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Towards an HIV-1 cure: measuring the latent reservoir

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Abstract

The latent reservoir of HIV-1 in resting memory CD4⁺ T cells serves as a major barrier to curing HIV-1 infection. While many PCR- and culture-based assays have been used to measure the size of the latent reservoir, correlation between results of different assays is poor and recent studies indicate that no available assay provides an accurate measurement of reservoir size. The discrepancies between assays are a hurdle to clinical trials that aim to measure the efficacy of HIV-1 eradication strategies. Here we describe the advantages and disadvantages of various approaches to measure the latent reservoir.

Keywords

HIV-1; Cure; Latency; Assays

The establishment of HIV-1 latency

HIV-1 currently infects more than 35 million people worldwide, with over 1.1 million people infected in the United States alone (<http://www.cdc.gov/hiv/statistics/basics/>). Although combination antiretroviral therapy (ART) is effective in suppressing HIV-1 replication by blocking various steps in the viral life cycle, it is not curative due to the existence of a latent viral reservoir in resting memory CD4⁺ T cells [1–6]. The latent reservoir (LR) has an extremely long half-life (44 months), necessitating lifelong treatment [7]. The LR may be established when HIV-1 infects fully or partially activated CD4⁺ T cells that then return to a resting memory state as part of the normal physiological process after encountering cognate antigens [8,9]. Thus, latent proviruses are found predominantly in different subsets of resting memory cells, but not in naïve cells [2,10–12]. Resting memory CD4⁺ T cells are largely nonpermissive for viral gene expression, and the viral genome persists in a DNA form as an integrated provirus that is not actively transcribed [13]. For

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this reason, latently infected cells are not targeted by the immune system or ART. The LR in resting memory CD4⁺ T cells is widely recognized as a major barrier to HIV-1 eradication.

Measurement and composition of the latent reservoir

The LR was first measured and characterized in the mid-1990s using a viral outgrowth assay (VOA), which is still considered the gold standard for measuring the frequency of resting CD4⁺ T cells that carry replication-competent proviruses in a latent state [1,4,14]. The VOA makes use of phytohemagglutinin (PHA), a strong T cell mitogen, to globally activate resting CD4⁺ T cells in a manner that mimics antigen-driven activation. This renders the cells permissive for viral gene expression and induces release of HIV-1 from latently infected cells. Since the development of the VOA, other assays such as PCR-based ones that measure total HIV-1 proviral DNA, have been advanced as simpler measures of the LR [15–17]. Although both culture- and PCR-based methods are commonly used, there is little correlation between the two types of assays. Based on the VOA, approximately 1 per 10⁶ resting CD4⁺ T cells contain replication-competent virus, but PCR-based assays show that on average 300 per 10⁶ resting CD4⁺ T cells contain HIV-1 proviral DNA [18].

These discrepancies are attributed to the heterogeneous nature of the proviruses in resting CD4⁺ T cells. These proviruses can be divided into two groups: those that are induced to release replication-competent virus after one round of T cell activation (induced proviruses) and those that are not (non-induced proviruses), as is shown in Figure 1. The VOA measures only the former population, whereas PCR-based approaches detect both groups of proviruses. This partially explains the lack of correlation between the two types of assays. Interestingly, most noninduced proviruses are defective [19]. The majority contain large internal deletions that arise by copy choice recombination between homologous regions in genomic viral RNA during reverse transcription [19–21]. Other defective proviruses contain inactivating G to A nucleotide substitutions introduced by APOBEC3G, a cytidine deaminase that is part of the host innate immune defense system [22–24]. A small percentage of proviruses contain other inactivating defects such as packaging signal deletions, small nucleotide insertions or deletions (INDELS), and missense mutations. Defective proviruses will not produce infectious virus in the VOA but can be detected by PCR assays, depending on the assay specifics. In addition, a small percentage of noninduced proviruses have intact genomes and are potentially replication-competent [19]. These proviruses are termed intact, noninduced proviruses (INPs). In order to achieve a sterilizing HIV-1 cure, all of the proviruses capable of reinitiating infection must be purged from the LR.

Accurately measuring changes in the size of latent reservoir

Currently, the most widely discussed approach to eliminate the LR is to reverse latency pharmacologically so that the infected cells can be eliminated. This approach is termed ‘shock and kill’ [25–27]. The rationale is to first force HIV-1 out of latency by inducing expression of HIV-1 genes since high levels of certain HIV-1 proteins can cause death of the infected T cells through cytopathic effects. In addition, presentation of peptides derived from viral proteins can lead to lysis of infected cells by virus-specific cytolytic T

lymphocytes (CTL). Cell surface expression of the HIV-1 envelope glycoprotein (Env) can, in principle, target infected cells for destruction by antibody-dependent cell-mediated cytotoxicity (ADCC). In addition, downregulation of MHC class I molecules by the HIV-1 accessory factor Nef can render productively infected cells susceptible to lysis by natural killer (NK) cells [28].

Clinical trials involving the ‘shock and kill’ strategy are in progress. Histone deacetylase inhibitors (HDACis) reverse HIV-1 latency in model systems and are being tested in infected individuals on ART [29–35]. However, recent studies have shown a discrepancy between the effects of latency reversing agents (LRAs) in *in vitro* HIV-1 latency models and in *ex vivo* assays with cells from HIV-1 infected individuals [36–38]. In order to determine if these LRAs are effective, it is important to develop techniques that accurately measure changes in the LR size *in vivo*.

