

Comparison of methods to quantify inducible HIV-1 outgrowth

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ABSTRACT

The quantitative viral outgrowth assay (qVOA) is the gold standard for measuring inducible, replication-competent HIV-1. Using MOLT4-R5 and SupT1-R5 cell lines instead of allogeneic blasts and HIV-1 RNA detection rather than p24 enzyme-immunoassay (EIA) has been proposed to improve the sensitivity of the qVOA. It is unclear, however, how these alternative approaches affect qVOA performance. We compared three qVOA methods across 15 persons with HIV-1 on suppressive antiretroviral therapy and found that the MOLT4-R5 method yielded a significantly higher proportion of p24-positive wells (42%) than both the allogeneic blast (29%) and SupT1-R5 (32%) assays. Additionally, 5 of 7 qVOAs that were negative by p24 EIA showed viral outgrowth by HIV-1 RNA quantification (>10-fold increase within 7 days). These findings reveal the potential for underestimation of the latent, inducible reservoir by qVOA depending on the target cells used and the measure of viral outgrowth. Use of MOLT4-R5 cells with both p24 EIA and HIV-1 RNA to detect viral outgrowth was the most sensitive method.

Introduction

HIV-1 infection has not been curable because of the persistence of a long-lived reservoir of cells carrying replication-competent proviruses. Quantifying the size of this reservoir is essential to measuring the impact of curative efforts. Various techniques have been used to measure the reservoir, including quantitative PCR (qPCR) assays for total or “sequence intact” proviral DNA, and culture-based quantitative assays of inducible viral outgrowth (qVOA).

The qVOA has been a traditional gold standard for estimating the size of the inducible, replication-competent HIV-1 reservoir. By stimulating purified, serially-diluted CD4⁺ T-cells from persons with HIV-1 in an environment conducive to viral outgrowth, qVOAs are capable of estimating the frequency of infection of host cells with inducible, replication-competent virus. However, the incomplete reversal of latent proviruses by single or even multiple stimulations within a qVOA renders it an underestimation.^{1,2} Although other methods to quantify the size of the reservoir by measuring sequence intact proviruses as opposed

to *in vitro* inducibility have emerged,^{3,4} the qVOA still remains relevant in its ability to detect inducible, replication-competent virus that is devoid of known or unknown lethal mutations. Specifically, PCR- or sequenced-based assays cannot prove replication-competence or inducibility of proviruses, whereas qVOA can establish both. Further, sequence analysis of replication-competent virus that grows in culture can be used to identify donor-specific attributes of the inducible HIV-1 reservoir, such as the clonality of cells carrying inducible, replication-competent provirus.^{5–7}

Traditional qVOA methods that include serial dilutions of CD4⁺ T-cells, stimulation with gamma-irradiated peripheral blood mononuclear cells (PBMCs), and amplification in allogeneic T-cell blasts from healthy donors are costly and time-consuming. The assay requires weekly addition of HIV-negative allogeneic blasts, which limits the capacity of most laboratories to run qVOAs. Efforts to improve the standardization of this assay by stimulation with CD3/28 beads and amplification of viral outgrowth through cell lines permissive to HIV-1 replication have been evaluated.^{8–10} In fact, multiple cell lines, such as the genetically

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engineered MOLT4-R5 and SupT1-R5 cell lines, have separately demonstrated similar sensitivity compared to allogeneic blasts,^{10,11} but comparison across these different methods has not been assessed.

Measuring p24 by standard EIA on days 14 or 21 of qVOA is the most common method to detect viral outgrowth. Detection of HIV-1 RNA by qRT-PCR on day 7 of qVOA has been shown to correlate with p24 detection on day 14.¹² Because traditional qVOA methods provide a minimal estimation of the size of the viral reservoir, it remains an important goal to improve the sensitivity of qVOAs. One approach shown to yield higher infectious units per million (IUPM) cells compared to p24 EIA is quantitation of HIV-1 RNA in culture supernatant using qRT-PCR.¹³ The finding that quantitation of HIV-1 RNA increases sensitivity implies that viral outgrowth may be occurring in otherwise p24-negative cultures.

In the current study, we sought to determine which target cell population (MOLT4-R5, SupT1-R5, or allogeneic blasts) was most sensitive for detecting inducible viral outgrowth. We also investigated whether qVOA cultures that were negative for p24 would show evidence of viral outgrowth detectable as logarithmic increases in HIV-1 RNA in culture supernatants. We hypothesized that using cell lines for targets and detecting viral outgrowth by HIV-1 RNA would enhance qVOA sensitivity.

