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Modification of the Abbott RealTime assay for detection of HIV-1 plasma RNA viral loads less than one copy per milliliter

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Abstract/Summary

Although commercial tests are approved for detection of HIV-1 plasma viral loads 20 copies per milliliter (ml), only one specialized research assay has been reported to detect plasma viral loads as low as 1 copy/ml. This manuscript describes a method of concentrating HIV-1 virions from up to 30ml of plasma, which can be combined with a commercial viral load test to create a widely-available, reproducible assay for quantifying plasma HIV RNA levels less than 1 copy/ml. Using this pre-analytically modified assay, samples with a known level of 0.5 copy/ml were detected in 8 of 12 replicates (mean 0.47 copy/ml; 95% confidence interval (CI) 0.14-0.81 copy/ml) and samples with a known level of 1.0 copy/ml were detected in 13 of 13 replicates (mean 1.96 copy/ml; 95% CI 1.42-2.50 copy/ml). By concentrating virus from 30ml of plasma, HIV RNA could be measured in 16 of 19 samples (84%) from 12 of 12 subjects (mean 2.77 copy/ml; 95% CI 0.86-4.68 copy/ml). The measured viral load correlated inversely ($r = -0.78$; $p = 0.028$) with the total duration of viral suppression (viral load < 40 copies/ml).

Keywords

HIV; plasma RNA; viral load; single copy; Abbott

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1. Introduction

Although combined antiretroviral therapy (ART) can reduce the plasma HIV-1 viral load below the detection limit of available commercial assays (<20 copies/ml), with the use of more sensitive tests, it is possible to detect residual HIV-1 RNA in the plasma of almost all patients on ART (Dornadula et al., 1999; Havlir et al., 2001; Maldarelli et al., 2007; Schockmel et al., 1997). It remains to be determined whether such low-level viremia correlates with response to treatment, durability of treatment, or prognosis, as has been shown for higher viral loads (Katzenstein et al., 1996; Mellors et al., 1996; O'Brien et al., 1996; Ruiz et al., 1996; Saag et al., 1996). Other potential applications for detecting low levels of virus include screening for HIV, detection of drug resistant variants, and evaluation of novel antiretroviral therapies.

A number of nucleic acid-based detection methods can be used to detect low levels of HIV-1 RNA or DNA, including PCR (standard, quantitative competitive, real time, in situ), branched DNA signal amplification (bDNA), strand displacement amplification (SDA), transcription-mediated amplification (TMA), and nucleic acid sequence-based amplification (NASBA). In the U.S., FDA-approved assays to measure plasma viral loads make use of real time PCR (the Abbott RealTime HIV-1 Assay [Abbott] (Swanson et al., 2007; Tang et al., 2007) and Roche Cobas Taqman HIV-1 Test [Roche] (Pas et al.; Sun et al., 1998)), bDNA (Versant HIV-1 RNA 3.0 Assay [Chiron/Bayer] (Collins et al., 1997; Dewar et al., 1994; Kern et al., 1996)), and NASBA (Nuclisense HIV-1 QT [bioMérieux] (van Gemen et al., 1993)). These commercially available assays are approved to quantify levels of HIV RNA >40 (Abbott), 20 (Roche Cobas v2.0), 50-75 (Chiron/Bayer), or 176 copies/ml (bioMérieux), though actual limits of detection may be lower. For example, the Abbott RealTime (Abbott) assay will detect 20 copies/ml in 50 of 57 (88%) cases, 10 copies/ml in 38 of 56 (68%) cases, and 5 copies/ml in 30 of 57 (53%) cases (package insert).