Significant progress has been made in developing culture- and PCR-based assays since the initial discovery of the LR in the 1990s. However, a detailed comparison of several current assays has shown no precise correlation [18]. Available assays are discussed below, and their advantages and disadvantages are summarized in Table 1. A high-throughput, reliable, and sensitive assay that can accurately measure the true size of the LR is urgently needed. Such an assay is essential for determining the efficacy of pharmacological agents used in current or future clinical trials and for reliably concluding that a sterilizing cure has been achieved in HIV-1-infected patients undergoing eradication treatments.

Culture-based assays to measure the latent reservoir

The LR was initially defined and measured using the VOA [1,4]. This assay is still considered the ‘gold standard’ for measuring the latent reservoir. In this assay, resting CD4⁺ T cells are isolated from patients on ART and plated in fivefold serial dilutions. Phytohemagglutinin (PHA), a lectin, is added along with irradiated allogeneic peripheral blood mononuclear cells (PBMCs) to induce global T cell activation. The PBMCs ensure successful activation since they contain macrophages and dendritic cells, which act as antigen-presenting cells and enhance the PHA-driven activation of T lymphocytes [14]. These conditions give uniform activation of resting CD4⁺ T cells as assessed by CFSE dilution [39]. Initially, CD4⁺ lymphoblasts from HIV-1-negative donors were used to expand virus released from the patient cells to detectable levels. This process can be simplified by using the continuously proliferating cell line, MOLT-4/CCR5, which expresses high levels of CD4 and CXCR4 and has been stably transfected with CCR5, a co-receptor for HIV-1 viral entry [40]. The patient cells are co-cultured with lymphoblasts or MOLT-4/CCR5 cells for two to three weeks and then culture supernatants are analyzed for free virus using an ELISA assay for HIV-1 p24 antigen. Alternatively, a highly sensitive RT-PCR assay for virus in the supernatant can detect viral outgrowth as early as seven days after activation [40].

The VOA has a number of advantages, including the ability to detect individual latently infected cells. The clonal viruses that grow out in wells seeded at limiting dilution can be characterized by sequencing and functional studies. The VOA has the additional advantage

of not detecting defective proviruses, which vastly outnumber replication-competent proviruses and which are detected in standard DNA PCR assays [19]. However, some recent studies have shown that the VOA underestimates the true size of the LR because viral outgrowth is not observed in all wells that contain cells with intact proviruses. When wells negative for viral outgrowth were stimulated a second time with PHA, outgrowth of replication-competent virus was observed [19]. There are several potential explanations for the initial failure of these INPs to give rise to viral outgrowth following cellular activation. Viral induction could represent a stochastic process such that each intact provirus has only a finite probability of becoming induced following cellular activation. Most HIV-1 proviruses integrate into introns of actively expressed cellular genes [41], and transcriptional interference from the host gene could reduce the probability that a given intact provirus will be induced by a single round of T cell activation. However, the same provirus may be induced by subsequent rounds of T cell activation. Additionally, some of these INPs could have been successfully induced to produce viral RNA and release infectious virions. However, the released virus may have failed to establish a spreading infection that is required for detection by the VOA. For these reasons, the LR may actually be larger than the average value of one replication-competent provirus per 10^6 resting $CD4^+$ T cells, as measured by the VOA [19]. Thus, the VOA is best regarded as a definitive minimal estimate of the frequency of latently infected cells.

In addition to underestimating the size of the LR, the VOA can be problematic because it uses large volumes of blood (120–180 mL), is labor intensive and expensive, and requires 1–3 weeks of culture in a BSL3 laboratory. Hence there has been great interest in developing alternative assays.

T cell activation assays with viral RNA readout

Other culture-based assays used to measure the LR include ones in which patient resting $CD4^+$ T cells are plated and maximally activated at limiting dilution such that there is less than one virus-producing cell per well. Following maximal T cell activation, the frequency of cells producing viral RNA is directly measured by a quantitative PCR (qPCR) specific for either cell-associated unspliced mRNA [37], multiply spliced mRNA [39], or HIV-1 mRNA in the culture supernatant (presumably reflecting virion release) [37,40]. These assays measure different forms of RNA present throughout the HIV-1 life cycle. It is important to identify what form of HIV-1 RNA is being detected since these represent different stages in replication. Early in the replication cycle, multiply spliced mRNA transcripts are generated, but with time the transcripts are increasingly exported as unspliced and singly spliced mRNA species. Some mRNA transcripts are translated to make viral proteins necessary for virion assembly while other full-length transcripts are packaged into newly assembled virions for subsequent infection. When virions are released from the infected cell, certain assays can measure the two full-length mRNA copies that are present within a single virion. A recent study compared virion release with intracellular RNA measurements and found that, on average, 1.5% of proviruses could be reactivated to produce HIV-1 virions, whereas 6.8% and 8.2% of proviruses from two different patients could be reactivated to produce unspliced cell-associated HIV-1 RNA [37]. Since HIV-1 mRNA present in the culture supernatant reflects virus production, this measurement is likely a more accurate

quantification of the latent reservoir. In summary, these RNA-based assays allow for detection of infected cells with no need for outgrowth of the virus before PCR measurement, shortening the length of required culture time. However, the same caveats that apply to the VOA also apply to these assays. Since they only involve a single round of T cell activation, they do not detect INPs within the LR. Additionally, some defective proviruses may be able to produce RNA and even release virions even though they are unable to produce infectious virions. These defective proviruses could be detected by these assays. Thus, this class of assays may be confounded by both false negative and false positive results and do not accurately measure the size of the LR.