Materials and methods

Participants

All donors were on long-term (>5 years) antiretroviral therapy (ART). Participants 1 through 3 are from a local cohort at the University of Pittsburgh Medical Center (UPMC) HIV-AIDS Program and participants 4 through 15 are from the AIDS Clinical Trials Group (ACTG) HIV Reservoir Cohort A5321/41s. The first three donors were included because they were previously shown to have infectious unit per million (IUPM) values > 1.0 using allogeneic blasts. Both sets of donors were from studies approved by Institutional Review Boards and all donors gave written informed consent.

Isolation of total CD4⁺ T-cells

PBMCs that were isolated from leukapheresis product by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare) were cryopreserved. Cells were later thawed, washed with PBS +2% FBS (StemCell), and enriched for CD4⁺ T-cells by negative selection (Easy-Sep, Stemcell).

Quantitative viral outgrowth assays

qVOA protocols were adapted from published literature.^{10,11} For all three assays, HIV + CD4⁺ T-cells were suspended in Super T-cell Media (STCM),^{10,15} serially diluted from 1.0×10^6 cells/mL to 0.3×10^6 cells/mL and 0.1×10^6 cells/mL, and plated in up to 6 wells per dilution depending on available cell yield. Cultures were stimulated with 10-fold gamma-irradiated PBMCs ("feeder" cells) and 1 µg/mL PHA (Remel) from an HIV-negative participant on the first day of the assay and washed on the second day before addition of target cells¹⁵ (Fig. 1).

Allogeneic blasts were prepared from PHA-activated PBMCs obtained from an HIV-negative donor for addition as target cells.¹⁰ 4×10^6 blasts were added to the 1×10^6 CD4⁺ cells/mL dilution, and 1×10^6 blasts were added to the 0.3×10^6 cells/mL and 0.1×10^6 cells/mL dilutions. On days 7 and 14 of the culture, half of the cells and media were discarded from each qVOA well and replaced with additional similarly prepared allogeneic blasts.

MOLT4-R5 cells were prepared and added once to culture wells in accordance to published methods.¹⁰ 4×10^6 MOLT4-R5 targets were added to the 1×10^6 CD4⁺ cells/mL dilution, and 1×10^6 targets were added to the 0.3×10^6 cells/mL and 0.1×10^6 CD4⁺ cells/mL dilutions. To extend the assay from 14 to 21 days, day 5 culture splitting was repeated on days 12 and 19, and day 9 culture splitting was repeated on day 16.

SupT1-R5 cells were prepared, added once to culture wells, and split in accordance to published methods.¹¹ 1×10^6 SupT1-R5 targets were added to the 1×10^6 CD4⁺ cells/mL dilution, and 0.5×10^6 targets were added to the 0.3×10^6 cells/mL and 0.1×10^6 cells/mL dilutions. 24-well plates instead of 12-well plates were used for CD4⁺ T-cell dilutions higher than 1×10^6 cells/mL.

Calculation of IUPM using p24 EIA

An Alliance HIV-1 EIA Kit (PerkinElmer) and SpectraMax i3x (Molecular Devices) were used to detect p24 capsid protein. Absorbance values above three-fold the average media control absorbance were designated as p24-positive. Maximum likelihood statistics were used to calculate infection frequency as IUPM CD4⁺ T-cells.¹⁶

Calculation of IUPM using HIV-1 RNA

HIV-1 RNA was measured in p24-negative supernatant using the HIV-1 Aptima kit and Hologic Panther system. Supernatants were tested from 12 of the 15 donors; the remaining 3 donors had no p24-negative

