New assays for monitoring residual HIV burden in effectively treated individuals

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**Purpose of review**
Measurements of HIV burden have relied upon quantification of viral nucleic acids by real-time PCR (qPCR). To develop and test strategies for eradication, new methods are needed to better characterize residual cellular reservoirs in patients on suppressive antiretroviral therapy (ART). This review summarizes recent advances that may lead to clinically useful tests with improved sensitivity, reproducibility and throughput.

**Recent Findings**
HIV DNA remains the most sensitive measure of residual infection, but its low levels are difficult to differentiate from assay noise by qPCR. Digital PCR has begun to improve the precision of existing real-time assays, but there remains a need to distinguish replication-competent proviruses. Rapid technological progress in single-cell analysis is beginning to offer new approaches, notably CyTOF and microengraving, which could provide vastly more information about the composition of the latent reservoir.

**Summary**
To investigate and assess therapies directed towards eradication, improved assays that simultaneously offer high sensitivity, precision and information content will be needed.

**Keywords**
cytometry via time of flight, droplet digital PCR, digital PCR, eradication, HIV DNA, HIV latency, microengraving, single-cell analysis

**INTRODUCTION**
The increasing interest in research towards eradication of HIV has emphasized the need for new assays to monitor persistent disease burden in infected patients on effective combination antiretroviral therapy. Currently available assays offer high sensitivity or specificity, but not both. Assays that measure various forms of HIV DNA are typically the most sensitive, but these fail to distinguish defective proviral genomes or other replication-incompetent forms. Terminal-dilution coculture assays\cite{1–3} of replication-competent virus consistently detect only one infectious unit per hundreds of HIV proviral genomes\cite{4,*}, suggesting that these laborious assays might have a sensitivity of 1% or less. The basis for this discrepancy remains unclear. For effective monitoring in clinical trials, a single assay or a validated suite of assays should be cost-effective, should provide simultaneously high sensitivity and specificity and must be highly reproducible both within and between laboratories. No assay can overcome the limited sampling of perhaps 10\textsuperscript{6} latently infected cells\cite{5} diluted throughout the body that one specimen provides, but procedures that can be applied to a variety of specimen types may be particularly valuable for eradication studies.

No assay meeting all these criteria currently exists. Recent efforts to quantitatively compare different assays help clarify the current challenge. Eriksson et al.\cite{4,*} analysed samples from patients on suppressive ART in parallel by several methods. The results of different types of assays correlated poorly. On average, hundreds of copies of total and integrated HIV DNA were detected per replication-competent latently infected cell. This is consistent with prior results\cite{1} but clearly inconsistent with the recent demonstration that cells containing even two integrated proviruses are rare \textit{in vivo}\cite{6,*}. Yukl et al.\cite{7,*} used a similar panel of assays to look

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for evidence of residual infection in the ‘Berlin patient’ and concluded that many popular methods based on PCR are prone to contamination at levels that cloud interpretation of this putative eradication.

Quantitative assays currently used in translational studies measure the total size of the latent cell pool, despite the fact that this cell population is known to be heterogeneous with regard to cell-surface phenotype [8], antigen specificity [9], response to treatment, clearance dynamics [10] and anatomic compartment. As efforts to improve well established assays continue, rapid technological advance in nucleic acid sequencing and in single-cell analysis have begun to open new approaches. Highly multiplexed characterization of lymphocytes (cytometry via time of flight, CyTOF) [11,12] and massively parallel culture of individual lymphocytes [13–15,16] have been used to characterize subtle changes in dynamic properties of lymphocytes in HIV-infected patients. These rapidly advancing methods may soon reshape how latency is characterized both experimentally and conceptually.

QUANTIFICATION OF HIV RNA AND HIV DNA

For patients achieving suppression of viraemia, HIV nucleic acids associated with CD4⁺ T lymphocytes generally offer the most sensitive marker of residual disease burden. Although HIV RNA is measured most frequently, its value is limited in most patients who have achieved virologic suppression. Although residual viraemia can be detected below the standard limit of detection (50 copies/ml or less), the source and significance of this residual viraemia are unclear. In addition to these biological considerations, an often overlooked limitation to these assays is the high signal-to-noise ratio near the limit of detection (Fig. 1). Ultracentrifugation of large volumes in assays improves the limit of detection [17] to as low as 1 copy/ml [18], but the accuracy and precision remain limited by intrinsic assay characteristics.