PCR-based assays to measure the latent reservoir

PCR-based assays are commonly used to measure persistent HIV-1 and provide a complementary approach to the VOA. These methods provide a quicker and easier way to study viral persistence and can be applied to a variety of immune cell types. The most common PCR method for measuring the LR is a qPCR for proviral DNA in either unfractionated PBMCs [15,42–46], CD4⁺ T cells [47,48], or resting CD4⁺ T cells [49]. All methods involve isolating the desired cell populations from the peripheral blood of infected individuals and subsequently extracting the DNA. A qPCR using primers located in conserved regions of the HIV-1 genome is carried out on the DNA extracts. The number of infected cells is calculated using a standard curve constructed with known copy numbers of proviral DNA, typically from a plasmid standard such as pNL4-3. A qPCR assay for a cellular gene present in two copies per diploid genome (frequently the ribonuclease RNase P), is used to determine the total number of cells in the sample. The proviral DNA copy number, combined with the total number of cells present, can give an estimate of the frequency of cells that harbor HIV-1 DNA. These methods have also been adapted to measure proviral DNA in the gut-associated lymphoid tissue (GALT) [50–52], a site which contains a high frequency of infected cells, as well as in CD4⁺ T cells in the bone marrow [53]. Other PCR methods use the droplet digital PCR technique to measure HIV-1 DNA in CD4⁺ T cells [17,18] as well as in PBMCs [18,54,55]. This method permits absolute quantification of HIV-1 DNA rather than relative quantification derived from a standard curve, as with qPCR.

In measuring the LR, it is important to detect only stably integrated proviruses and not extra-chromosomal HIV-1 DNA forms, which are unstable or replication-defective. Pioneering studies by Stevenson and colleagues showed that most of the HIV-1 DNA in resting CD4⁺ T cells from viremic patients is in a linear, unintegrated form which represents the end product of reverse transcription prior to integration [56]. Following cellular activation, this unintegrated viral DNA is integrated and transcribed, ultimately giving rise to infectious virus. Since the VOA involves cellular activation, it also detects cells with linear, unintegrated HIV-1 DNA if carried out on samples from viremic patients. Several studies suggest that in the absence of integration, the linear, unintegrated form of the viral genome is labile [57,58]. Therefore, cells with unintegrated HIV-1 DNA should not be considered as part of the stable LR. Following initiation of ART, labile, unintegrated viral genomes in recently infected cells decay, and after six months, the frequency of cells detected in the VOA falls to a stable plateau [59]. Importantly, because it can detect cells with linear

unintegrated viral genomes, the VOA does not give a reliable estimate of the LR in untreated patients or in patients who have been on therapy for less than six months.

Due to the problem of linear, unintegrated HIV-1 DNA, there has been considerable interest in PCR assays that can distinguish integrated viral genomes from unintegrated genomes. The most common method used for this purpose is Alu-PCR, which has been applied to either purified CD4⁺ T cells or PBMCs [16,60–63]. Alu-PCR selectively amplifies integrated HIV-1 genomes by using one primer targeting Alu elements, which are found in high copy numbers in the human genome, and a second primer located in the HIV-1 *gag* gene. A nested real-time PCR is then performed using a second set of primers in the HIV-1 LTR. Controls in which the Alu primer is excluded are important to demonstrate that the observed signal is in fact coming from integrated HIV-1 DNA. Proviruses integrated in close proximity to Alu elements give shorter first round PCR products and will amplify to a greater extent. A standard curve with a mixture of DNA from cells with different HIV-1 integration sites is used to account for differential amplification of proviruses integrated at different distances from an Alu element, and a correction factor is used to account for proviruses that are too far from an Alu sequence to be detected. When compared to droplet digital PCR for HIV-1 DNA in patients on suppressive ART, there was a strong correlation, indicating that the majority of HIV-1 DNA in ART patients is integrated [18]. This conclusion is further supported by additional studies showing that unintegrated forms of HIV-1 DNA are typically found in low levels in patients on suppressive ART [42,45,62]. Alternative methods for measuring integrated proviruses have also been developed and include linker ligation PCR [64] and inverse PCR [2].

Recently, two different research groups [65,66] have improved methods to study the specific integration sites of proviruses. Their findings show that a substantial fraction of proviruses (40% in one study) [65] are integrated into the genomes of cells that have undergone clonal expansion after infection. In one patient, approximately half of the infected cells contained a single viral clone with the same integration site [65]. In addition, integration sites were shown to be favored in genes that are associated with cancer [65,66]. These studies suggest that clonal expansion may occur as a result of integration into growth promoting genes [65], which will cause an expansion in the number of CD4⁺ T cells carrying HIV-1 proviruses. However, it remains unknown whether or not the proviruses present in these expanded clones are replication-competent. PCR-based assays will detect clonally expanded proviruses regardless of whether or not they are replication-competent, while culture-based assays will only detect outgrowth of replication-competent virus.

Total HIV-1 DNA PCR measurements can also detect other forms of unintegrated proviruses that are not a part of the LR. Two-long terminal repeat circles (2-LTR circles) result when integration fails and non-homologous end-joining (NHEJ) occurs between the two LTRs of linear, unintegrated HIV-1 DNA [67]. 1-LTR circles also are present during the course of HIV-1 infection and arise when homologous recombination occurs between linear HIV-1 DNAs at the LTRs [68]. This results in a circularized form of HIV-1 containing only a single LTR. Neither of these forms can integrate nor produce infectious virus. Although these forms are not considered to be part of the LR, 2-LTR circles have been used as a measure of recent infection or ongoing replication, based on the assumption

that 2-LTR circles are labile. However, the stability of 2-LTR circles remains controversial [43,69–71]. Several assays have been developed to study 2-LTR circles, including a droplet digital PCR assay on resting CD4⁺ T cells and PBMCs [18,55], as well as a qPCR assay on PBMCs [43,72,73] and CD4⁺ T cells [69]. These assays use primers flanking the 2-LTR circle junction. 1-LTR circles are more difficult to quantify using PCR [2,74,75]. It remains unclear how measurements of these replication-defective circular forms will contribute to our understanding of HIV-1 reservoirs.