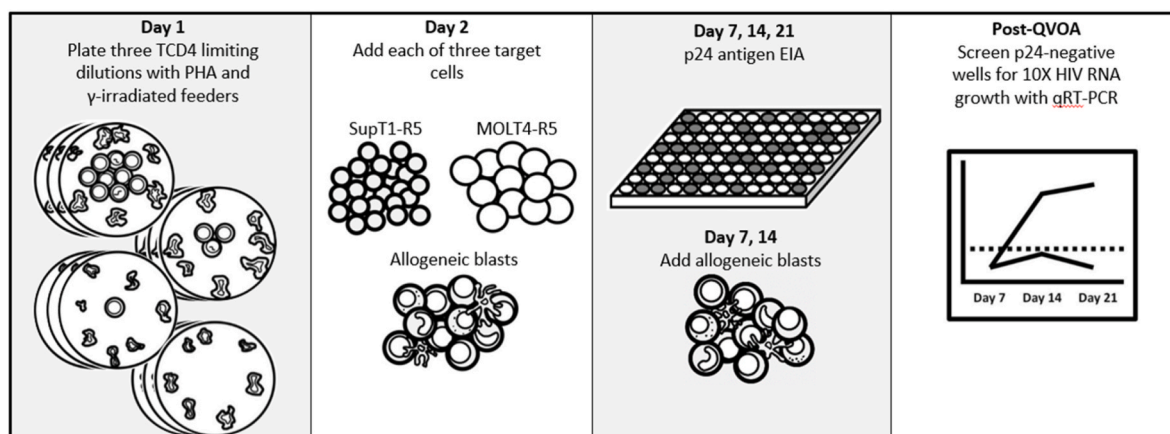


Fig. 1. Triplicate qVOA Set-up and Workflow. Total CD4⁺ T-cells were prepared in a limiting dilution in the presence of PHA and gamma-irradiated feeders on Day 1 before addition of targets cells (PHA-activated allogeneic blasts, SupT1-R5 cells, or MOLT4-R5 cells) on Day 2. Allogeneic blasts were replenished on Days 7 and 14, and p24 measured on Days 7, 14, and 21. Day 21 p24-negative wells were screened for quantitative HIV-1 RNA with qRT-PCR, from which Day 7 and 14 samples were quantified to measure RNA kinetics.

wells or the samples were lost during a transient platform malfunction. Samples were diluted to a volume of 700 μ L with the Specimen Diluent (Hologic) and tested once. Wells positive for quantitative HIV-1 RNA on day 21 were similarly tested from day 7 and 14 time points of the qVOA. Individual wells were designated as "RNA-positive" after screening for an increase in HIV-1 RNA copies/mL of at least 10-fold over a 7-day period and² the absence of HIV-1 RNA copies/mL decrease over 21 days. Such wells were added to p24-positive wells to re-calculate IUPMs using the online calculator.¹⁶

Statistics

Pearson's coefficient was used to calculate all correlations. The differences between IUPMs were compared using Wilcoxon matched-pairs signed rank tests. The differences between overall proportions of positive wells were compared using two-tailed paired T-tests. All values below the lower limit of detection (LoD) or lower limit of quantitation (LLOQ) were assigned values of $\frac{1}{2}$ of the limit.

Results

MOLT4-R5, SupT1-R5, and allogeneic blast assays provide comparable IUPMs when measured by p24-antigen EIA

A summary of demographic, immunologic, and virologic characteristics of the 15 participants can be seen in Table 1. All participants were on suppressive antiretroviral therapy (ART) for at least 5 years prior to this study (median 9 years), with a median plasma HIV-1 RNA of <50 cps/mL at entry. The median CD4⁺ T-cell count was 774 cells/mm³. The ACTG A5321 participants 4–15 had sustained and well-documented HIV-1 RNA values < 50 copies/mL at all time points at or after week 48 of ART. Cell-associated integrase HIV-1 DNA (CAD) and RNA (CAR) were measured for participants 4–15; median values were 735 copies/million PBMC and 114.1 copies/million PBMC, respectively.¹⁴ The median intact HIV-1 provirus was 27.6 copies per million PBMC.⁴

Because Day 21 IUPMs were significantly higher than those measured on Day 14 for all three assays (Wilcoxon test, $p < 0.005$),

IUPMs from Day 21 were used as the primary comparative endpoint. Fig. 2A shows Day 21 IUPM values obtained from each of the three different cell types used for qVOA (allogeneic blasts [Standard]; MOLTR-R5; SupT1-R5). Of the forty-five qVOAs performed, ten assays were below the limit of detection (four Standard, three MOLTR-R5, three SupT1-R5), and five were detected above the upper limit of quantitation (one Standard, three MOLTR-R5, one SupT1-R5). There were no significant differences in IUPMs obtained by detection of p24-antigen alone on Day 21 for the three different cell types (Wilcoxon test, $p > 0.05$). Strong correlations (Fig. 2B) were seen between the allogeneic blast and MOLTR-R5 protocol (Pearson's correlation coefficient, $r = 0.6066$, $p < 0.05$), between the blast and the SupT1-R5 protocol ($r = 0.8860$, $p < 0.0001$), and between both cell line protocols ($r = 0.7972$, $p < 0.001$).

To verify that the virus in the qVOAs was replicating, the kinetics of p24 EIA readouts were assessed. Figure S1 shows the growth in p24 over time in qVOAs marked as p24-positive or below the limit of detection on day 21. All positive qVOAs showed increases in p24 levels from day 7 to values exceeding 2 pg/mL by day 21, indicative of viral replication.

MOLT4-R5 assay provides significantly higher proportion of positive wells compared to blast and SupT1-R5 assays when measured by p24-antigen EIA.

