LTRs to amplify near-full-length HIV proviral DNA from single templates [28••, 45, 46]. Sanger or next-generation sequencing is used to sequence the amplified proviruses. These methods are labor- and cost-intensive, but can provide important details on the types of defective proviral genomes present, in addition to quantifying the number of intact proviruses. Results from these studies have demonstrated that qPCR/ddPCR quantification of HIV proviral DNA significantly overestimates the size of the intact HIV reservoir.

Cell-Associated HIV RNA Assays

Within HIV-infected cells, several forms of RNA exist: multiply spliced (ms), incompletely spliced (is) and unspliced (us) [47]. Initially, msRNA transcripts are generated, encoding for regulatory proteins, such as *Tat* and *Rev*. As the infection proceeds, there is a shift towards isRNA and usRNA, encoding for the full viral genome to be packaged, as well as structural and accessory viral proteins. Assays that measure unspliced HIV RNA have been the most commonly used platform for previous clinical studies, as illustrated for studies determining the effect of HIV latency-reversing agents (Table 1).

Total HIV RNA transcripts can be isolated and quantified by RT-qPCR or ddPCR, using primers and probes targeting the LTR region [20–22]. To assay usRNA species, probe and primers targeting viral *gag*, located downstream of the major 5′ splice donor site (D1), are used. To target the msRNA species, probe and primers encoding for *tat* and *rev* are used [48]. We have reported that levels of cell-associated (CA)-usRNA are predictive of the timing of viral rebound after treatment interruption [49].

HIV RNA copy numbers are normalized to cellular input, either by running a parallel qPCR for a control gene (such as β-actin [50] or CCR5 [51]) or estimated by total extracted RNA amounts, assuming that 1 ng RNA corresponds to approximately 1000 cells [51]. Some groups also report the average transcription per infected cell, calculated as the CA-HIV RNA/DNA ratio [21]. One major limitation of CA-RNA quantification methods is that defective CA-RNA species are also present, similar to HIV DNA [52] and thus RT-qPCR likely overestimates the number of intact HIV transcripts.

Another recently described technique is the PrimeFlow RNA Assay, which is based on fluorescence in situ RNA hybridization using a set of 20–40 probes. Simultaneous use of PrimeFlow and p24 protein staining via flow cytometry was successfully reported in cell lines and in vitro infected cells [53]. The PrimeFlow assay is reported to detect different classes of HIV RNA transcripts with a limit of detection (LOD) of 10–100 infected cells within 10^6 total cells [54].

Furthermore, a recently published paper used this dual technique to detect and characterize infected CD4 T cells from the peripheral blood of both suppressed and non-suppressed patients [55]. The assay can also be used with chromogenic detection, allowing microscopic imaging of vRNA-positive cells, termed RNAscope. vRNA and vDNA double-positive cells, as well as vDNA positive vRNA negative cells, were identified in lymphoid tissue sections from macaques using this technique [44••]. A potential limitation of this assay is the requirement of large volumes of blood or difficult-to-sample tissue sections.

Ultrasensitive Plasma Residual Viremia Assays

Single-Copy Assay (SCA)

To characterize residual plasma viremia, several assays were developed to detect a single copy or less of HIV-1 RNA per milliliter of plasma. The SCA assay consists of an initial nucleic acid extraction through pelleting of virions by ultracentrifugation. To monitor this step of viral RNA recovery from the plasma and control for possible reverse transcriptase qPCR (RT-qPCR) inhibition, samples are spiked with an internal control standard, a known quantity of replication competent avian sarcoma (RCAS) virions. Following a proteinase K digestion of proteins, nucleic acids are precipitated. HIV-1 RNA is quantified via an RT-qPCR reaction using primers and probe targeting either the *gag* (gSCA) or integrase (iSCA) regions [56]. The gSCA was the original version of the SCA, but inefficient amplification of viral RNA in 15–30% of clinical plasma samples led to the need for initial confirmation of primer/probe efficiency for each participant sample [57]. The newer iSCA assay uses primers and probe targeting the more conserved integrase region and with significantly decreased rates of primer/probe sequence mismatches with patient samples [57]. For a more sensitive assay, a modification to iSCA, called mega-iSCA, was developed, which assays large volumes of plasma (20–35 ml) with a LOD down to <0.1 copies/ml.

The HIV Molecular and Monitoring Core *gag* assay (HMMC*gag*) is an assay developed at NCI Frederick and represents a variant of the SCA, using primers and probe targeting the beginning of the *gag* gene. The assay's design relies on a hybrid of qPCR and digital PCR format to maximize the advantages and minimize the disadvantages of both assay formats. The assay is set up in multiple replicates and for samples where all reactions are positive for amplification; viral load is calculated based on the standard curve. But, for samples for which some reactions are not positive for amplification, the viral load is instead calculated based on the Poisson distribution for the frequency of positive wells [58].