It is important to understand the relationship between PCR-based assays for proviral DNA and other assays of the latent reservoir. As discussed above, PCR assays for HIV-1 proviruses give infected cell frequencies that are at least two logs higher than those obtained by the VOA [18]. This is due to the fact that PCR methods detect many defective proviruses as well as replication-competent proviruses. A recent study by Ho and colleagues [19] characterized INPs and found that almost 90% of these proviruses are defective and contain large internal deletions, APOBEC3G-induced hypermutations, or other inactivating mutations (Figure 1). Detection of these defective proviruses helps explain the overestimation of the LR size by PCR-based assays.

An additional concern with PCR-based assays is that they may fail to detect changes in LR size since most of the PCR signal is from defective proviruses that may respond differently to LRAs. Many defective proviruses may be unable to produce viral proteins even if latency is successfully reversed. Elimination of infected cells is likely dependent upon viral protein production, and thus cells containing defective proviruses may not be eliminated even by successful strategies. Thus, large numbers of cells with defective viruses could potentially mask successful clearance of latently infected cells by eradication strategies. In summary, the VOA underestimates the true size of the LR since one round of activation does not induce outgrowth of all replication-competent proviruses. On the other hand, PCR assays for HIV-1 DNA dramatically overestimate the LR since they cannot discriminate between defective and replication-competent proviruses.

Measuring residual viremia

While ART is effective in suppressing viremia to below the clinical limit of detection (50 copies of HIV-1 RNA/mL of plasma), HIV-1 RNA can still be detected at low levels in the plasma, indicating persistent residual viremia [76]. To measure residual viremia, Palmer and colleagues developed a highly sensitive single-copy assay (SCA). This assay can detect HIV-1 RNA down to one copy per mL of plasma [77–79]. The SCA is an RT-PCR assay that uses primers located in a conserved region of *gag* in the HIV-1 genome. It measures ongoing viral production from CD4⁺ T cells or from other stable reservoirs [80]. Another version of the SCA that uses a larger volume of plasma and primers located in a conserved region of integrase has recently been described [81].

Sequence analysis of residual viremia show that it is archival in character and sensitive to the current ART drugs [82,83]. These results suggested that residual viremia results from release of virus from stable reservoirs rather than ongoing viral replication. This hypothesis was substantiated in 2009 by the addition of a fourth drug to the three drug ART regimens of

patients who had stable suppression of viremia to below 50 copies/mL. This addition did not further reduce residual viremia [84] and further treatment intensification studies have confirmed this result [45,85–88]. Thus, residual viremia reflects virus production by a stable reservoir of cells infected prior to the initiation of therapy. In this sense, it can be used as a measure of viral reservoirs. However, the nature of the cells that produce the residual viremia is not yet clear. It is certainly possible that residual viremia results from the activation of a small fraction of the resting CD4⁺ T cells that constitute the latent reservoir. However, residual viremia in patients on ART is frequently oligoclonal [80,89]. Despite the enormous sequence diversification that occurs during HIV-1 infection, the residual viremia in many patients is dominated by a single clone, termed a predominant plasma clone (PPC). Proviruses with the same sequence can be found in resting CD4⁺ T cells in the blood, but there is not a 1:1 correspondence between plasma and cellular sequences [80,90]. This may reflect the large fraction of defective proviruses [19] or possibly the presence of an additional reservoir. The relationship between the PPC and the expanded clones of infected cells described above remains unclear. Thus, the relationship between residual viremia and the latent reservoir is complex. While the SCA is a useful tool for measuring viral persistence in the plasma, it may not accurately quantify changes in the LR in patients in eradication trials.

Treatment interruption and time to rebound as a measurement of latent reservoir size

Initially, following the introduction of ART, it was thought that only a few years of treatment would be sufficient to cure HIV-1 infection [91]. However, the discovery of the LR and its long-term stability indicates that patients must remain on ART for their entire lives to prevent a rebound in viremia [7], which typically occurs within a few weeks of stopping treatment [92]. As viral rebound reflects release of virus from stable reservoirs, there is a growing trend to evaluate reservoir reductions in HIV-1 eradication studies by stopping ART and observing the time it takes for viral rebound to occur [54,93–95]. This was done in the case of the two HIV-1 positive patients who received allogeneic stem cell transplants from CCR5-wild type donors while on ART. The transplants were done to treat Hodgkin's lymphoma in one case and diffuse large B-cell lymphoma and subsequent Hodgkin's lymphoma in the other case [95]. Although the patients (known as the 'Boston patients') had undetectable levels of HIV-1 DNA, both experienced a rebound of viremia several months after stopping ART [96]. In another example, a baby born to an HIV-positive mother (the 'Mississippi baby'), was given ART 30 hours after birth and, subsequently, viremia fell to undetectable levels and remained below the limit of detection. ART was discontinued against medical advice 18 months after initiation of treatment [97]. The baby showed no signs of virus in her blood following treatment cessation for over two years, but had a sudden rebound in viremia after 27 months off ART [98]. Interestingly, in all three cases, HIV-1-specific immune responses were absent. The long-term persistence of replication-competent HIV-1 in the absence of HIV-1-specific immune responses provides dramatic proof that the virus can persist *in vivo* in a latent form. These examples demonstrate that with a very small LR resulting from transplantation or early treatment, there is a substantial delay in the time to rebound.