In addition to comparing inter-assay IUPMs, overall proportions of p24-positive wells were examined. Higher proportions of positive qVOA wells would indicate increased sensitivity for viral outgrowth and would also provide a larger number of wells from which further characterization of infectious virus could occur. Fig. 3 illustrates that at each culture time point, the proportion of wells with p24-detectable virus in the allogeneic blast and SupT1-R5 protocols were not significantly different from each other (two-tailed paired *t*-test, $p > 0.05$). Both the standard and the SupT1-R5 protocols tended to amplify infectious HIV-1 in a higher number of qVOA wells than the MOLTR-R5 protocol on day 7 of the cultures. However, by days 14 and 21, the MOLTR-R5 protocol amplified virus in a significantly higher number of qVOA wells than the standard and SupT1-R5 protocols ($p < 0.05$).

Table 1

Participant demographics and characteristics.

Participant	Age	Sex	Race	Years on ART	Current CD4 ⁺ T cell count (cells/mm ³)	Current HIV-1 RNA (cps/mL) ^a	CAR ^b cps/M PBMC ¹⁴	CAD ^c cps/M PBMC ¹⁴	IPD ^d per 1 M cells ⁴	Current ARV regimen
1	73	Male	White	10	380	48	N/A	N/A	N/A	FTC TDF EFV
2	59	Male	Black	19	1023	TND	N/A	N/A	N/A	ABC 3 TC EFV
3	43	Male	White	9	416	TND	N/A	N/A	N/A	FTC TDF RAL
4	58	Male	White	9.4	672	<40	14.4	165.2	148.6	FTC TDF EFV
5	48	Male	Hispanic	6.7	957	<40	2.0	158.2	71.1	ABC 3 TC DTG
6	48	Male	Black	12.2	523	<40	26.5	291.6	181.5	FTC TDF EFV
7	44	Female	Hispanic	9	1017	<40	<X	26.4	27.8	FTC TDF EFV
8	59	Male	White	8.9	642	<40	1.3	12.3	27.4	FTC TDF RTV ATV
9	49	Male	Black	17.4	1117	<40	74.0	529.6	211.6	FTC TDF EFV
10	71	Female	White	7.1	573	<40	<X	70.0	0.0	FTC TDF RAL
11	40	Male	Hispanic	8.9	1013	<40	10.1	38.4	18.5	FTC TDF EFV
12	53	Male	White	5.2	1092	<40	1.4	31.3	1.9	FTC TDF RPV
13	56	Male	White	7.5	577	<40	4.8	43.9	16.9	ABC 3 TC RTV DRV
14	38	Male	White	10.6	876	<40	9.9	196.6	40.1	RTV ATV ABC 3 TC TAF
15	51	Male	White	13.9	1098	<40	51.4	178.7	0.0	ABC 3 TC DTG

TND, target not detected; N/A, not available.

3 TC, lamivudine; ABC, abacavir; ATV, atazanavir; DTG, dolutegravir; DRV, darunavir; EFV, efavirenz; FTC, emtricitabine; RAL, raltegravir; RPV, rilpivirine; RTV, ritonavir; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate.

^a HIV-1 RNA (cps/mL) measured by COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test for participants 1–3; HIV-1 RNA (cps/mL) measured by Abbott RealTime HIV-1 Viral Load assay for participants 4–15.

^b CAR, Cell-associated integrase HIV-1 RNA.

^c CAD, Cell-associated integrase HIV-1 DNA.

^d IPD, Intact HIV-1 Proviral DNA.