The sensitivity of viral load testing can be improved by using assays that can detect one or a few copies, running samples in replicate, and concentrating virus from a larger volume of plasma. Real time PCR has been used to detect single copies of different viruses from body fluids and tissues (Mackay et al., 2002). Many investigators have described assays capable of detecting a single copy of HIV-1 proviral DNA (Boni et al., 1993; Boni et al., 2004; Bush et al., 1992; McClure et al., 2000; Nuovo, 2000; Saha et al., 2001; Simmonds et al., 1990; Spann et al., 1991; Wieland et al., 1996; Yourno, 1993; Yourno and Conroy, 1992; Zazzi et al., 1992; Zazzi et al., 1993) or HIV RNA (Fischer et al., 2008; Kaiser et al., 2007; Kumar et al., 2007; Palmer et al., 2003) from clinical samples, including peripheral blood mononuclear cells, dried blood spots, and brain tissue. Replicate sampling has been used in single copy assays (Palmer et al., 2003), but can also be used to improve the sensitivity of assays with higher limits of detection. For example, the semiquantitative TMA assay has a 50% detection limit of 3.6-14 copies (Busch et al., 2005; Lelie et al., 2002), but when used in quadruplicate, the detection limit can be reduced to <3.5 copy/ml (Hatano et al., 2009).

Finally, the sensitivity can be increased by concentrating virus from a larger volume of plasma. Using high speed centrifugation to pellet virions from up to 7ml, several groups have modified existing commercial assays (Havlir et al., 2001; Schockmel et al., 1997) or created in-house assays (Dornadula et al., 1999; Palmer et al., 2003) with detection limit of 20 copy/ml (Schockmel et al., 1997), <5 copy/ml (Dornadula et al., 1999), 2.5 copy/ml (Havlir et al., 2001), or 1 copy/ml (Palmer et al., 2003). However, centrifugation of higher plasma volumes often results in precipitation of a poorly-soluble pellet (perhaps composed of plasma lipids and lipoproteins) that seems to variably interfere with nucleic acid extraction efficiencies or with PCR reactions (unpublished observation). Given that density cushions of sucrose and iodixanol have been used to pellet and purify virions from culture

supernatants (Dettenhofer and Yu, 1999), it was hypothesized that an iodixanol cushion could be used to pellet virions from higher volumes of plasma while excluding plasma constituents that affect RNA extraction or PCR efficiencies. This manuscript describes a method for concentrating virus from up to 30ml of plasma, which can be used in conjunction with the Abbot Real Time HIV-1 Assay to create an easy-to-use, widely-available, reproducible test for quantifying plasma viral loads less than 1 copy/ml.

2. Materials and methods

HIV-positive adults with known, detectable viral load (>40 copies/ml) or undetectable viral load were recruited from two clinic-based cohorts in San Francisco (the PLUS study and the SCOPE cohort). The study was approved by the local Institutional Review Board of the University of California, San Francisco (UCSF). All participants gave informed consent.

Blood (approximately 68ml) was collected in eight 8.5ml BD Vacutainer ACD tubes with solution A (BD Vacutainer, Franklin Lakes, USA), combined, and spun at 1,000g for 10 minutes without brake. The plasma was carefully pipetted off (keeping at least 2ml from the buffy coat), combined, spun again at 1,000g for 10 minutes without brake, poured into a fresh 50ml conical tube, and stored at 4°C overnight for subsequent pelleting of virions.

To determine the efficacy of concentration across a range of copy numbers, different volumes of HIV-positive plasma from participants with known viral load (quantified using the Abbott assay) were spiked into 30ml aliquots of HIV-negative donor plasma to achieve a series of final HIV concentrations ranging from 0.5 to 50 copy/ml (experiment 1) or 0.9 to 1345 copies/ml (experiment 2). A third experiment was done to determine if the modified assay could detect very low levels of viremia. For this experiment, HIV-positive plasma from one of two additional subjects with known viral load (quantified at least twice using the Abbott assay) was spiked into replicate 30ml aliquots of HIV-negative donor plasma to obtain 10 replicates of 0.5 copy/ml and 10 replicates of 1.0 copy/ml (5 replicates from each subject).

A fourth experiment was performed to determine the performance characteristics in clinical samples. Blood was obtained from 12 fasting ART-treated individuals with viral loads that were undetectable using the Abbott assay. For 7 of these participants, plasma was obtained at two different time points, approximately two weeks apart, during which time there was no significant change in clinical status. Simultaneous blood samples were sent to the clinical lab for measurement of routine viral load using the Abbott assay.