Although treatment interruption is ultimately the only way to determine if a patient is cured, there are precautions and ethical issues to consider when using ‘time to rebound’ as a measurement of LR size. Drug resistance can occur if the interruption or subsequent reinitiation of ART is done in a way that allows suboptimal drug concentrations to be present for a significant period of time [99,100]. One of the Boston patients developed a new resistance mutation while restarting therapy [96]. The Strategies for Management of Antiretroviral Therapy (SMART) study, conducted in 2006, compared the effects of episodic treatment interruption to continuous ART and found that the patients who underwent treatment interruption experienced higher levels of morbidity, opportunistic diseases, and malignancies compared to those on continuous ART [101–104]. Additional difficulties with this approach include the enormous variability in time to rebound. If the LR is reduced significantly in size but not entirely eliminated, rebound could be governed by stochastic processes and may occur unpredictably months or years after treatment interruption [105]. Thus, treatment interruption is problematic in evaluating reservoir reductions.

Current measurements in eradication clinical trials

As LRAs are meant to induce transcription of the latent provirus, methods to detect changes in HIV-1 RNA levels are used to evaluate their efficacy in eradication studies. Methods using qPCR have been developed for measuring steady state HIV-1 RNA levels in PBMCs [44,51], resting CD4⁺ T cells [29], and in the GALT [18,51]. Droplet digital PCR has also been used to quantify HIV-1 RNA [106], and a recently described method detects polyadenylated HIV-1 mRNAs in resting CD4⁺ T cells [36]. Most studies also incorporate careful measurements of HIV-1 RNA in the plasma to detect transient increases in viral production. Recently, small increases in HIV-1 RNA in the detectable range have been documented in patients receiving the HDACi panobinostat [35]. In addition to determining whether or not a LRA has been effective in ‘shocking’ latently infected cells to produce viral RNA, it is also important to determine whether or not the infected cells have been ‘killed’ and eliminated from the reservoir [107]. In order to do this, an accurate measure of the LR before and after treatment(s) is necessary. Currently, the VOA provides the most definitive way to do this. To date, no significant reduction in the LR has been achieved by any ‘shock and kill’ strategy. Current and future eradication trials involve HDACis [32,35,108], gene therapies [54], and treatment intensifications [109]. Most current and future trials will measure LRA efficacy by decreases in the frequency of latently infected cells as measured by the VOA, increases in transient LRA-induced cell-associated HIV-1 RNA, or the appearance of plasma HIV-1 RNA. Some studies also measure total and integrated HIV-1 DNA or utilize a combination of multiple measures (Table 2). In interpreting the outcomes of these trials, it will be important to keep in mind the differences in what is actually measured by different assays of the LR.

Concluding remarks

Following the discovery of the LR in resting memory CD4⁺ T cells and its long-term stability, identifying ways to eliminate the LR has become a research priority. It is equally important to develop better ways to measure the LR [110], since current and future

eradication trials lack a universal measure to assess whether or not a decrease in the LR has been achieved. Although many assays have been developed to quantify the LR, there is little correlation between the methods [18] and none measure the true LR size as is shown in Figure 2. Additionally, there are also disadvantages to both PCR- and culture-based assays that need to be considered when measuring the LR or evaluating eradication therapies. The VOA is still considered the gold standard for measuring the LR since it provides a definitive minimal estimate of reservoir size. However it does not detect all latently infected cells that contain replication-competent virus [19]. Underestimating the size of the LR may give the false impression that a patient is ‘cured’ when the patient may still have latently infected cells. If taken off ART, a patient may experience a rebound in viremia, as was the case with the Boston patients [96] and the Mississippi baby [98]. PCR-based assays are also commonly used to quantify proviral DNA, but they cannot distinguish between replication-competent and defective proviruses. A failure to distinguish between the two would make it impossible to know when the LR has been successfully cleared and when or if patients can safely interrupt therapy. Treatment interruption is also used to measure a reduction in reservoir size; however, there are many concerns with this approach including increased risks for drug resistance and disease progression. Thus, there remains a need for assays that can measure the effects of therapeutic interventions on reservoir size without the need for treatment interruption. When applied to the ‘shock and kill’ strategy, neither culture- or PCR-based assays can adequately measure both the ‘shock’ and the ‘kill’ aspects. However, it may not be necessary to use the same assay to measure both the ‘shock’ and the ‘kill’. Certain assays may be better suited to measure each aspect. For instance, RNA-based culture assays may provide a rapid measure for the ‘shock’, but assays such as the VOA may be best suited for measuring the ‘kill’. In the absence of a universal assay, clinical trials are using a combination of culture- and PCR-based methods to identify and measure changes in the LR (Table 2). However, the conclusions drawn from these trials will vary depending on the measurements used. This consideration and other outstanding questions regarding measuring the latent reservoir are highlighted in Box 1. Without a high throughput, sensitive, and well-validated assay, it will remain difficult for researchers to identify novel LRAs, move forward with clinical trials, and develop strategies to eradicate HIV-1.

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Box 1**Outstanding questions**

- Can all intact proviruses be reactivated, and if not, what factors govern reactivation?
- If latency can be successfully reversed, will the immune system effectively ‘kill’ infected cells?
- In the shock and kill strategy, is it necessary to have the same assay measure both the shock and the kill, or can different assays be effectively used for each aspect?
- What qualities and features would be necessary in a universal assay to measure the true size of the latent reservoir?
- What magnitude of change in the latent reservoir can be accurately measured by the current assays and what degree of decrease will be required to substantially delay viral rebound?