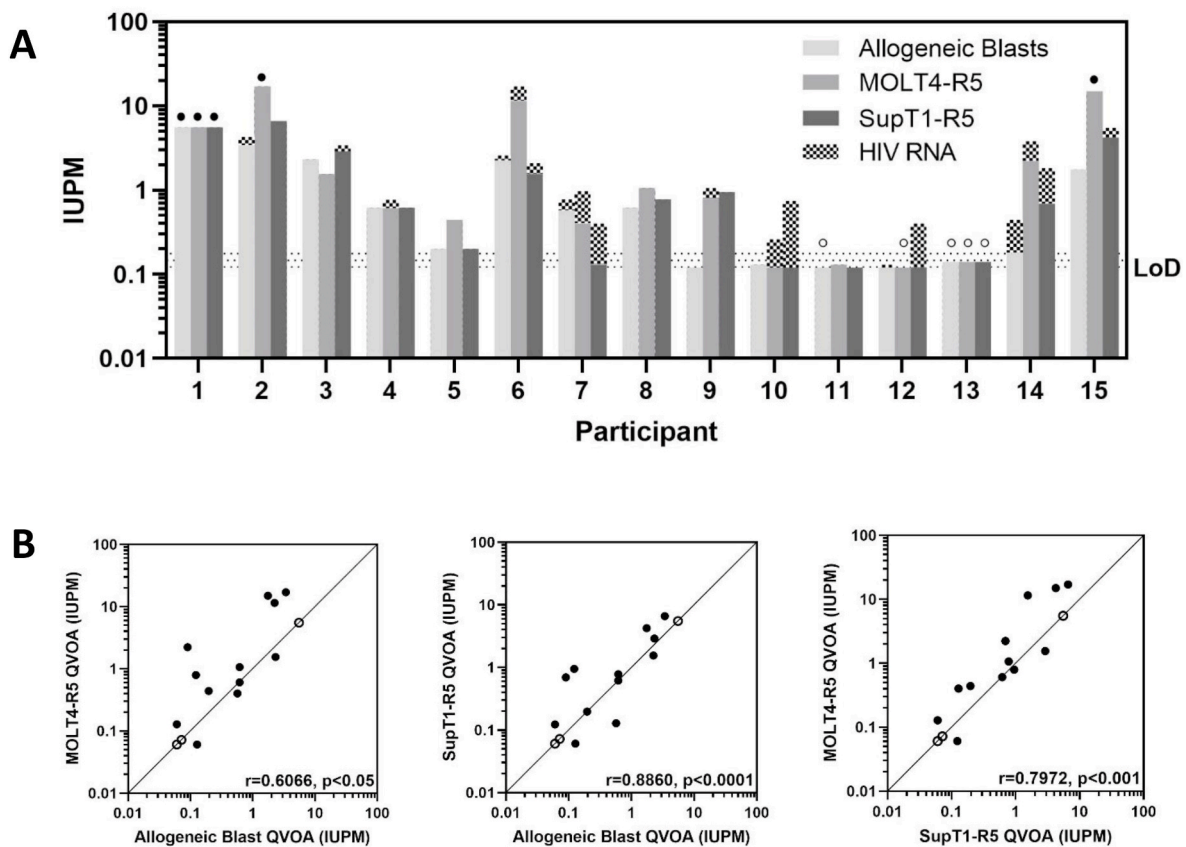


Fig. 2. Day 21 IUPMs from allogeneic blast, MOLT4-R5, and SupT1-R5 qVOAs. (A) Day 21 IUPMs across 15 participants calculated by p24 EIA from allogeneic blast (light gray), MOLT4-R5 (medium gray), and SupT1-R5 (dark gray) assays. Assays with no positive wells (open circle) or all positive wells (closed circles) are indicated. (B) Pearson's correlation coefficient r was calculated between each pair of protocols. White circles indicate when both assays had an IUPM below the LoD or above the upper limit of quantitation.

Isothermal amplification of HIV-1 can detect viral growth not detected by p24 EIA

We explored the possibility that some qVOA wells had HIV-1 outgrowth that was undetectable by p24 EIA, an observation made elsewhere to achieve faster and more sensitive quantitation methods.^{12,13,17} To minimize misclassification of viral expression without replication, 10-fold increases in HIV-1 RNA cps/mL over a 7-day period were used as a requirement for evidence of viral growth.

Of 33 qVOAs assessed, 19 contained at least one RNA-positive p24-negative well. Of 390 p24-negative wells, 101 (26%) had detectable HIV-1 RNA and 32 (8%) were designated as RNA-positive by their kinetics (Figure S2). 6 of these 32 wells were from the allogeneic blast assay, 9 from the MOLT4 assay, and 17 from the SupT1 assay. Overall, 10-fold RNA growth was detected in 5 of 7 qVOAs with IUPMs that were below the limit of detection by p24 EIA alone.

HIV-1 outgrowth detected by HIV-1 RNA suggests MOLT4-R5 and SupT1-R5 cell lines are more sensitive for IUPM detection than allogeneic blasts.

Wells that were p24-negative but showed evidence of HIV-1 outgrowth by HIV-1 RNA were classified as positive and IUPMs were recalculated and can be seen in Fig. 2A. Incorporation of RNA-positive wells revealed significantly higher IUPMs in assays performed with MOLT4-R5 (Wilcoxon test, $p < 0.01$) and SupT1-R5 ($p < 0.05$) cell lines compared with the allogeneic blast protocol.