Transcription-Mediated Amplification (TMA)

TMA is a qualitative nucleic acid amplification test, commonly used in blood donor screening [59], with a binary positive or negative result [6, 8, 51, 60]. The assay is commercially available through Procleix Ultrio Plus and is widely used in Europe. First, viral RNA is captured by oligonucleotides containing a T7 promoter primer site, which is subsequently captured by magnetic particles to allow for the separation of viral RNA. This is followed by an isothermal transcription-mediated amplification step using reverse transcriptase and T7 RNA polymerase leading to the exponential production of RNA amplicons. Finally, the amplicons are detected via a fluorescent probe. When run in a single replicate, the assay has a sensitivity of 3.6 HIV copies/ml at a 50% limit of detection [61]. When the assay is performed in quadruplicate, the sensitivity drops down to less than 3.5 HIV copies/ml [62].

Modified Abbott qPCR Assay for Low-Copy Detection

The conventional Abbott qPCR assay is an automated platform to measure HIV-1 viral load in plasma samples using volumes of up to 1 ml. The assay utilizes primers and probe targeting the integrase region of *pol*, with a unique probe design, being partially double-stranded and tolerating mismatches. Thus, the assay can be used for quantifying HIV-1 group M (subtype A-H), group N and group O isolates [63]. The LOD for the conventional assay is 40 copies/ml. The modified version of this assay relies on concentrating virus from a high volume of plasma (up to 30 ml) by ultracentrifugation on an iodixanol density cushion. The concentrated pellet is resuspended and HIV-1 RNA is quantified using the Abbott HIV-1 qPCR assay with a reported LOD of less than 1 copy/ml [64].

Quantitative Viral Outgrowth Assay (QVOA)

While nucleic acid-based measures of HIV reservoir size have some advantages, they are unable to quantify the size of the replication-competent reservoir. QVOA, or the Infectious Units Per Million (IUPM) assay, has historically been the gold standard for detection of the replication-competent HIV reservoir [65, 66]. Using a limiting dilution culture format, QVOA measures the number of wells containing detectable HIV-derived p24 antigen released in the supernatant after resting cells are subjected to one round of stimulation [65, 66]. Previous reports show that QVOA is relatively robust, and with sufficient cell numbers, HIV-1 can be recovered from the majority of HIV-1-infected participants on suppressive antiretroviral therapy [2, 65, 67, 68]. QVOA was used to demonstrate the high stability and low decay rate of the latent virus reservoir in suppressed patients [68],

showing generally less than a twofold variation between longitudinal measurements [69].

The main advantage of this assay is that it detects only the replication-competent virus reservoir. However, QVOA underestimates the reservoir size because at any given time, only a subset of the replication-competent reservoir is activated. Studies have calculated that only 1% of cells harboring HIV provirus release infectious virions after being subjected to maximum in vitro activation [46]. A large proportion of those non-induced cells harbor defective copies of the integrated HIV provirus, but a subset represents a population of cells with intact virus, which are not activated in any single round of activation. Some reports demonstrated that QVOA did not strongly correlate with the frequency of cells harboring intact proviruses and likely underestimates the total replication-competent virus reservoir by approximately 25-fold [28••, 46]. Thus, QVOA should be thought of as the lower-bound estimate of the replication-competent reservoir. QVOA has not been useful in in vivo studies measuring effect of latency reversal agents (LRAs) on the size of the virus reservoir, which highlights its limited sensitivity [6, 8]. It has been suggested that a more than sixfold difference between longitudinal QVOA measurements could be used to reliably detect a change in reservoir size with high confidence [69]. Other limitations of QVOA include the requirement of a large sample volume and that it is both time- and resource-intensive. Of note, integrated HIV DNA levels has been found to be significantly correlated with QVOA [70••].

Inducible HIV RNA Assays

The quantification of the replication-competent HIV reservoir is challenging due to the overwhelming proportion of infected cells that harbor replication-deficient proviruses and the low frequency of transcriptionally active cells in those on chronic suppressive ART. The inducible HIV RNA assays seek to bridge the divide between the nucleic acid-based measurements of the HIV reservoir (CA-DNA or CA-RNA) and QVOA. They provide a more accurate reading of the inducible HIV reservoir than the CA-RNA assay alone while potentially providing the frequency of HIV-expressing cells in an assay that is far more rapid and scalable than QVOA. However, replication-defective proviruses may lead to RNA transcripts [52], and one limitation of all assays measuring HIV RNA levels is the inability to fully define the replication-competent fraction.