Highlights

- A high throughput and well-validated assay is needed to accurately measure the HIV-1 latent reservoir.
- PCR-based assays overestimate the true size of the latent reservoir.
- Culture-based assays underestimate the size of the latent reservoir.
- No two HIV-1 eradication clinical trials use the same measurement.

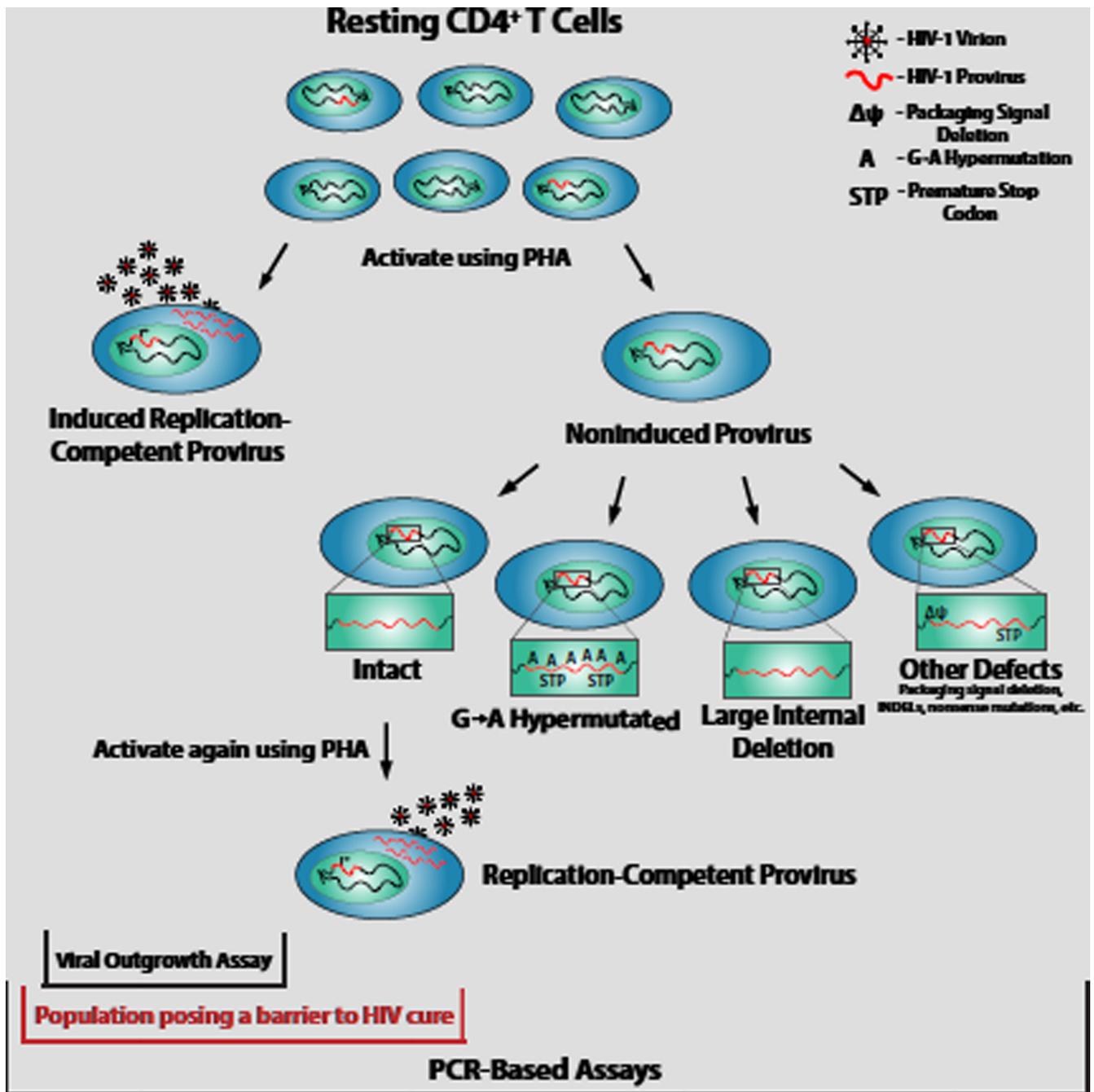


Figure 1. Profile of different types of HIV-1 proviruses in resting CD4⁺ T cells

The major types of proviruses are shown, including those that pose a barrier to an HIV-1 cure and those that do not. Following a single round of T cell activation, some proviruses are induced to produce virions, which can go on to infect other cells (induced, replication-competent proviruses). Proviruses that are not induced to produce replication-competent virions following a single round of T cell activation are termed noninduced proviruses. Many of these noninduced proviruses are defective and contain large internal deletions, G→A hypermutations or other inactivating defects. However, some noninduced proviruses

have fully intact genomes and, upon subsequent rounds of cellular activation, can produce virions. These proviruses are termed intact, noninduced proviruses (INPs). Culture-based assays detect only induced replication-competent proviruses, while PCR-based assays detect all types of proviruses. Only induced replication-competent proviruses and INPs pose a barrier to an HIV-1 cure.