Day 21 proportions of qVOA wells positive by p24 were also appended with RNA-positive outgrowth wells (Fig. 3). The proportion of qVOA positive wells in the standard assay increased from 29% to 31%, in the MOLT4-R5 assay from 42% to 46%, and in the SupT1-R5 assay

from 32% to 39%. After incorporation of HIV-1 RNA, both the SupT1-R5 and the MOLT4-R5 assays had significantly higher overall proportions of positive wells than the allogeneic blast assay (paired T-test, $p < 0.05$ and $p < 0.01$, respectively).

qVOA weakly correlates with the intact proviral DNA assay (IPDA)

Because the qVOA is widely understood to provide only a minimum estimate of the size of the reservoir due to incomplete induction, extinction events, or failure to reach a critical threshold for growth in cell culture,¹⁸ it is of great interest to examine the extent to which our qVOA dataset aligns with methods designed to detect more intact proviruses. Accordingly, IUPMs from the 12 ACTG participants across all three cell types used for qVOA protocols were compared to IPDA and total cell-associated integrase HIV-1 DNA (CAD) in cells from the same collection time point.

Correlations between IPDA and qVOA are seen in Fig. 4. Initial statistical correlations suggested little to no relationship between IPDA and qVOAs using allogeneic blasts (Pearson's $r = 0.2870$, $p = 0.3657$), MOLT4-R5 ($r = 0.1188$, $p = 0.7131$), and SupT1-R5 cells ($r = 0.05856$, $p = 0.8565$). However, among the 12 participants, one had an IPDA value of 0 despite having detectable IUPMs in each qVOA assay. It is likely this false-negative IPDA measurement is caused by primer/probe mismatches, resulting in amplification failure.¹⁹ After removing this outlier from the comparisons in Fig. 4, the IPDA was found to have a modest but not significant correlation with qVOAs in allogeneic blasts (Pearson's $r = 0.5173$, $p = 0.1032$) and in MOLT4-R5 cells ($r = 0.5218$, $p = 0.099678$), but a stronger correlation was found with qVOA in SupT1-R5 cells ($r = 0.7824$, $p = 0.0044$). Inclusion of wells with

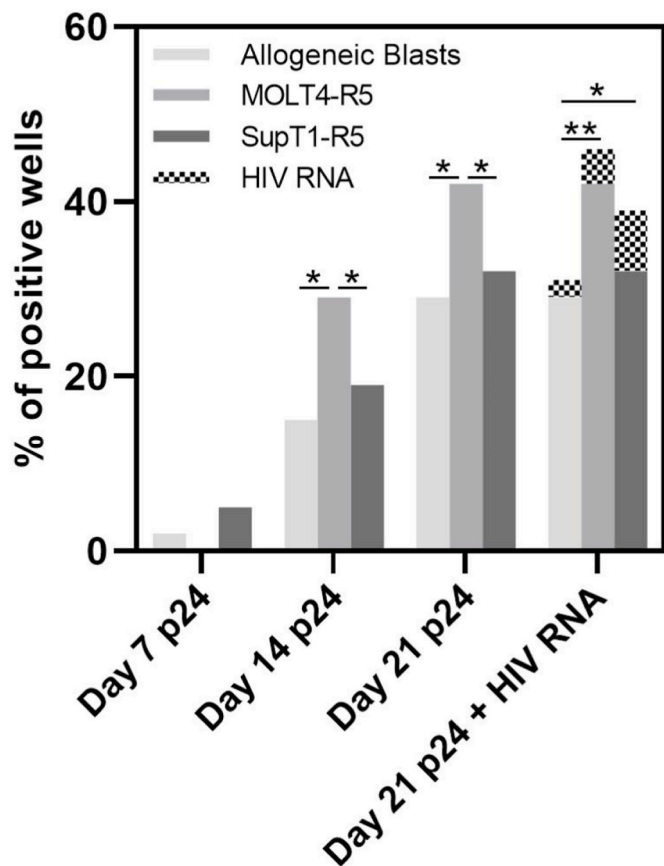


Fig. 3. Proportions of positive wells from MOLT4-R5, SupT1-R5, and allogeneic blast qVOA protocols. The proportion of positive wells from each qVOA was calculated and aggregated at each assay time point. Overall proportions of p24-positive wells from allogeneic blast (light gray), SupT1-R5 (medium gray), and MOLT4-R5 (dark gray) qVOA protocols were compared on a pair-wise basis. (* $p < 0.05$, ** $p < 0.01$, two-tailed paired T-test). Day 21 results were supplemented to include wells containing HIV-1 RNA that increased 10-fold over any 1-week period.

evidence of outgrowth by HIV-1 RNA did not improve correlations between IPDA and qVOA (Figure S3).

There was no evidence of correlation between cell-associated DNA from PBMC and qVOA IUPMs in any cell type calculated with or without wells with evidence of outgrowth by HIV-1 RNA (Figure S4).

