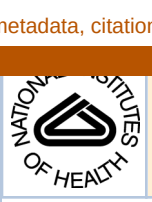

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Performance Characteristics of the Cavidi ExaVir Viral Load Assay and the Ultra-Sensitive P24 Assay Relative to the Roche Monitor HIV-1 RNA Assay

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Abstract

Background—The Cavidi viral load assay and the ultra-sensitive p24 antigen assay (Up24 Ag) have been suggested as more feasible alternatives to PCR-based HIV viral load assays for use in monitoring patients infected with HIV-1 in resource-limited settings.

Objectives—To describe the performance of the Cavidi ExaVir Load™ assay (version 2.0) and two versions of the Up24 antigen assay and to characterize their agreement with the Roche Monitor HIV-1 RNA assay (version 1.5).

Study Design—Observational study using a convenience sample of 342 plasma specimens from 108 patients enrolled in two ACTG clinical trials to evaluate the performance characteristics of the Up24 Ag assay using two different lysis buffers and the Cavidi ExaVir Load™ assay.

Results—In analysis of agreement with the Roche assay, the Cavidi assay demonstrated superiority to the Up24 Ag assays in accuracy and precision, as well as sensitivity, specificity, and positive and negative predictive values for HIV-1 RNA ≥400, ≥1000 and ≥5000 copies/mL. Logistic performance curves indicated that the Cavidi assay was superior to the Up24 assays for viral loads greater than 650 copies/mL.

Conclusions—The results suggest that the Cavidi ExaVir Load assay could be used for monitoring HIV-1 viral load in resource-limited settings.

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The authors disclose no conflicts of interest.

Keywords

Cavidi; p24 antigen; viral load; resource limited setting

Background

HIV-1 plasma viral load assays and CD4 cell counts have proven useful in initiating therapeutic regimens and assessing adherence and response to treatment. Adequately sensitive assays are essential for prompt detection of virologic treatment failure, and thereby, prevention of the evolution and transmission of drug-resistant strains of HIV. As access to antiretroviral (ARV) drugs expands, attention has turned to the feasibility of performing viral load assays in resource-limited countries. Cost and technical complexity tend to limit feasibility of amplification-based methods currently approved by the FDA. The Cavidi ExaVir Load™ assay[1–17] of reverse transcriptase (RT) activity, and the ultra-sensitive p24 antigen (Up24 Ag) assay[8, 16, 18–21] are noted for their lower cost and simpler technical requirements. Furthermore, RT assays can be used to evaluate phenotypic drug resistance[22–24].

Objectives

We evaluated agreement between the Roche HIV RNA Amplicor Monitor assay (version 1.5) and three alternative assays: the Cavidi assay, the Up24 Ag assay using the manufacturer's lysis buffer (Up24-m), the Up24 Ag assay adding an external buffer for enhanced lytic reaction (Up24-JS)[21].

Study Design

This study was approved by the Adult AIDS Clinical Trials Group (ACTG) as New Works Concept Sheet 227 and by the Institutional Review Board of The University of North Carolina at Chapel Hill (UNC).

Clinical Samples

We obtained baseline plasma samples from 23 participants in ACTG protocol 201[25,26] and longitudinally-collected samples from 85 participants in ACTG protocol 307[27,28]. The Roche RNA assay was always performed. Other assays were prioritized if sample volume was limited: Cavidi RT, Up24-JS, then Up24-m. Prioritization resulted in some assay values being intentionally missing. For 273 specimens, volume was sufficient for all assays. For 36, neither Up24 antigen assay was performed. For 32, the Up24-m assay was not performed. For one, both Up24 assays were performed but the Cavidi assay was not because less than 1 ml of plasma was available.

Roche HIV RNA Amplicor Monitor Assay, Version 1.5

Plasma viral loads were measured using the Amplicor HIV-1 Monitor™ assay, version 1.5 (Roche Molecular Systems, Branchburg, NJ.) The lower limit of quantification (LLQ) was 400 copies/mL.