For all four experiments, virions were pelleted by centrifugation on a density cushion. Plasma was diluted 1:1 with PBS to reduce the density (authors note: subsequent experiments have suggested that this step is not necessary), divided in two portions, and gently layered onto 10ml of 6% iodixanol (OptiPrep Density Gradient Medium [Sigma-Aldrich, St. Louis, USA] diluted 1:10 in PBS) in 50ml polypropylene tubes (Beckton, Palo Alto, USA). Samples were then centrifuged at 47,810g (20,000rpm in SH3000 rotor of a Sorvall RC6 floor centrifuge) for 3hrs at 4°C without braking. Viral pellets were resuspended in a total of 1000 μ l of PBS and frozen at -80°C.

Samples were thawed and the HIV-1 RNA was measured according to the Abbott m2000 RealTime assay protocol. Copy values were extrapolated from the Ct values of the standards and then adjusted for the concentration factor. Linear regressions (best-fit method) and correlations (Pearson r for data meeting normality tests; Spearman r for other data) were performed using GraphPad Prism 5.0.

3. Results

Two experiments were performed to measure the efficacy of recovery from a range of HIV concentrations (from 0.5 to 1345 copies/ml). In these two experiments, all samples gave detectable results and there was a strong linear correlation between the number of HIV RNA copies that had been spiked into donor plasma (input) and the number of copies measured from the pelleted and resuspended virions (output) ($r^2 = 0.998$, $P=0.001$ for the first experiment and $r^2 = 0.97$, $P<0.0001$ for the second experiment; see Figure 1A-B). The slopes of the linear regression lines were 0.80 and 0.81, respectively.

Next, the assay was tested using samples with very low levels of HIV. For samples with a calculated input of 0.5 copy/ml, HIV RNA was detected in 6 of 10 replicates (Table 1), with a mean measured output of 0.28 copy/ml (95% CI 0.08-0.48 copy/ml). For samples with a calculated input of 1.0 copy/ml, HIV RNA was detected in 10 of 10 replicates (Table 1), with a mean value of 1.71 copy/ml (95% CI 1.21-2.20 copy/ml). Including the low copy samples from the first two experiments, samples with a calculated input of 0.5 copy/ml were detected in 8 of 12 replicates from 3 of 3 subjects (mean 0.47 copy/ml; 95% CI 0.14-0.81 copy/ml), and 1.0 copy/ml samples were detected in 13 of 13 replicates from 4 of 4 subjects (mean: 1.96 copy/ml; 95% CI 1.42-2.50 copy/ml).

Finally, the assay was tested on a set of clinical samples from treated patients. Seven individuals were tested twice and 5 were tested once (total of 19 samples). HIV RNA was not detected in any study participant by the clinical lab or the Abbott assay as applied to unspun plasma. In contrast, after concentrating virus from up to 30ml of plasma, HIV RNA was detected in 16 of 19 samples (84%) from 12 of 12 (100%) study participants (Table 2). The calculated viral loads ranged from 0.4 to 11.4 copy/ml (mean 2.8 copy/ml; 95% CI 0.9-4.7 copy/ml).

For 10 of 12 participants, clinical data was available on the duration of the last period over which the viral load was consistently undetectable by commercial assays. For these 10 participants, there was a trend towards an inverse correlation between the plasma viral load as measured from 30ml of plasma (mean of available time points) and the duration of most recent viral suppression (Spearman $r = -0.61$; $p=0.067$). Because some of the participants had prior “blips” of detectable virus preceded by additional periods of suppression, an alternative duration was calculated using the total period of suppression, as defined by the sum of the lengths of all time periods with two adjacent viral loads that were undetectable by commercial assays. For the 8 participants for whom data was available since the first period of suppression, the viral load measured from 30ml correlated inversely (Pearson $r = -0.82$, $p=0.013$) with the total duration of viral suppression.

4. Discussion

By pelleting virions from up to 30ml of plasma using centrifugation on an iodixanol cushion, the Abbott RealTime assay was adapted to detect plasma viral loads less than 1 copy/ml. Only one other published assay (Palmer et al., 2003) has reported the ability to detect plasma viral loads of approximately 1 copy/ml, and this assay relies on single copy detection from the equivalent of 1.3ml of plasma (3 replicates, each with 10 μ l out of 55 μ l extracted from 7ml of plasma). The ability to concentrate virions from a larger volume (30ml) may reduce the proportionally greater effects of random distribution that occur at low copy numbers (Poisson effects) and could reduce the limit of detection. While the concentration method described above could be combined with the detection platform of Palmer et al (2003), the concentrated virus can also be used for subsequent testing by commercial viral load assays, thereby making use of the desirable characteristics of these

assays, such as wide availability, extensive testing, standardization, reproducibility, automation, and the capability to detect plasma virus from a variety of HIV-1 subtypes. When combined with the Abbott assay, as described above, the result is a widely-available, easily-standardized, sensitive method for detecting plasma viral loads <1 copy/ml.