Discussion

The qVOA is widely used to assess the impact of experimental

“curative” interventions on the latent HIV-1 reservoir. The standard protocol for this assay incorporates weekly additions of CD8-depleted PHA-stimulated blasts from healthy, HIV-1 negative donors, which provide allogeneic stimulation as well as target cells for viral propagation. Cell lines have been substituted for allogeneic blasts to decrease dependence on normal donor cells. Although previous studies have shown comparable results with cell lines, no direct comparison across MOLT4-R5, SupT1-R5, and allogeneic blast procedures had been performed before the current study.

Here, we performed each of these three qVOAs in parallel and compared IUPMs and overall proportions of wells containing outgrowth at three weekly time points. We found that day 21 IUPMs were significantly higher than day 14 IUPMs, indicating that some sensitivity may be lost by ending the assay at too early a time point. The significantly higher IUPMs on day 21 suggests that the 3 week-long assay retains an advantage for inducible reservoir quantitation. When measuring outgrowth by p24 EIA alone, the MOLT4-R5 assay had a significantly greater proportion of positive wells than the SupT1-R5 or allogeneic blast assays, suggesting that the MOLT protocol is the most sensitive for viral outgrowth detection.

By isothermal amplification of HIV-1 RNA using an automated platform (Hologic Panther), we observed that HIV-1 RNA increased by at least 10-fold over at least one week in 8% of all p24-negative wells tested, and in 5 of the 7 qVOAs that were negative for IUPMs by p24 alone, supporting previous findings that HIV-1 RNA detection may enhance sensitivity of reservoir quantitation.^{12,13,17} Because we analyzed the weekly kinetics of HIV-1 RNA in cell culture supernatant, maintaining a 21-day culture allows for extended observation of HIV-1 outgrowth that would otherwise be missed using p24 EIA readouts.

Overall, the inducible HIV-1 reservoir estimations obtained by p24 detection in MOLT4-R5 or SupT1-R5 cell lines correlated well with the standard qVOA using blasts as has been reported.^{11,12} However, incorporating HIV-1 RNA increases as evidence of outgrowth showed that the two cell lines were more sensitive for IUPM detection than allogeneic blasts. A similar trend was seen when quantifying the overall proportions of positive wells: both the MOLT4-R5 and SupT1-R5 protocols had a significantly greater proportion of positive wells than did their allogeneic blast counterpart. While these results further confirm that either cell line would be preferable to allogeneic blasts when quantifying IUPM based on the presence of RNA growth, the MOLT4-R5 cell line may in fact be the most advantageous of the three cell types. Since cellular proliferation and clonal populations contribute at least in part to the long-lived reservoir, it is important that outgrowth cultures efficiently amplify virus in as many culture wells as possible. These cultures will maximize our ability to detect clones or specific viral variants found in rebound viremia after stopping antiretroviral therapy. In addition, heightened sensitivity for inducible, replication-competent virus is important for measuring the efficacy of curative treatments for HIV-1.

Although the SupT1-R5 and allogeneic blast methods amplified virus

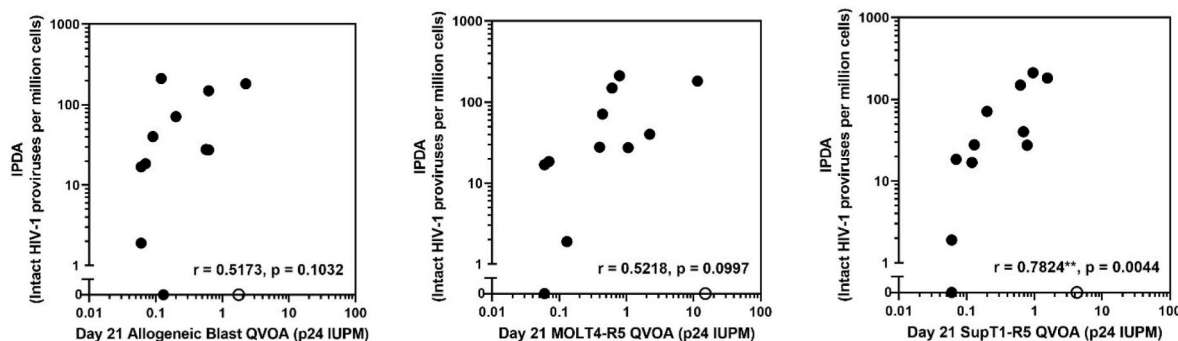


Fig. 4. IPDA and qVOA relationship. Pearson’s correlation coefficients were calculated between IPDA and qVOA IUPMs obtained by using allogeneic blasts, MOLT4-R5, or SupT1-R5 cells after removal of an outlier (open circle) in which IPDA was negative and qVOA was positive by all three assays.