Cavidi ExaVir Load™ Assay

The Cavidi assay (Cavidi AB, Uppsala, Sweden) was performed following the package insert. For each sample, RT activity was measured relative to a serially diluted reference enzyme standard of known concentration. Results were reported as fentograms RT per milliliter (fg/mL) and converted to HIV-1 RNA copies per mL equivalents (cps/mL eqs)

using the ExaVir ™ Load kit version 2.0 and ExaVir ™ Load Analyzer software version 1.61

Ultra-Sensitive p24 Antigen Assays

Samples were assayed by Up24-m and Up24-JS on the same plate following the standard procedure using the HIV-1 p24 enzyme-linked immunosorbent (ELISA) assay kit (catalog no. NEK050 / 050A / 050 / B HIV-1 ELISA; Perkin Elmer Life Sciences, Inc, Waltham, MA) and the p24-specific viral load ELAST® amplification system kit (catalog no. NEP116VL). Specimens were assayed using the manufacturer's buffer (Up24-m assay) and separately using a previously described external buffer[21] (Up24-JS assay). For Up24-m, 250ul of assay dissociation buffer was added to 50ul of sample, followed by boiling for 5min. For Up24-JS, 25ul of buffer was first added to 50ul of the sample, incubated for 10min, followed by addition of 225ul of assay dissociation buffer and boiling for 5min. Samples were cooled and processed per package inserts. Sample absorbance was measured in kinetic and endpoint modes using Quanti-Kin detection software (Rilab, Genoa, Italy) and reported as p24 antigen fg/mL.

Statistical Analysis Strategy

Analysis of inter-assay agreement on $\log_{10}$ scale focused on Bland-Altman plots and other graphical figures computed using bivariate longitudinal mixed-effects models for assay pairs. Log_{10} assay pairs were assumed to follow a bivariate Gaussian distribution characterized by two sets of inter- and intra- subject variance components. The subject-specific random effect for one assay was assumed to be correlated with that of the other assay. The models were fitted via an algorithm designed to cope with a mix of missing, left-censored, and complete values, while accounting for correlation within persons and between assays, and avoiding use of imputation methods.[29–32]. Every pair of values was included in the fitting of these models unless both values were intentionally missing. Parameter estimates obtained were then used to compute confidence intervals, estimates, tests, and predicted locations of left-censored and missing values. Descriptive assay-specific univariate linear mixed-effects model were also fitted.

In analyses of agreement in terms of dichotomized assay values, logistic models for detection of HIV-1, conditional on the magnitude of viral load according to the Roche assay as a covariate, were fitted using a GEE-1 algorithm[33] assuming exchangeable covariance structure. Similar models for binary indicators of whether the assays exceeded a given threshold (e.g., 5000 cp/mL) were fitted conditional on an indicator of whether the Roche assay exceeded that same threshold.

Sensitivity analyses were performed to examine robustness of primary results to reasonable perturbations of assumptions and methods. All computations were performed using SAS 9.1.3 (SAS Institute, Inc., Cary, NC.)

Results

The Longitudinal Assay Data

HIV-1 was detected by the Roche assay in 80.7% (276/342) of samples. Among samples with assay values not missing by design, HIV-1 was detected in 75.4% (257/341), 74.8% (229/306), and 66% (181/274) by the Cavidi, Up24-JS, and Up24-m assays, respectively (Figure 1). Fully observed pairs of values (black dots) are represented in Figure 1 along with model-based predictions for the left-censored values (open circles) and values missing by design (open squares.) Most samples (88.7%, 244/275) with HIV-1 RNA above the limit of detection by Roche also had detectable HIV-1 RT by Cavidi (Figure 1). In comparison,

85.6% (208/243) and 78.4% (171/218) of samples with HIV-1RNA above the limit of detection by Roche also had detectable HIV-1 by Up24-JS and Up24-m, respectively.