Additional changes may serve to increase further the performance of the modified assay. Although the data suggest that HIV-1 virions are pelleted reproducibly and efficiently during centrifugation, a control virus particle, such as that used for the Abbott detection, could be introduced as an internal control before centrifugation to account for any variation in pelleting and resuspension. This study did not attempt to concentrate virus from more than 30ml, but it is likely that larger volumes could be used for centrifugation, which would lower further the limit of detection. However, such gains may be marginal relative to the increased volume of blood required. Alternatively, the pelleted virus could be used in a different assay with single copy sensitivity. In this way, the theoretical detection limit could be reduced to 0.03 copy/ml, though at the expense of the availability and reproducibility of the Abbott detection system.

By concentrating virus from up to 30ml of plasma, HIV RNA levels could be measured in 12 of 12 subjects whose viral loads had been undetectable using the conventional Abbott assay. In this relatively small number of participants with varying total duration of viral suppression (range: 1.2 to 11.2 years; median 6.3 years), the measured viral loads tended to correlate inversely with the duration of suppression on ART. Studies using other assays have suggested that the residual plasma virus declines to a plateau value by 1-2 years of ART (Havlir et al., 2003; Maldarelli et al., 2007; Hatano et al., 2009). If indeed there is an inverse correlation between the viral load and the duration of suppression, this finding raises the possibility that some patients may have a more extended decline in plasma virus that could be measured by sensitive assays over longer durations of therapy. This finding will need to be confirmed in a dedicated study, preferably a prospective study with a larger number of participants and a continuous period of suppression.

It remains to be determined whether such low levels of virus have prognostic significance. Residual viral loads of <50 copy/ml have been correlated with increased risk of virologic failure (Geretti et al., 2010) as well as poor CD4+ T cell reconstitution on ART (Mavigner et al., 2009). Several recent studies have reported that low-level viremia correlates with blood levels of soluble immune activation markers (Ostrowski et al., 2008) and T cell activation markers (Havlir et al., 2003; Mavigner et al., 2009; Yukl et al., 2010), though other studies did not find an association with T cell activation (Gandhi et al.; Hatano et al., 2010). Other possible clinical applications for techniques to concentrate virus include screening of the blood bank, confirming HIV infection in HIV vaccine recipients, and detecting rare drug resistant variants.

Assays with this capability should also prove indispensable for HIV research on persistence and eradication. Given the recent resurgence of interest in curing HIV infection (Richman et al., 2009; Trono et al., 2010), there is a growing need for techniques to quantify persistent virus in a reproducible and high-throughput manner. Techniques for HIV concentration and low-level detection can be used to better evaluate responses to ART, to characterize the residual virus, and to monitor new therapies aimed at eradication. The ultrasensitive, widely-available, reproducible assay described in this manuscript could be applied to address such questions in clinical and basic HIV research.

