Ultrasensitive P24 Digital ELISA Can Lead to an Overestimate of HIV Reservoir Size

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Introduction

The quantitative viral outgrowth assay (qVOA) is currently the gold standard to estimate the minimal size of the HIV reservoir. It is based on the frequency of cells harboring inducible, replication-competent proviruses that can be recovered after 14 to 21 days of culture (1). The ultrasensitive SIMOA p24 assay, developed by Quanterix, can quantify p24 with a limit of detection up to 1000 times lower than standard ELISA (2, 3). We investigated whether the SIMOA p24 commercial kit can be used as a reliable read out to calculate the frequency of infected cells in qVOA experiments. In addition, we tested if SIMOA can be biased by defective proviruses still capable of producing p24.

Objectives

- To investigate if digital ELISA can be reliably applied to qVOA experiments to improve the detection of inducible, replication competent proviruses.
- To determine whether artefactual signal due to background and/or inducible but defective proviruses can cause detection of low level p24, leading to an overestimate of HIV reservoir size.
- To address if such ultrasensitive assay and a low cell input can potentially contribute to false enrichment of replication competent HIV in rare cell populations.

Materials and Methods

Peripheral blood mononuclear cells (PBMC) and total CD4+ T-cells from 6 patients on suppressive ART were sorted based on CD32, a surface marker recently characterized by Descours *et al* (4). CD4+CD32- cells (14-30x10^6) were plated in 5-fold dilutions. CD4+CD32+ cells, present at very low frequency, were plated in replicate with low cell input (2.2-62 x 10^3 total cells). qVOAs were conducted as described by Laird *et al* (5). The SIMOA p24 2.0 commercial kit was used to assay supernatants collected at days 5, 9, 14 and 21. The Lower limit of quantification (0.01 pg/ml) was used to define wells as positive for p24. We used droplet digital PCR to quantify cell associated HIV DNA and RNA, and performed RNA single genome sequencing from cell associated RNA. HIV sequences, partial or recovered in full length with three overlapping amplicons, where analyzed as in Bruner *et al* (6) to assess replication competence and the potential to express *gag* gene products.

Results

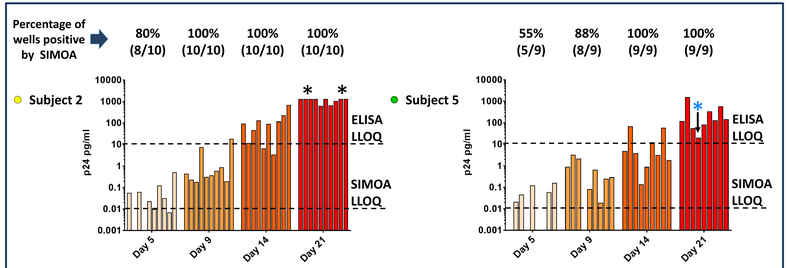


Figure 1 – SIMOA p24 assay allows earlier detection of viral outgrowth compared to standard ELISA

Bar graphs represent p24 values by SIMOA in longitudinal samples from wells positive at day 21 by standard ELISA. Dashed lines indicate the lower limits of quantification for both assays. The black star symbol represents values above the upper limit of quantification for standard ELISA. The blue star symbol highlights one well with delayed outgrowth.

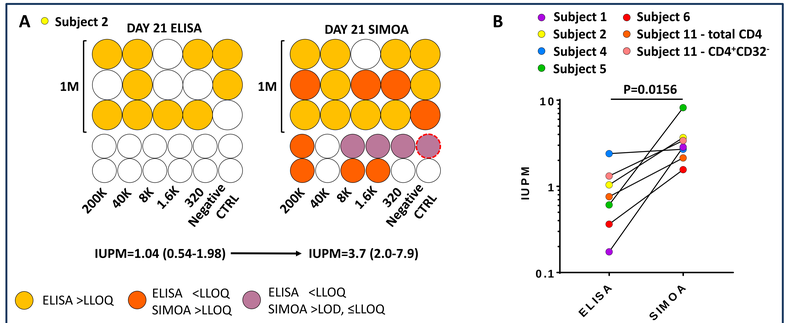


Figure 2 – SIMOA p24 assay detects more wells as positive, leading to higher frequency of IUPM

A) Representative qVOA experiment performed with a total of 15.5 x10^6 $CD4^+CD32^-$ cells. Culture supernatants were measured with both standard ELISA and SIMOA. Only wells with values above LLOQ were used to calculate IUPM. B) IUPM frequencies based on ELISA versus SIMOA from 7 qVOA experiments. P values calculated with non parametric Wilcoxon t-test.

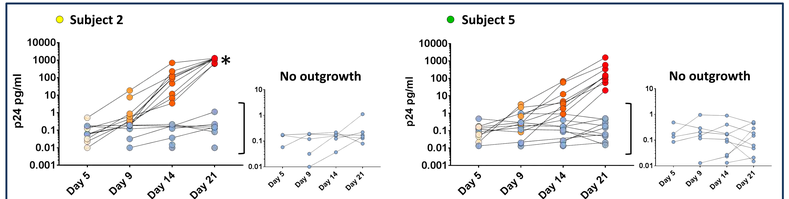


Figure 3 – Only a fraction of wells positive by p24 SIMOA shows exponential viral outgrowth

P24 values above LLOQ measured by SIMOA from day 5 to day 21. Wells with exponential outgrowth are shown in shades of orange. Wells without outgrowth (negative by standard ELISA) are shown in blue. The black star symbols represents values above the upper limit of quantification by standard ELISA.