Estimates of the mean (and total variance) for the underlying distributions of Roche, Cavidi, Up24-JS, and Up24-m $\log_{10}$ values were 3.83 (1.6), 3.67 (1.8), 3.65 (2.1) and 3.29 (1.7), respectively (Table 1). The estimate of intra-assay correlation between two Roche assays performed at different times for an arbitrary patient was 0.78, while the estimates for Cavidi, Up24-JS, and Up24-m assays were 0.80, 0.88, and 0.83, respectively. The estimate of inter-assay correlation between Roche and Cavidi assays for an arbitrary patient at an arbitrary time ($r = 0.78$) was larger than that for Up24-JS ($r = 0.71$) and Up24-m ($r = 0.68$).

Agreement on Viral Load

In Bland-Altman plots (Figure 2) for agreement with the Roche assay, the Up24 assays exhibited deviations from the Roche assay that were more dispersed than those for the Cavidi assay; i.e., the variance of the deviations from the Roche assay was smaller for the Cavidi assay (0.73), than for the Up24-JS (1.10) and Up24-m (1.09) assays (Table 1). Estimates of mean difference between Roche assays and alternative assays indicated underestimation by the Cavidi (0.15 $\log_{10}$), Up24-JS (0.13 $\log_{10}$), and Up24-m (0.48 $\log_{10}$) assays (Table 1); here, bias of Cavidi was small but deemed reproducible ($p < 0.0033$ for the test of “no bias”.) Association between magnitude of bias and magnitude of viral load was not detected for Cavidi, Up24-JS or Up24-m assay (Table 1 and Figure 2). The fitted models also provided information about calibration; e.g., given any Cavidi assay value, the best linear unbiased predictor of the corresponding $\log_{10}$ Roche assay was$1.07 + 0.75 \log_{10}$ Cavidi. The null hypothesis “calibration is unnecessary” was rejected for Cavidi, Up24-JS and Up24-m (each $p < 0.0001$).

Agreement on Dichotomized Values

Relative to the Roche assay as a gold standard, Cavidi was superior to both Up24 assays in terms of sensitivity, specificity, and positive (PPV) and negative predictive value (NPV) (Table 2). Estimates of PPV indicated the Roche assay is highly likely to exceed a given threshold whenever the other assay has done so. Given a Cavidi assay < 5000 cp/mL, for example, the Roche assay is likely to be < 5000 cp/mL (NPV = 87%); in contrast, values for the Up24 assays below this level do not ensure that the Roche assay value will be < 5000 cp/mL (NPV = 62%, 53%).

Performance in Detection of HIV-1

Estimates of logistic performance curves representing the probability of detecting HIV-1 at given levels of the Roche assay value indicated that Cavidi, Up24-JS, and Up24-m assays are all likely to detect HIV-1 when viral load (per Roche) is greater than 4.5 $\log_{10}$ (Figure 3). For all viral load values greater than 2.8 $\log_{10}$, the performance curve for Cavidi was superior to the curves for Up24-JS and Up24-m assays.

Agreement on Viral Load Change-from-Baseline

Longitudinal data from 71 subjects (234 evaluations) allowed comparison of assays in terms of change-from-baseline (Δ) scores (Figure 4). Model-based estimates of mean Δ for Roche was -0.51 $\log_{10}$ cp/mL. Similar declines were evident for Cavidi (-0.36), Up24-JS (-0.44), and Up24-m (-0.52). Estimated correlation with ΔRoche was greatest for ΔCavidi ($r = 0.87$) and less for ΔUp24-JS ($r = 0.33$), and ΔUp24-m ($r = 0.22$).

Comparison of Other Characteristics of the Assays

The four assays were compared in terms of cost, turn-around- times, storage requirements, additional reagent required, equipment, and degree of technologic complexity (Table 3). The p24 antigen assay was the least expensive ($5 – $7 USD) and least complex, but still required storage at 4°C and equipment such as a heat block, a 37°C incubator, and a ELISA plate shaker, washer, and reader. The most complex and expensive ($20–50 USD) was the Roche assay which also required equipment such as a thermocycler, ELISA plate washer and reader, 37° C incubator, and a matrix automated pipette. The Cavidi assay was intermediate in cost and complexity, but required 2–3 days to complete.