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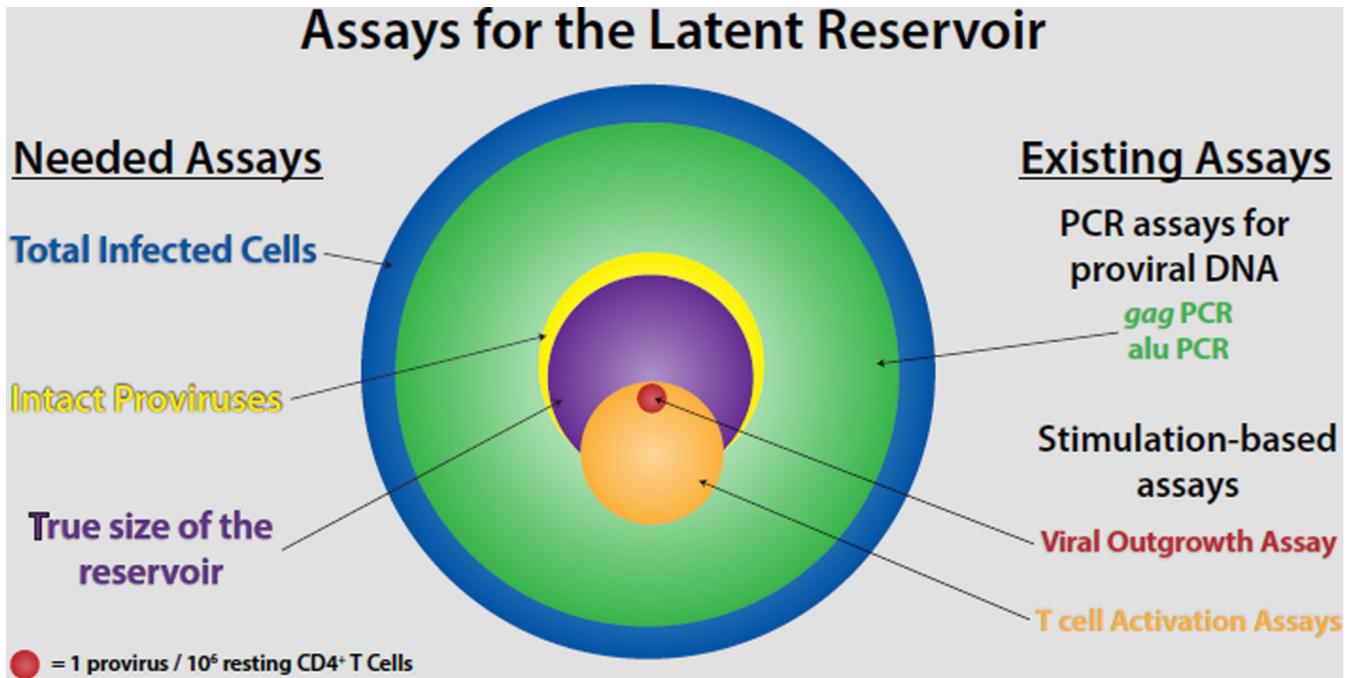


Figure 2. Venn diagram comparison of proviral populations measured by different methods for assessing the latent reservoir

Typical or estimated results from different PCR- and culture-based assays are shown relative to a prediction of the true latent reservoir size. The frequency of infected cells detected with different assays is represented by the area of each circle. PCR-based assays overestimate the LR since most of the templates amplified represent defective viruses [18]. Additionally, some proviruses are likely deleted in primer binding sites for the PCR so PCR-based assays likely underestimate the total number of infected cells. The VOA underestimates the LR size because not every replication-competent virus is induced by a single round of T cell activation [19]. Assays involving T cell activation with a viral RNA readout give intermediate values but suffer from both of the problems mentioned above; some of the viral RNA detected is derived from defective proviruses and not all of the replication-competent proviruses are induced by a single round of activation [37,39,40]. There is a need for assays that measure only those proviruses that pose a threat to an HIV-1 cure.

Table 1

Comparison of assays for measuring the HIV-1 latent reservoir

Assay	What it measures	What it excludes	Detection Method	Advantages	Disadvantages	Refs
Viral outgrowth assay (VOA)	Replication-competent virus induced by single round of T cell activation. Can in principle detect linear unintegrated HIV-1 DNA in recently infected cells. For this reason, the VOA is only useful as a measure of the LR in patients who have had prolonged suppression of viral replication on ART.	Defective proviruses, noninduced replication-competent proviruses (INPs), 1- and 2-LTR circles	p24 ELISA or RT-PCR	Allows for quantification of induced replication-competent proviruses without detection of defective proviruses	<ul style="list-style-type: none"> - Expensive and requires a large blood volume (120–180 mL) - Time consuming (requires 2–3 weeks tissue culture in a BSL3 laboratory) - Limited dynamic range because levels in most patient on ART are close to the limit of detection with the standard sample volume (180 mL blood) - Does not detect all proviruses that potentially pose a barrier to a cure 	[1,4,14,19,40]
T cell activation assays with viral RNA readout	Fraction of proviruses that can be induced to make either cell associated unspliced or multiply spliced HIV-1 RNA or full length HIV-1 mRNA released into the culture supernatant (presumably as virions)	Some defective proviruses, intact proviruses not induced to make HIV-1 RNA after a single round of activation (INPs)	RT-qPCR	<ul style="list-style-type: none"> - No need for growth of virus before measurement - Less culture time is required than the VOA 	<ul style="list-style-type: none"> - Requires 2–7 days of culture in a BSL3 laboratory - Does not detect all proviruses that potentially pose a barrier to a cure - May detect some defective proviruses 	[37,39,40]
qPCR for HIV-1 DNA	Total proviral DNA including replication-competent proviruses, and defective proviruses, and unintegrated forms of HIV-1 DNA	Proviruses with deletions in amplified regions	qPCR	Easy, quick, and does not require any extended culture time in a BSL3 laboratory	<ul style="list-style-type: none"> - Detects many proviruses that do not pose a barrier to an HIV-1 cure - Provides quantitation relative to a standard curve, but not an absolute value. Different qPCR assays use different standards 	[15,42–53]
Droplet digital PCR (ddPCR) for HIV-1 DNA	Total proviral DNA including replication-competent proviruses, and defective proviruses, and unintegrated forms of HIV-1 DNA	Proviruses with deletions in amplified regions	ddPCR	Highly precise; provides an absolute quantitation rather than a relative one	<ul style="list-style-type: none"> - Detects many proviruses that do not pose a barrier to an HIV-1 cure 	[17,18,54,55]
Alu PCR for Integrated HIV-1 DNA	Integrated proviral DNA	Unintegrated proviral DNA including 1- and 2-LTR circles, and linear unintegrated HIV-1 DNA	qPCR	Useful in measuring integrated proviruses in untreated patients, in which large amounts of unintegrated DNA can confound total proviral DNA measurements	<ul style="list-style-type: none"> - Does not detect proviruses that are too far from an Alu sequence to be amplified, but uses a correction factor to account for these; also detects defective proviruses 	[16,60–63]