DIGITAL PCR

In contrast, HIV DNA can be readily detected in the vast majority of patients, even after many years of ART. This includes total HIV DNA, typically measured using PCR primers and probes in HIV pol or gag, and integrated or episomal forms. Because there is no clinically approved assay for HIV DNA, methods are not standardized between laboratories. Real-time PCR has been used most frequently, but this approach limits assay accuracy by exponentially amplifying noise.

Digital PCR has recently been proposed as an alternative to real-time with potentially improved accuracy and precision [19]. In this method, each sample is divided into thousands of independent microscopic reactions prior to PCR amplification. Commercially available devices achieve this micro-partitioning in different ways; emulsification into droplets is shown in Fig. 2. The concentration of the target template is determined by counting the number of partitioned droplets with positive endpoint fluorescence (Fig. 2). Digital PCR provides an increased precision of four-fold to over 20-fold versus real-time [20], using identical quantities of clinical samples from peripheral blood.
If the number of HIV copies per assayed sample is sufficiently small, ‘digital’ PCR can effectively be performed in a 96-well plate. This approach has been painstakingly applied by Yu et al. [21] to quantify integrated HIV DNA. In effect, this is a low-throughput implementation of digital PCR. Recently, it was extended to measure integrated HIV DNA in parallel with either 2-long terminal repeat (LTR) circles [22] or total HIV DNA [23]. The only study [4] that has compared this assay with droplet digital PCR (ddPCR) found a remarkably strong correlation ($R = 0.8$) between the two in patients on suppressive antiretroviral therapy for at least 1 year. Thus, these two new assays for HIV DNA may provide equivalent information for eradication studies.

### SINGLE-CELL ANALYSIS

Latently infected cells are highly heterogeneous and very scarce. Even the most sensitive assays detect only several hundred infected cells in a typical peripheral blood sample. It is therefore both desirable and potentially feasible to analyse this small cell population extensively, but first, the cells must be identified by screening millions of lymphocytes.

Flow cytometry can achieve adequate throughput, but there is no published method to identify infected cells. One assay in development detects induced intracellular expression of p24 antigen [24]. As for any single-cell analysis, the limit of detection is constrained by the number of cells that can be practically analysed. The high throughput of modern flow cytometers makes it feasible to analyse tens of millions of cells, and thus to detect induced p24 expression at levels below one per million lymphocytes.

Many alternative approaches to high-throughput single-cell analysis are now available. Among these, CyTOF and microengraving have already been used to characterize primary lymphocytes in unprecedented detail and are particularly promising for the near future. CyTOF couples flow cytometry, which analyses single cells, with time-of-flight (TOF) mass spectrometry, which analyses single molecules [11,25]. In this method, detection antibodies are conjugated to stable isotopes of transition metals, instead of fluorescent labels. This allows multiplexed detection and quantification of over 30 different molecules without the complexity of fluorescence compensation needed for multiparameter flow cytometry [25].

CyTOF has been used to characterize leucocyte phenotype in terms of over 30 different cellular proteins measured simultaneously [12,25]. Although CyTOF requires cell destruction for analysis, it has been used to characterize cell function by simultaneously measuring phosphorylated and unphosphorylated forms of key proteins in signal transduction pathways [26]. This analysis of intracellular phosphorylation was sufficient to classify cells, that is to predict the cell surface phenotype. Whether similar changes distinguish latently infected cells is not yet clear, but CyTOF provides a method to identify any such signatures.