Discussion

The Cavidi assay agreed with the Roche assay more closely than either Up24 Ag assays. Use of the alternate “JS” lysis buffer improved performance of the Up24 Ag assay as demonstrated previously[21]. Relative to the Roche RNA assay as a gold standard, the Cavidi RT assay exhibited greater accuracy and precision than the Up24 assays. Up24-m substantially underestimated viral load (mean difference > 0.3 $\log_{10}$) while Up24-JS and Cavidi RT assays did not. The differences between the Roche and Cavidi values were less dispersed than for Up24-JS and Up24-m. The results also suggest that refinement in calibration of the Cavidi assay (using 1.07 + 0.75 $\log_{10}$ Cavidi) might improve agreement with the Roche assay. Agreement was slightly better on the change-from-baseline scale, although for the Up24 assays it appeared that mean deviation from the Roche assay depends on the magnitude of underlying viral load.

The WHO recently issued new treatment guidelines suggesting that antiretroviral therapies be changed when viral load exceeds 5000 cp/mL.[34] The performance of the Cavidi assay was superior to that of the Up24 assays when considering a dichotomized assay value.

Three analytes were measured: HIV-1 RNA, HIV-1 RT, p24 antigen. While RT concentrations were converted to cp/mL equivalents, the p24 antigen levels were expressed as fg/mL. In addition, both the RNA and RT assays measure virion-associated components, while the Up24 antigen assay can measure both virion-associated and free or immune-complex-bound p24 antigen in plasma. The Cavidi RT assay was in closer agreement with the Roche RNA assay than either of the Up24 antigen assays.

Primary analyses of assay agreement and correlation relied on methods that used all data and did not rely on imputation.[29–32] Single-imputation frequently cited in the assay-comparison literature yields biased estimates of means, variances, standard errors, and correlations of interest; e.g., had we imputed a value of 1.0 for all left-censored assay values prior to fitting the models, we would have obtained smaller estimates of the inter- and intra-assay correlations.

This study was limited to individuals infected with subtype B. Inferences about how the assays perform when applied to other subtypes would involve extrapolation. However, the Cavidi RT assay does not depend on particular sequences (RNA assays) or antigenic epitopes (p24 antigen assay) and therefore should be applicable to any lentivirus, including HIV-2[1]. In Western Africa where co-infection with HIV-1 and HIV-2 is not uncommon, Cavidi would provide a sum of both viral loads; the other assays do not detect HIV-2. Our results are consistent with results from previous studies for subtype B3–5,8–11, 20,21, as well as with results from studies that included other subtypes[6,7,9,12,13–16,19]. The Cavidi RT assay is currently in use in Botswana for monitoring HIV-1 viral load[12].

This study used the standard Roche Monitor assay (LLQ = 400 cp/mL) instead of the ultrasensitive Roche assay (LLQ = 50 cp/mL) which would have allowed additional inferences about assay performance in the range of 50 to 400 cp/mL. However, plasma sample volumes were not sufficient for performing the more sensitive assay and the other assays as well. This work was performed prior to release of version 3 of the Cavidi assay[4,9]. It is plausible that use of that version would produce more accurate results.

Unit costs of the assays we performed ranged from $5 to $50 per plasma sample and all required refrigeration and use of specialized equipment that runs on electricity. Infrastructure is often lacking in resource limited settings' and skilled technologists are seldom available except in a few central hospitals, reference laboratories or research centers[35]. Because the turn-around time for a typical assay is at least 4–8 hours, results would not be available on the day of collection even at the testing facility. Point of care viral load assays are desperately needed that can provide a result while the patient waits [35, 36].

In conclusion, the performance characteristics of the Cavidi RT assay observed in this study lend support to its use in monitoring HIV-1 viral load in resource-limited settings.