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References

- Boni J, Emmerich BS, Leib SL, Wiestler OD, Schupbach J, Kleihues P. PCR identification of HIV-1 DNA sequences in brain tissue of patients with AIDS encephalopathy. *Neurology*. 1993; 43:1813–7. [PubMed: 8414037]
- Boni J, Shah C, Flepp M, Luthy R, Schupbach J. Detection of low copy numbers of HIV-1 proviral DNA in patient PBMCs by a high-input, sequence-capture PCR (Mega-PCR). *J Med Virol*. 2004; 72:1–9. [PubMed: 14635004]
- Busch MP, Glynn SA, Wright DJ, Hirschhorn D, Laycock ME, McAuley J, Tu Y, Giachetti C, Gallarda J, Heitman J, Kleinman SH. Relative sensitivities of licensed nucleic acid amplification tests for detection of viremia in early human immunodeficiency virus and hepatitis C virus infection. *Transfusion*. 2005; 45:1853–63. [PubMed: 16371038]
- Bush CE, Di Michele LJ, Peterson WR, Sherman DG, Godsey JH. Solid-phase time-resolved fluorescence detection of human immunodeficiency virus polymerase chain reaction amplification products. *Anal Biochem*. 1992; 202:146–51. [PubMed: 1621975]
- Collins ML, Irvine B, Tyner D, Fine E, Zayati C, Chang C, Horn T, Ahle D, Detmer J, Shen LP, Kolberg J, Bushnell S, Urdea MS, Ho DD. A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml. *Nucleic Acids Res*. 1997; 25:2979–84. [PubMed: 9224596]
- Dettenhofer M, Yu XF. Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. *J Virol*. 1999; 73:1460–7. [PubMed: 9882352]
- Dewar RL, Highbarger C, Sarmiento MD, Todd JA, Vasudevachari MB, Davey RT, Kovacs JA, Salzman NP, Lane HC, Urdea MS. Application of branched DNA signal amplification to monitor human immunodeficiency virus type 1 burden in human plasma. *Journal of Infectious Diseases*. 1994; 170:172–179.
- Dornadula G, Zhang H, VanUitert B, Stern J, Livornese L, Ingberman MJ, Witek J, Kedanis RJ, Natkin J, DeSimone J, Pomerantz R. Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy. *Journal of the American Medical Association*. 1999; 282:1627–1632. [PubMed: 10553788]
- Fischer M, Joos B, Niederost B, Kaiser P, Hafner R, von Wyl V, Ackermann M, Weber R, Gunthard HF. Biphasic decay kinetics suggest progressive slowing in turnover of latently HIV-1 infected cells during antiretroviral therapy. *Retrovirology*. 2008; 5:107. [PubMed: 19036147]
- Gandhi RT, Zheng L, Bosch RJ, Chan ES, Margolis DM, Read S, Kallungal B, Palmer S, Medvik K, Lederman MM, Alatrakchi N, Jacobson JM, Wiegand A, Kearney M, Coffin JM, Mellors JW, Eron JJ. The effect of raltegravir intensification on low-level residual viremia in HIV-infected patients on antiretroviral therapy: a randomized controlled trial. *PLoS Med*. 2010; 7(8)
- Geretti AM, Doyle T, Smith C, Garcia A, Labbett W, Johnson M, Phillips A. Association between low-level viremia below 50 copies/ml and risk of virologic rebound in HIV-infected patients receiving HAART. 17th annual Conference on Retroviruses and Opportunistic Infections (CROI 2010); 2010. Abstract 505
- Hatano H, Delwart EL, Norris PJ, Lee TH, Dunn-Williams J, Hunt PW, Hoh R, Stramer SL, Linnen JM, McCune JM, Martin JN, Busch MP, Deeks SG. Evidence for persistent low-level viremia in individuals who control human immunodeficiency virus in the absence of antiretroviral therapy. *J Virol*. 2009; 83:329–35. [PubMed: 18945778]
- Hatano H, Delwart EL, Norris PJ, Lee TH, Neilands TB, Kelley CF, Hunt PW, Hoh R, Linnen JM, Martin JN, Busch MP, Deeks SG. Evidence of persistent low-level viremia in long-term HAART-suppressed, HIV-infected individuals. *AIDS*. 24:2535–9. [PubMed: 20651585]
- Hatano H, Hayes T, Dahl V, Sinclair E, Lee T-H, Hunt P, Palmer S, Busch M, Shacklett B, Deeks S. Raltegravir intensification in antiretroviral-treated patients exhibiting a suboptimal CD4+ T cell response. 17th annual Conference on Retroviruses and Opportunistic Infections (CROI 2010); 2010. Abstract 101LB