to p24-detectable levels in a similar number of qVOA wells, the SupT1-R5 protocol had 17 p24-negative wells containing low-level RNA outgrowth - almost 3 times as many compared to the standard assay. The presence of RNA outgrowth in these wells suggests that while SupT1-R5 cells may not amplify virus as efficiently as the MOLT4-R5 cells, SupT1 cells can support lower levels of HIV-1 amplification. The divergent results between the cell lines may be due to intrinsic cellular differences, the specifics of the protocols (cell input number, media change schedule, etc.), or a combination of both.

While IUPMs from each of these qVOAs should be interpreted as a minimum estimation of the size of the reservoir due to incomplete induction, it is likely that PCR-based assays such as the IPDA can detect intact proviruses not necessarily induced by the single round of stimulation in the qVOA - as has been reported.⁴ In order to compare the relative coverage of the viral landscape provided by the qVOA and the IPDA, we compared IUPMs measured across the three distinct qVOAs to IPDA and CAD values measured from the same 12 participants.

We found that IPDA moderately, but insignificantly, correlated with IUPMs from the allogeneic blast and MOLT4-R5 qVOAs, but was more strongly correlated with SupT1-R5-based IUPMs. Although inclusion of RNA-positive wells did not improve the correlations, the relationship between qVOA and IPDA appeared to be stronger than the correlations between qVOA and total HIV-1 integrase DNA. This finding is consistent with most of proviral DNA being defective.¹ While these findings are consistent with the IPDA's intended purpose of representing a broader landscape of the infectious viral reservoir by including intact proviruses not induced by the qVOA, unforeseen mismatches between intact proviruses and primers/probes sequences may lead to falsely low IPDA values. Such mismatches are uncommon (<10%) and they can be recognized by amplification failure of specific primer/probe sets.¹⁹

This study is limited by a few assumptions that can be investigated through further work. First, the differences in the protocols, excluding target cells, may have affected the sensitivity of the assays. While methods could have been unified to form a single protocol among the qVOAs, we felt it appropriate to follow the methods reported for each target cell type. In addition, the requirement that a 10-fold increase of HIV-1 RNA from week-to-week is indicative of ongoing replication is an assumption. Characterization of sequences in weekly qVOA wells could elucidate whether ongoing replication is indeed occurring in these wells. While the 10-fold weekly increase in RNA on its own cannot serve as a sufficient indicator of viral outgrowth, using this measurement as a screening tool to identify virus undetected by p24 EIA could be important for reservoir analytics - especially for samples in which qVOA assays are negative by traditional methods using allogeneic blasts as target cells. Finally, the relatively small sample size of the study likely limited the detection of correlations between IPDA and IUPM, although a major strength of this study was the same-day, parallel performance of all three assays from the same population of purified cells.

How the results from these assays would compare with other methods to characterize the latent reservoir would be worth investigating in the future. The recently reported differentiation QVOA (dQVOA) amplifies infectious virus from resting CD4⁺ T-cells that are differentiated into effector T-cells to enhance latency reversal.²⁰ While amplification without targets in the dQVOA was similar to amplification with allogeneic blasts, it remains to be seen whether a more highly permissive cell line protocol, such as the ones examined in this study, in combination with a differentiated effector CD4 T-cell reservoir might further improve the sensitivity of that outgrowth assay.

The qVOA remains vital in assessing curative efforts for HIV-1 for its capacity to detect inducible, replication-competent virus. The results presented here suggest that MOLT4-R5 cells and combined measures of p24 and HIV-1 RNA as indicators of viral outgrowth provide an advantage over standard qVOA protocols using allogeneic blasts.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: • P. Nathan Enick: none • Joseph P. Brooker: none • Camille M. Tumiotto: none • Brittany T. Staines: none • Joseph J. Eron: none • Deborah K. McMahon: none • Rajesh T. Gandhi: Dr. Gandhi has served as a Scientific Advisory Board member within the last three years for Merck and Gilead Sciences, Inc. • John W. Mellors: Dr. Mellors serves or has served as a consultant for Gilead Sciences, Inc., Merck, and Accelevir Diagnostics; owns share options in Co-Crystal Pharmaceuticals, Inc.; and, is a part-time employee and shareholder of Abound Bio, Inc. His holdings and roles in Co-Crystal Pharmaceuticals and Abound Bio are unrelated to the current work. • Michele D. Sobolewski: none

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jve.2021.100043>.

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