Cell-Associated HIV RNA

This assay measures the copy number of cell-associated viral RNA after stimulation. Briefly, CD4 T cells are stimulated, followed by cell lysis and RNA extraction. Levels of usRNA and msRNA can be measured using RT-qPCR [71, 72]. The extent of HIV-1 transcription after activation is based on levels of msRNA, like *rev* and *tat*. A limitation of this assay is the requirement of RNA extraction, where potential loss of viral RNA may occur. This was addressed in the newer TILDA assay, which will be discussed subsequently.

Supernatant RNA

This assay relies on measuring the production of viral particles in culture supernatants of stimulated cells. CD4 T cells isolated from patients are stimulated (using PHA [73], CD3/CD28 beads [74] or latency reversal agents [74]), releasing infectious virions in the supernatant. At different time points post-stimulation, viral RNA is isolated from the cellular supernatant using a commercial RNA isolation kit, and levels of HIV RNA are assayed using RT-qPCR. Similar to the inducible CA-RNA assay, the limitation of this assay is that it requires an RNA extraction step.

Tat/rev Induced Limiting Dilution Assay (TILDA)

TILDA is an assay that measures the frequency of cells with inducible HIV msRNA [71, 75]. Briefly, about one million CD4 T cells are stimulated with PMA and ionomycin for 12 h, a time point chosen based on kinetic studies demonstrating maximal RNA production of *tat* and *rev*. After distribution in a limiting dilution format directly in the reaction buffer, samples are subjected to RT-qPCR for *tat* and *rev* transcripts. *Tat*/*rev* was observed to be frequently missing in defective proviruses with internal deletions. Thus, this assay is attractive as it is less likely to be measuring defective RNA species [28••]. Using the maximum likelihood method, the frequency of cells producing HIV msRNA post-stimulation can be calculated based on the number of positive wells [76]. Results from TILDA correlated with those obtained through measuring integrated viral DNA. The TILDA assay is less sample-intensive than QVOA and does not involve an RNA extraction step or amplification of virus replication, avoiding the need for a prolonged culture time [76]. However, it is important to note that not all cells producing msRNA are releasing intact infectious virions. Compared to nucleic acid-quantification assays, this technique is more resource-intensive.

Protein-Based Assays

There are few high-throughput and sensitive assays for detecting HIV protein production. Investigators at Merck recently reported the development of an ultrasensitive immunoassay to quantify p24 and the uncleaved p55 levels in cell lysates and in media from cultured patient cells. It is reported that the

assay can detect protein levels down to 14 fg/ml with a dynamic range of >4 $\log_{10}$, but does require the use of specialized equipment (Quanterix Simoa technology) [77].

Murine Viral Outgrowth Assay (MVOA)

The murine viral outgrowth assay (MVOA) is a binary endpoint assay that uses a mouse model to determine whether patient-derived cells harbor infectious virus [78]. In this assay, either whole PBMCs or sorted CD4 T cells are injected into NOD/Prkdc^{scid}/gamma-chain knockout (NSG) mice. Some mice are further subjected to CD8 T cell depletion or subjected to T cell stimulation via injection of an anti-CD3 antibody. Over time, HIV RNA from the plasma of xenografted mice is isolated and quantified by RT-qPCR. The assay was successful in recovering virus from patient cells, including an elite controller, who had negative QVOA results [78]. It can be used to survey a large number of patient cells, requiring one mouse per 10–50 million CD4 T cells. However, it suffers from drawbacks related to the inherent heterogeneity of human cell engraftment in the murine host and the lack of a quantitative readout.

Conclusion

The development and evaluation of HIV curative strategies rely upon our ability to accurately and precisely quantify the size of the remaining HIV reservoir. At this time, all current HIV reservoir assays have drawbacks such that combinations of assays are generally needed to gain a more comprehensive view of the HIV reservoir. Techniques that quantify levels of HIV cell-associated DNA are high-throughput, but significantly overestimate the size of the intact or true viral reservoir. While the QVOA assay has historically been considered the gold standard for measuring the size of the replication-competent reservoir, this assay is challenging to perform and is useful only for determining the lower bound for the size of the replication-competent reservoir. Quantifying the number of intact proviruses by sequencing appears to provide the best current estimate of the HIV reservoir's potential true size, but this assay is still relatively new and is both labor-intensive and expensive, calling into question its scalability in large clinical studies. Newer assays for the single-cell measurement of HIV-expressing cells and the high-throughput quantification of HIV protein levels represent promising technologies, but still require additional validation. The development of a rapid, high-throughput assay that can sensitively quantify the levels of the replication-competent HIV reservoir remains the holy grail of HIV reservoir assays and would accelerate the journey to our ultimate goal of finding an effective HIV curative strategy.

Compliance with Ethical Standards

Conflict of Interest JL has received research funding and consulted for Gilead and Merck. JL was funded in part by a grant from NIH/NIAID (125109).

Human and Animal Rights and Informed Consent There were no human or animal experiments performed for the purpose of this review.

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