- Havlir DV, Bassett R, Levitan D, Gilbert P, Tebas P, Collier AC, Hirsch MS, Ignacio C, Condra J, Gunthard HF, Richman DD, Wong JK. Prevalence and predictive value of intermittent viremia with combination hiv therapy. *Jama*. 2001; 286:171–9. [PubMed: 11448280]
- Havlir DV, Strain MC, Clerici M, Ignacio C, Trabattoni D, Ferrante P, Wong JK. Productive infection maintains a dynamic steady state of residual viremia in human immunodeficiency virus type 1-infected persons treated with suppressive antiretroviral therapy for five years. *J Virol*. 2003; 77:11212–9. [PubMed: 14512569]
- Kaiser P, Joos B, Niederost B, Weber R, Gunthard HF, Fischer M. Productive human immunodeficiency virus type 1 infection in peripheral blood predominantly takes place in CD4/CD8 double-negative T lymphocytes. *J Virol*. 2007; 81:9693–706. [PubMed: 17609262]
- Katzenstein DA, Hammer SM, Hughes MD, Gundacker H, Jackson JB, Fiscus S, Rasheed S, Elbeik T, Reichman R, Japour A, Merigan TC, Hirsch MS. The relation of virologic and immunologic markers to clinical outcomes after nucleoside therapy in HIV-infected adults with 200 to 500 CD4 cells per cubic millimeter. AIDS Clinical Trials Group Study 175 Virology Study Team. *N Engl J Med*. 1996; 335:1091–8. [PubMed: 8813039]
- Kern D, Collins M, Fultz T, Detmer J, Hamren S, Peterkin JJ, Sheridan P, Urdea M, White R, Yeghiazarian T, Todd J. An enhanced-sensitivity branched-DNA assay for quantification of human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol*. 1996; 34:3196–202. [PubMed: 8940471]
- Kumar AM, Borodowsky I, Fernandez B, Gonzalez L, Kumar M. Human immunodeficiency virus type 1 RNA Levels in different regions of human brain: quantification using real-time reverse transcriptase-polymerase chain reaction. *J Neurovirol*. 2007; 13:210–24. [PubMed: 17613711]
- Lelie PN, van Drimmelen HA, Cuypers HT, Best SJ, Stramer SL, Hyland C, Allain JP, Monchamont P, Defer C, Nubling M, Glauser A, da Silva Cardoso M, Viret JF, Lankinen MH, Grillner L, Wirthmuller U, Coste J, Schottstedt V, Masecar B, Dax EM. Sensitivity of HCV RNA and HIV RNA blood screening assays. *Transfusion*. 2002; 42:527–36. [PubMed: 12084160]
- Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. *Nucleic Acids Res*. 2002; 30:1292–305. [PubMed: 11884626]
- Maldarelli F, Palmer S, King MS, Wiegand A, Polis MA, Mican J, Kovacs JA, Davey RT, Rock-Kress D, Dewar R, Liu S, Metcalf JA, Rehm C, Brun SC, Hanna GJ, Kempf DJ, Coffin JM, Mellors JW. ART suppresses plasma HIV-1 RNA to a stable set point predicted by pretherapy viremia. *PLoS Pathog*. 2007; 3:e46. [PubMed: 17411338]
- Mavigner M, Delobel P, Cazabat M, Dubois M, L'Faqihi-Olive FE, Raymond S, Pasquier C, Marchou B, Massip P, Izopet J. HIV-1 residual viremia correlates with persistent T-cell activation in poor immunological responders to combination antiretroviral therapy. *PLoS One*. 2009; 4:e7658. [PubMed: 19876401]
- McClure P, Curran R, Boneham S, Ball JK. A polymerase chain reaction method for the amplification of full-length envelope genes of HIV-1 from DNA samples containing single molecules of HIV-1 provirus. *J Virol Methods*. 2000; 88:73–80. [PubMed: 10921844]
- Mellors JW, Rinaldo CR Jr, Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science*. 1996; 272:1167–1170. [PubMed: 8638160]
- Nuovo GJ. In situ strand displacement amplification: an improved technique for the detection of low copy nucleic acids. *Diagn Mol Pathol*. 2000; 9:195–202. [PubMed: 11129443]
- O'Brien WA, Hartigan PM, Martin D, Esinhart J, Hill A, Benoit S, Rubin M, Simberkoff MF, Hamilton JD, AIDS.; t.V.C.S.G.o. Changes in plasma HIV-1 RNA and CD4+ lymphocyte count relative to treatment and progression to AIDS. *New England Journal of Medicine*. 1996; 334:426–431. [PubMed: 8552144]
- Ostrowski SR, Katzenstein TL, Pedersen BK, Gerstoft J, Ullum H. Residual viraemia in HIV-1-infected patients with plasma viral load ≤ 20 copies/ml is associated with increased blood levels of soluble immune activation markers. *Scand J Immunol*. 2008; 68:652–60. [PubMed: 19055701]
- Palmer S, Wiegand AP, Maldarelli F, Bazmi H, Mican JM, Polis M, Dewar RL, Planta A, Liu S, Metcalf JA, Mellors JW, Coffin JM. New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol*. 2003; 41:4531–6. [PubMed: 14532178]