Abbreviations

Cp/mL	copies/mL
ELISA	enzyme-linked immunosorbent assay
FDA	US Food and Drug Administration
HIV	Human immunodeficiency virus
LLQ	Lower limit of quantitation
NPV	Negative predictive value
PPV	Positive predictive value
RT	Reverse transcriptase
Up24	Ultrasensitive p24 antigen
Δ	Change from baseline

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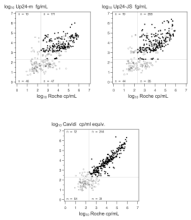

Figure 1.
Viral load measurements by Roche, Cavidi, Up24-JS and Up24-m assays for N = 342, 341, 306, and 274 plasma samples, respectively, are plotted: observed pairs of values (•), pairs with one or both values left-censored (○), pairs with one value missing (□). Missing by design and left-censored values were predicted using parameter estimates from bivariate mixed-effects models.

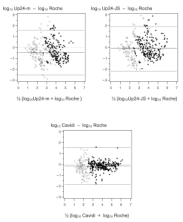

Figure 2.

Bland-Altman plots for analysis of agreement between the Roche Monitor assay and the Cavidi, Up24-JS, and Up24-m assays are shown. For every pair of values plotted in Figure 1 (including pairs with both values observed (•), and pairs with intentionally missing (□) or left-censored (○) values) the average and difference of the two assay values was computed and plotted here along with model-based upper and lower 95% prediction limits (a.k.a. "limits of agreement"). The estimate of mean difference is indicated by a solid line segment. For Cavidi, Up24-JS, and Up24-m, both assay values were observed (•) for 244, 208, and 171 plasma samples, respectively.

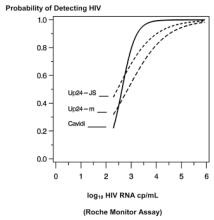

Figure 3.

Each logistic-linear performance curve provides estimates of the probability of detecting HIV-1 at given levels of viral load measured by the $\log_{10}$ Roche Monitor. The relative positions of the curves favor the Cavidi RT assay for viral loads greater than 2.8.