Assay	What it measures	What it excludes	Detection Method	Advantages	Disadvantages	Refs
qPCR for 2-LTR circles	2-LTR circles	Integrated proviral DNA	qPCR	Used to measure ongoing replication based on the controversial assumption that the circles are labile	Does not detect integrated provirus, which is the only form that can contribute to latency	[43,69,72,73]
Droplet digital PCR for 2-LTR circles	2-LTR circles	Integrated proviral DNA	ddPCR	Highly precise; provides an absolute quantitation rather than a relative one; used to measure ongoing replication	Does not detect integrated provirus, which is the only form that can contribute to latency	[18,55]
Single-copy assay (SCA)	Residual viremia	Proviral DNA within cells or compartments	RT-PCR	Valuable as a measure of ongoing virus production in the setting of ART	<ul style="list-style-type: none"> - Time consuming and does not directly measure the LR as the residual viremia may also originate from other reservoirs. - Limited dynamic range because levels in most patients on ART are close to the limit of detection with the standard sample volume (8 ml plasma). 	[77-81]

Table 2

Measurements used in select recent and future HIV-1 eradication clinical trials

Name	Clinical Trial ID	Sponsor	Category	Measures	Estimated completion
Lisinopril	NCT01535235	University of California, San Francisco	Angiotensin-converting-enzyme inhibitor	Change in RNA in total CD4s and in GALT, and DNA in total CD4s and GALT	August 2014
Disulfiram	NCT01944371	University of California, San Francisco/Monash University/American Foundation for AIDS Research (amfAR)	Acetaldehyde dehydrogenase inhibitor	Cell-associated RNA in total and resting CD4s, plasma RNA by SCA, proviral DNA, mRNA expression	August 2014
Romidepsin	NCT01933594	AIDS Clinical Trials Group/National Institute of Allergy and Infectious Diseases (NIAID)/Gilead	HDAC inhibitor	Cell-associated RNA in total and resting CD4s, plasma RNA by SCA, total and 2-LTR DNA circles in resting and total CD4s	January 2015
GTU-multiHIV + LIPO-5	NCT01492985	French National Institute for Health and Medical Research-French National Agency for Research on AIDS and Viral Hepatitis (Inserm-ANRS)	DNA + lipopeptide vaccines	Plasma RNA by SCA during and after stopping treatment, and proviral DNA	January 2015
Vacc-4x + Romidepsin	NCT02092116	Bionor Immuno AS/Celgene	Vaccine + HDAC inhibitor, stopping HAART treatment	VOA, total and integrated DNA in CD4s, cell-associated unspliced RNA in CD4s, and plasma RNA	December 2015
CD4-ZETA +/- IL-2	NCT01013415	University of Pennsylvania	Modified T cells with or without IL-2	Plasma RNA by SCA, tissue RNA, frequency of latent replication-competent HIV-1 in PBMCs	December 2015
ULTRASTOP	NCT01876862	Objectif Recherche VACcin Sida (ORVACS)/Fondation Bettencourt Schueller	Highly active antiretroviral therapy (HAART) interruption	DNA in sorted CD4 subsets, plasma RNA by SCA, HIV-1 DNA in PBMCs and total CD4s, defective DNA sequences and presence of stop codons	December 2015
AGS-004	NCT02042248	University of North Carolina at Chapel Hill/Argos Therapeutics/U.S. National Institutes of Health (NIH)	Autologous dendritic cells and HIV strain	Plasma RNA by SCA and VOA	January 2016
Vorinostat	NCT01365065	Bayside/Merck	HDAC inhibitor	Plasma RNA by SCA and unspliced RNA in CD4s	March 2016

Name	Clinical Trial ID	Sponsor	Category	Measures	Estimated completion
BMS-936559	NCT02018510	NIAID	Anti-PDI antibody	Plasma RNA by SCA, total DNA, 2LTR circle DNA, cell-associated RNA, RNA/DNA ratios in total CD4s	April 2016
Peg-Interferon-a2b	NCT01935089	University of Pennsylvania	Cytokine	Alu-HIV <i>gag</i> PCR to detect change in HIV-1 DNA in total CD4s	July 2016
Poly-ICLC	NCT02071095	Campbell Foundation/Oncovir, Inc.	Toll-like receptor (TLR)-3 agonist	Cell-associated RNA, plasma RNA by SCA, and proviral DNA	June 2017
New Era Study	NCT00908544	MUC Research GmbH	2 Nucleoside Reverse Transcriptase Inhibitors (NRTI) + 1 protease inhibitor (PI)/Ritonavir + Maraviroc + Raltegravir	Cell-associated proviral DNA in PBMCs and total CD4s, plasma RNA by SCA	November 2019