- Pas S, Rossen JW, Schoener D, Thamke D, Pettersson A, Babel R, Schutten M. Performance evaluation of the new Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 test version 2.0 for quantification of human immunodeficiency virus type 1 RNA. *J Clin Microbiol.* 48:1195–200. [PubMed: 20164281]
- Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ. The challenge of finding a cure for HIV infection. *Science.* 2009; 323:1304–7. [PubMed: 19265012]
- Ruiz L, Romeu J, Clotet B, Balague M, Cabrera C, Sirera G, Ibanez A, Martinez-Picado J, Raventos A, Tural C, Segura A, Foz M. Quantitative HIV-1 RNA as a marker of clinical stability and survival in a cohort of 302 patients with a mean CD4 cell count of $300 \times 10^6/l$. *AIDS.* 1996; 10:F39–44. [PubMed: 8883577]
- Saag MS, Holodniy M, Kuritzkes DR, O'Brien WA, Coombs R, Poscher ME, Jacobsen DM, Shaw GM, Richman DD, Volberding PA. HIV viral load markers in clinical practice. *Nature Medicine.* 1996; 2:625–629.
- Saha BK, Tian B, Bucy RP. Quantitation of HIV-1 by real-time PCR with a unique fluorogenic probe. *J Virol Methods.* 2001; 93:33–42. [PubMed: 11311341]
- Schockmel GA, Yerly S, Perrin L. Detection of low HIV-1 RNA levels in plasma. *J Acquir Immune Defic Syndr Hum Retrovirol.* 1997; 14:179–83. [PubMed: 9052729]
- Simmonds P, Balfe P, Peutherer JF. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cell and at low-copy numbers. *Journal of Virology.* 1990; 64:864–872. [PubMed: 2296085]
- Spann W, Pachmann K, Zabnienska H, Pielmeier A, Emmerich B. In situ amplification of single copy gene segments in individual cells by the polymerase chain reaction. *Infection.* 1991; 19:242–4. [PubMed: 1917036]
- Sun R, Ku J, Jayakar H, Kuo JC, Brambilla D, Herman S, Rosenstrauss M, Spadoro J. Ultrasensitive reverse transcription-PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol.* 1998; 36:2964–9. [PubMed: 9738051]
- Swanson P, Huang S, Abravaya K, de Mendoza C, Soriano V, Devare SG, Hackett J Jr. Evaluation of performance across the dynamic range of the Abbott RealTime HIV-1 assay as compared to VERSANT HIV-1 RNA 3.0 and AMPLICOR HIV-1 MONITOR v1.5 using serial dilutions of 39 group M and O viruses. *J Virol Methods.* 2007; 141:49–57. [PubMed: 17184853]
- Tang N, Huang S, Salituro J, Mak WB, Cloherty G, Johanson J, Li YH, Schneider G, Robinson J, Hackett J Jr. Swanson P, Abravaya K. A RealTime HIV-1 viral load assay for automated quantitation of HIV-1 RNA in genetically diverse group M subtypes A–H, group O and group N samples. *J Virol Methods.* 2007; 146:236–45. [PubMed: 17707519]
- Trono D, Van Lint C, Rouzioux C, Verdin E, Barre-Sinoussi F, Chun TW, Chomont N. HIV persistence and the prospect of long-term drug-free remissions for HIV-infected individuals. *Science.* 329:174–80. [PubMed: 20616270]
- van Gemen B, Kievits T, Schukink R, van Strijp D, Malek LT, Sooknanan R, Huisman HG, Lens P. Quantification of HIV-1 RNA in plasma using NASBA during HIV-1 primary infection. *J Virol Methods.* 1993; 43:177–87. [PubMed: 8366168]
- Wieland U, Suhr H, Salzberger B, Eggers HJ, Braun RW, Kuhn JE. Quantification of HIV-1 proviral DNA and analysis of genomic diversity by polymerase chain reaction and temperature gradient gel electrophoresis. *J Virol Methods.* 1996; 57:127–39. [PubMed: 8801225]
- Youno J. Direct polymerase chain reaction for detection of human immunodeficiency virus in blood spot residues on filter paper after elution of antibodies: an adjunct to serological surveys for estimating vertical transmission rates among human immunodeficiency virus antibody-positive newborns. *J Clin Microbiol.* 1993; 31:1364–7. [PubMed: 8501243]
- Youno J, Conroy J. A novel polymerase chain reaction method for detection of human immunodeficiency virus in dried blood spots on filter paper. *J Clin Microbiol.* 1992; 30:2887–92. [PubMed: 1452659]
- Yukl SA, Gianella S, Sinclair E, Epling L, Li Q, Duan L, Choi AL, Girling V, Ho T, Li P, Fujimoto K, Lampiris H, Hare CB, Pandori M, Haase AT, Gunthard HF, Fischer M, Shergill AK, McQuaid K, Havlir DV, Wong JK. Differences in HIV burden and immune activation within the gut of HIV-