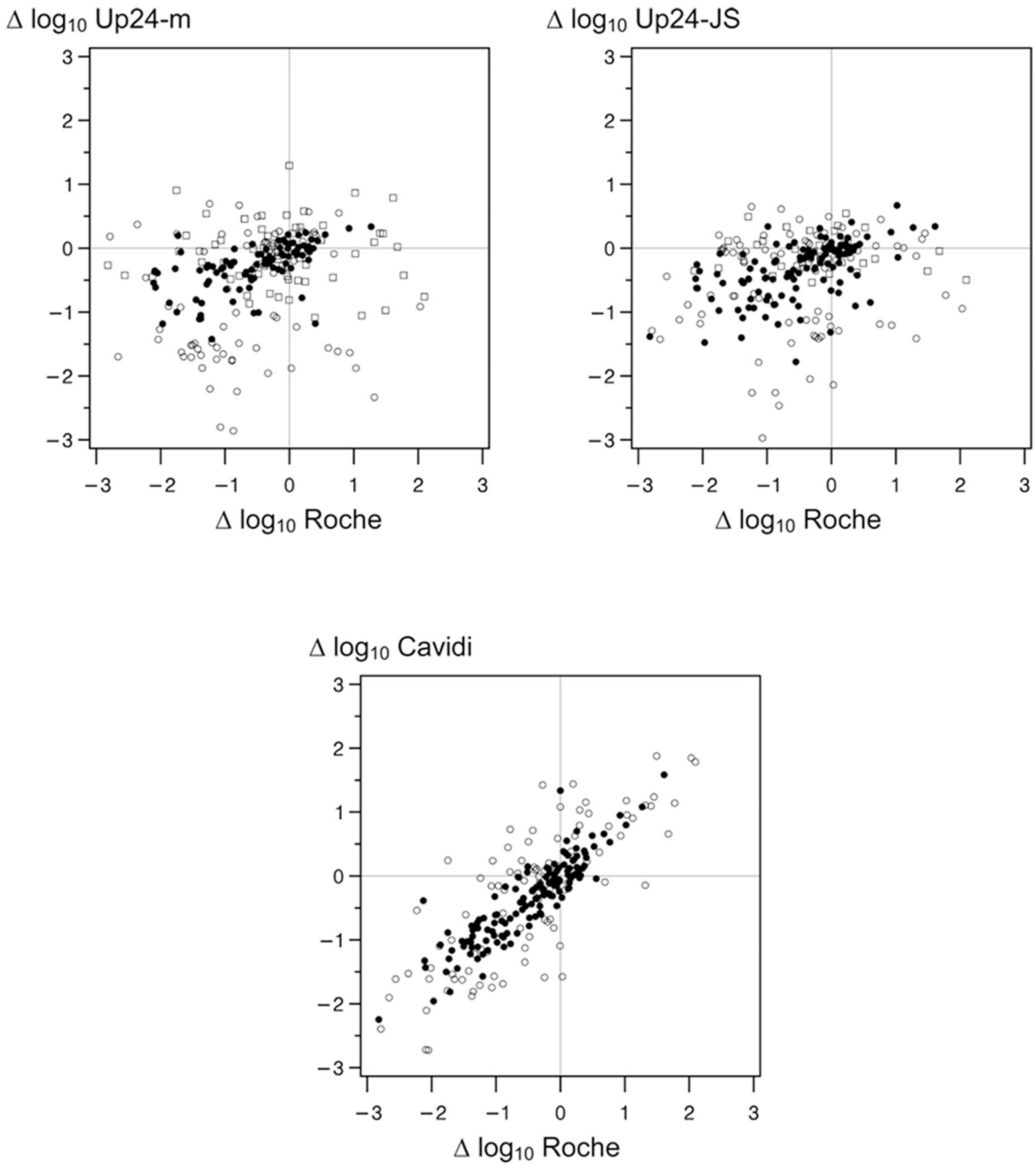

Figure 4.

Viral load change-from-baseline scores (Δ) for 234 plasma samples from 71 patients were obtained from observed assay values (•), and from predictions for assay values missing by design (□) or left-censored (○). Predicted values were computed using parameter estimates from bivariate mixed-effects models. Among [ΔCavidi, ΔRoche] pairs of scores, k = 137 were complete, c = 97 involved censored values, and m = 0 involved missing values. Among [ΔUp24-JS, ΔRoche] pairs, k = 117, c = 94, and m = 33. Among [ΔUp24-m, ΔRoche] pairs, k = 92, c = 77, and m = 65.

Table 1

Assay Agreement

Estimates are shown with 95% confidence intervals obtained from bivariate and univariate mixed-effects models.

	Roche RNA	Cavidi RT	Up24-JS	Up24-m
Mean of VL **	3.83 [3.60, 4.06]	3.67 [3.43, 3.91]	3.65 [3.38, 3.92]	3.29 [3.04, 3.54]
Total Variance of VL *	1.61 [1.22, 2.00]	1.80 [1.31, 2.20]	2.07 [1.48, 2.66]	1.72 [1.20, 2.24]
Intra-Assay Correlation *	0.78 [0.71,0.84]	0.80 [0.74, 0.86]	0.88 [0.84, 0.92]	0.83 [0.77, 0.89]
Inter-Assay Correlation *	--	0.78 [0.73, 0.84]	0.71 [0.63, 0.79]	0.68 [0.59, 0.76]
Mean of Differences (Roche minus Assay) **	--	0.15 [0.05, 0.26]	0.13 [−0.04, 0.31]	0.48 [0.30, 0.66]
Variance of Differences (Roche minus Assay) **	--	0.73 [0.63, 0.83]	1.10 [0.87, 1.32]	1.09 [0.87, 1.31]
Correlation of Differences with Averages *	--	0.07 [−0.07, 0.21]	0.15 [−0.03, 0.33]	0.03 [−0.16, 0.22]
Best Predictor of Roche H_0 "no calibration needed"	--	1.07 + 0.75 VL $p < 0.0001$	1.45 + 0.64 VL $p < 0.0001$	1.59 + 0.66 VL $p < 0.0001$

Comparison of entries within a row:

* The null hypothesis "no difference among the assays" was not rejected (i.e., $p \geq 0.05$)

** The null hypothesis "no difference among the assays" was rejected (i.e., $p < 0.05$)

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Table 2

Performance of Assays Relative to the Roche Monitor Assay

Entries for sensitivity (Sens), specificity (Spec), positive predictive value (PPV), and negative predictive value (NPV) are estimates on percent scale. They are shown with 95% confidence intervals, obtained by fitting 24 logistic-linear regression models appropriate for repeated measures.