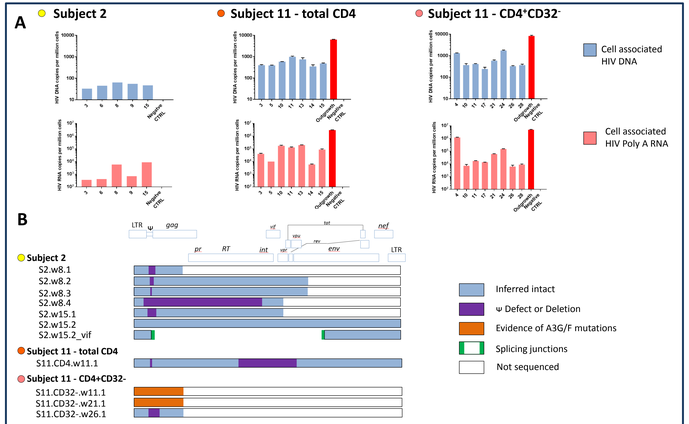


Figure 4 – Wells without viral outgrowth but positive by SIMOA have high levels of HIV-RNA, in some cases due to induced defective proviruses

A) Cell associated HIV DNA and RNA measured by ddPCR from wells without viral outgrowth. The x axis shows well number and controls.
B) Sequence map of HIV transcripts recovered from cell associated RNA in wells without exponential outgrowth.

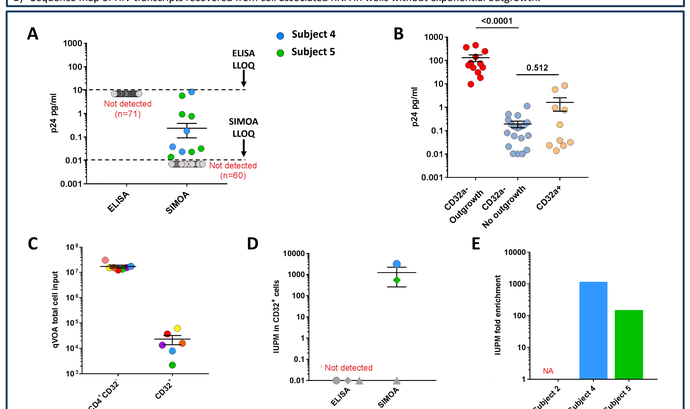


Figure 5 _ Ultrasensitive p24 measurements as a possible factor in overestimation of latent infection

A) p24 values from $CD32^+$ culture wells measured with ELISA versus SIMOA (Lower limit of quantification (LLOQ): 5-10 pg/mL and 0.01 pg/mL, respectively) B) p24 values in qVOA wells of $CD32^-$ with outgrowth (ELISA, red), $CD32^-$ without outgrowth (SIMOA, blue) and $CD32^+$ cultures (SIMOA, orange). C) Total cell input in $CD32^-$ and $CD32^+$ cultures. D) IUPM calculation based on ELISA versus SIMOA from 3 subjects. E) Fold enrichment of IUPM in $CD32^+$ cells.

The lower limit of quantification of SIMOA (0.01 pg/ml) allowed earlier p24 detection in $CD32^-$ wells that were positive by ELISA at day 21 (Fig.1), detecting p24 in 88-100% of these wells at day 9. When we calculated cell infection frequency based on qVOA wells defined as positive by ELISA versus SIMOA, the latter lead to a significant increase in IUPM (mean IUPM 0.9 and 3.5, respectively) (Fig.3). However, of the wells positive by SIMOA, only 53% (19/36) showed exponential viral outgrowth from day 5 to 21, while the others had low p24 levels (mean 0.21 pg/ml) with erratic kinetics (Fig.2).

In these wells with no outgrowth we found both HIV DNA and RNA, and sequencing of HIV transcripts revealed multiple variants with an intact *gag* ORF, but that also carried defects in the major splice donor site, hypermutation or large deletions (Fig.4), suggesting that the extreme sensitivity of SIMOA may detect low levels of p24 released from cells with defective proviruses (7). In the case of qVOA experiments with a low input of $CD32^+$ cells (all negative by ELISA, Fig.5A-C), SIMOA caused a dramatic overestimate of infected cell frequency in 2 out of 3 qVOAs (554 and 3184 IUPM), representing a possible explanation for the enrichment of HIV reservoir in $CD32^+$ cells reported by Descours *et al* (Fig.5D-E).

Conclusion

SIMOA allows earlier detection of p24 compared to ELISA. However, longitudinal sampling is necessary to distinguish viral outgrowth from false positive and low-level p24 produced by defective proviruses. Our results advice caution in using SIMOA on a single timepoint, as it can lead to an overestimate of IUPM, particularly for qVOA with low cell input.

References

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