positive patients receiving suppressive antiretroviral therapy. *J Infect Dis.* 2010; 202:1553–61. [PubMed: 20939732]

Zazzi M, Romano L, Brasini A, Valensin PE. Nested polymerase chain reaction for detection of human immunodeficiency virus type 1 DNA in clinical specimens. *J Med Virol.* 1992; 38:172–4. [PubMed: 1287130]

Zazzi M, Romano L, Brasini A, Valensin PE. Simultaneous amplification of multiple HIV-1 DNA sequences from clinical specimens by using nested-primer polymerase chain reaction. *AIDS Res Hum Retroviruses.* 1993; 9:315–20. [PubMed: 8512746]

Figure 1A-B.

Efficient recovery of HIV over a wide range of copy numbers after concentration from 30ml of plasma. In two separate experiments, shown in 1A and 1B, different volumes of HIV-positive plasma of known viral load (measured using the Abbott assay) were spiked into 30ml aliquots of HIV-negative donor plasma to get known copy numbers of HIV. The x-axis shows the $\log_{10}$ of the number of copies added to 30 ml of plasma (input). Virions were then pelleted by centrifugation on iodixanol, resuspended in 1 ml of PBS, and measured again using the Abbott assay. The y-axis shows the $\log_{10}$ of the number of copies in the 1 ml of PBS (output). In 1A, error bars indicate the SEM from two independent replicates.

Table 1

Recovery of low copy numbers of HIV after dilution in donor plasma and centrifugation

subject	Input (copies)	Input (copies/ml)	Output (copies)	Output (copies/ml)
A467	15	0.5	ND ^a	ND
	15	0.5	ND	ND
	15	0.5	ND	ND
	15	0.5	ND	ND
	15	0.5	6	0.2
A467	30	1	23	0.77
	30	1	15	0.5
	30	1	15	0.5
	30	1	15	0.5
	30	1	9	0.3
A470	15	0.5	26	0.87
	15	0.5	64	2.13
	15	0.5	25	0.83
	15	0.5	21	0.7
	15	0.5	44	1.47
A470	30	1	56	1.87
	30	1	77	2.57
	30	1	61	2.03
	30	1	64	2.13
	30	1	74	2.47

^aND = not detected

Table 2

Plasma HIV-1 RNA, as measured using the modified assay, in subjects with viral load <40 copies/ml

subject	week 0 copy/ml	week 2 copy/ml
A187	0.4	
A191	10.1	
A193	1.4	
A194	1.2	
A196	2.1	
A185	11.4	0.5
A186	6.3	4.5
A188	0.9	ND ^a
A189	4.8	1.7
A190	ND	0.5
A195	0.5	ND
A198	3.1	1.8

^aND = not detected