HIV-1 Detected	Cavidi	P24-JS	P24-m
Sens**	88 [82, 92]	82 [74, 88]	75 [66, 82]
Spec*	65 [53, 76]	44 [29, 59]	54 [39, 68]
PPV**	94 [89, 97]	91 [86, 95]	94 [87, 97]
NPV*	50 [38, 61]	45 [31, 60]	39 [26, 53]

Viral Load ≥ 400 cp/mL (2.6 $\log_{10}$)	Cavidi	P24-JS	P24-m
Sens**	88 [83, 92]	79 [70, 85]	73 [65, 81]
Spec**	72 [61, 81]	47 [32, 63]	62 [46, 76]
PPV**	94 [91, 97]	87 [79, 92]	90 [82, 94]
NPV**	69 [58, 78]	39 [24, 57]	38 [25, 53]

Viral Load ≥ 1000 cp/mL (3.0 $\log_{10}$)	Cavidi	P24-JS	P24-m
Sens*	83 [76, 89]	82 [74, 88]	77 [68, 84]
Spec**	89 [82, 94]	48 [35, 62]	58 [44, 72]
PPV**	94 [90, 97]	80 [72, 87]	81 [72, 88]
NPV**	70 [59, 79]	49 [33, 65]	42 [28, 57]

Viral Load ≥ 5000 cp/mL (3.7 $\log_{10}$)	Cavidi	P24-JS	P24-m
Sens**	83 [75, 89]	64 [53, 73]	43 [34, 53]
Spec**	96 [92, 98]	71 [58, 81]	65 [55, 74]
PPV**	95 [91, 98]	78 [66, 86]	73 [62, 82]
NPV**	87 [80, 92]	62 [50, 72]	53 [43, 62]

Comparison of entries within a row:

* The null hypothesis “no difference among the three assays” was not rejected (i.e., $p \geq 0.05$)

** The null hypothesis “no difference among the three assays” was rejected (i.e., $p < 0.05$)

Table 3

Comparison the Assays on Costs, Turn-Around Time, and Technical Ease

	Roche Assay: ***Amplicor Monitor 1.5***	**Cavidi Assay:** ***ExaVir Load***	**Perkin Elmer Assay:** ***Heat Dissociated Up24***[1]
Cost[2]	$20 – $50	$15 – $20	$5 – $7
Time	1 day	2–3 days	1 day
Complexity	High	Medium	Low to Medium
Storage	4°C	4°C and −20°C	4°C
Minimum Specimen	0.2 ml plasma	1.0 ml plasma	0.1 ml plasma
Additional Reagents[3]	Isopropyl Alcohol and Ethyl Alcohol	None	Optional external buffer [4]
Additional Equipment[5]	37°C oven ELISA Plate reader ELISA Plate washer Bio-safety Cabinet Thermocycler Matrix automated pipette	33°C oven Plate reader Water pump equipment to wash plates Bio-safety Cabinet or PCR hood Single channel pipette able to dispense 1.5ml[6]	37°C oven ELISA Plate reader ELISA Plate washer ELISA Plate shaker 100°C Heat block pH meter [4]

[1] With or without external buffer.

[2] Cost of kit for assaying one plasma specimen; does not include additional equipment, additional reagents, labor, quality assurance.

[3] Reagents not supplied in the kit.

[4] If using the external buffer: EDTA, Tris-HCL, SDS, Deoxycholic Acid, and a pH meter.

[5] Equipment needed in addition to pipettes, tips, gloves and gowns that are needed for all the assays.

[6] Recommended.