Microengraving involves simultaneous culture of single lymphocytes in an array of subnanolitre wells [27,28]. Secreted molecules are captured on an antibody-functionalized surface, which can be periodically replaced to measure secretion kinetics [29]. Medium can also be changed, allowing the responses of individual lymphocytes to different stimuli to be monitored for up to 2 weeks. After analysis of the capture surface, cells of special

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**FIGURE 2.** Droplet digital PCR. (a) The PCR mixture, which includes the target template and fluorescent hydrolysis probe, is emulsified into approximately 15 000 droplets. After thermal cycling, the number of fluorescent droplets is counted. The initial template concentration is then calculated, assuming random partitioning. (b) Replicate assay wells are plotted against one another to compare the precision of a digital PCR assay for HIV pol with an identical real-time PCR assay. For clinical samples (circles), the coefficient of variation was four-fold lower, on average. A larger reduction in the C.V., >20-fold, was seen for a 2-LTR assay in the same samples. C.V., coefficient of variation.
interest can be selectively recovered from an array [30]. This technology has recently been used for high-throughput quantitative analysis of individual primary T lymphocytes by measuring cytokine production kinetics [13,14,29,31], and by relating cytokine production to the cytolytic activity of both CD8+ T [14] and natural killer (NK) cells [32]. Nucleic acids can be amplified and detected by performing single-cell ‘digital’ PCR within the culture array. Microengraving has been used to clonally expand HIV-specific CD8+ T lymphocytes with high throughput [16*], but it might instead be used to characterize cellular reservoirs of HIV infection. Other microfluidic culture systems have the potential to provide similar information [33], but none of these has yet achieved the practicality and experimental throughput demonstrated in these recent microengraving studies.

Microengraving and CyTOF offer the obvious prospect of characterizing the diverse latently infected cell population, but it is also noteworthy that single-cell analysis inherently provides quantitative measurements of cell populations. Regardless of the parameters measured, any method that identifies latently infected cells individually would also provide a count of their number. These techniques should therefore measure the size of the latent reservoir with the same precision and accuracy as ddPCR, while providing enormous additional detail about the heterogeneity of this population. If the response of latently infected cells to proposed adjunctive therapies is as heterogeneous as the cellular phenotypes suggest, this additional information may prove essential for monitoring and evaluation of eradication studies.

CONCLUSION

Studies of HIV eradication will require assays capable of precisely quantifying changes in cellular reservoirs. Existing assays for total HIV DNA may greatly overestimate the amount of infectious virus, whereas current assays for replication-competent virus likely lack sensitivity and are clearly too noisy and too expensive for use in large clinical trials. Digital PCR improves the accuracy and precision of HIV DNA measurement over real-time PCR, and this general approach should be applicable to any quantification of any nucleic acid. Rich characterization of the latent reservoir by single-cell analysis methods such as CyTOF and microengraving may reveal new details about its heterogeneous composition. For the foreseeable future, a panel of assays will likely be necessary to provide sufficient sensitivity, precision and information for clinical studies of eradication.

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Conflicts of interest

None declared.

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

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

4. Eriksson S, Graf EH, Dahl V, et al. Comparative analysis of measures of viral reservoirs in HIV-1 eradication studies. PLoS Pathogens 2013 [in press]. This study performed the first comprehensive comparison of assays for HIV RNA, HIV DNA and infectious cell number. Twenty chronic and 10 acute patients were analysed in parallel. Overall, the three endpoints were very weakly correlated.
6. Josefsson L, King MS, Makitalo B, et al. Majority of CD4+ T cells from peripheral blood of HIV-1-infected individuals contain only one HIV DNA molecule. Proc Natl Acad Sci U S A 2011; 108:11199–11204. This study used painstaking sequential serial dilution of cells and cellular DNA to determine the number of copies of HIV DNA per infected cell. The estimated frequency of cells harbouring multiple copies of HIV DNA (reverse transcriptase) was less than 1% in all patients analysed. Technological advances should soon allow similar single-cell analyses with much higher throughput.

In this study, several different laboratories applied a panel of assays to analyse samples from the ‘Berlin patient’ for evidence of residual HIV infection. Residual nucleic acids were detected in a few peripheral blood samples, but the inconsistency of these results highlighted the need for rigorous, quantitative negative controls. Consistent evidence of residual infection was found in gut-associated lymphoid tissue.

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Strain and Richman


This study used microengraving to simultaneously analyse cytolytic activity and cytokine secretion by HIV-specific CD8^+ T lymphocytes. It illustrates the power of this platform and provides a convincing challenge to immunologic dogma about cytotoxic lymphocytes.


The performance of ddPCR is analysed and compared with real-time PCR for quantification of HIV pol and 2-LTR junctions. Digital PCR is consistently more precise, especially for 2-LTR circles.


