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Protocol

Immunomagnetic quantitative immuno-PCR for detection of less than one HIV-1 virion

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A B S T R A C T

Methods that allow the accurate and reliable detection of ultra-low molecular levels of proteins using techniques such as quantitative immuno-PCR (qIPCR) have demonstrated numerous technical difficulties. Protein detection methods lose specificity when the protein target is immersed within a matrix of thousands of molecules having wide ranges of concentrations. In addition, sensitivities are limited because of high background signals.

To validate the performance of an immunomagnetic bead qIPCR method designed to remove the 'matrix' effect for HIV-1 p24 antigen detection, regression analyses were performed using samples from patients infected with HIV-1 diluted to approximately 100–1000, 10–100, 1–10, and 0.1–1.0 HIV-1 p24 Ag molecules/reaction. The number of HIV-1 p24 Ag molecules was derived from quantified HIV-1 RNA determinations. The modified immunomagnetic qIPCR bead assay demonstrated a limit of quantification of 10–100 HIV-1 p24 molecules per reaction, with an average correlation coefficient of 0.948 ± 0.028 over a 4-log dynamic range. This method detects less than one HIV-1 virion (a limit of detection unreported previously for HIV-1), and thus, has the potential to identify HIV-1 infection and monitor the dynamics of the disease course earlier than nucleic acid methods.

The immunomagnetic qIPCR bead assay is a simple and inexpensive method for ultra-low protein detection of infectious agents, toxins, and cancer markers at a level unrecognized previously using any enzymatic or molecular method.

1. Introduction

The application of technologies that allow the accurate and reliable detection of ultra-low molecular levels of proteins using methods such as the quantitative immuno-polymerase chain reaction method (qIPCR) has significantly lagged behind the highly quantitative and sensitive molecular detection method for nucleic acids. Technical difficulties with the IPCR method have been presented in the scientific literature (McKie et al., 2002a,b) and occur primarily because of non-specific amplification (background noise) which significantly limits the sensitivity. It has been over 15 years since the first description of the conventional IPCR method (Sano et al., 1992); yet because of the technical difficulties, the IPCR method remains a research tool, unable to attain the performance characteristics required for use as a clinical diagnostic test.

The IPCR method has been shown to possess exquisite sensitivity and has been applied for the detection of a number of important analytes, including bacteria, viruses, toxins, and cancer markers (Niemeyer et al., 2005; Barletta, 2006; Barletta and Bartolome, 2007; Adler et al., 2008). Proteins of the human immunodeficiency virus (specifically HIV-1 p24 antigen (Ag)) have been detected by IPCR in the attogram (ag)/mL range and have been shown to offer earlier detection as compared with RT-PCR for HIV-1 RNA detection (Barletta et al., 2004). Other highly sensitive immuno-molecular assays such as the bio-barcode assay (BCA), use multiple particle types (e.g., silica, iron oxide magnetic and gold nanoparticles) and circumvent the use of PCR amplification as the final, signal-generating step (Nam et al., 2003, 2004, 2005). The BCA method has been used recently for the detection of HIV-1 p24 Ag (Tang et al., 2007) and was shown to display a limit of detection (LOD) equal to approximately 100 femtogram (fg)/mL (500 fM (femtoMolar)) which equates to 2000 HIV-1 RNA copies/mL. However, this level of virus detection is higher than that currently available through nucleic acid tests (NAT) which routinely detect 50–500 HIV-1 RNA copies/mL, or modified versions of the NAT assay that detect positives down to 12–25 HIV-1 RNA copies/mL (Roche Amplicor HIV-1 Monitor Test; version 1.5) (Yerly et al., 1999, 2000; Piwowar-Manning et al., 2003) or single-copy HIV-1 RNA/mL detection (Palmer et al., 2003). Additionally, conventional bio-barcode detection methods require expensive and sophisticated instrumentation (e.g., light-scattering measurement instruments, microarrays) and

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specialized procedures for labeling probes (e.g., gold nanoparticles). Other modifications of the assay utilize thin layer chromatography and graphic processing for colorimetric quantification (Nam et al., 2007). These features increase cost, limit portability, and complicate assay implementation.

A well-known limitation of protein detection methods is non-specificity which is readily apparent when testing plasma where the protein target is immersed in the heterogeneous milieu of thousands of protein molecules having a wide range of concentrations. That is, the LOD of many assays is negatively influenced when assessing protein analytes in plasma as compared with dilution of those proteins in a buffer (Linnet and Boyd, 2005; Rotmensch and Cole, 2000). It has also been demonstrated that the presence of high concentrations of HIV-1 anti-p24 antibody (Ab) will complex with and obscure detection of HIV-1 p24 Ag present in the sample (Lange et al., 1987; De Wolf et al., 1987; Pedersen et al., 1987; Schüpbach, 2002).

All samples used in these studies contained high concentrations of HIV-1 anti-p24 Ab (i.e., an average concentration of 130–164 ng/mL HIV-1 anti-p24 Ab). Samples from patients infected with HIV-1 may also have high levels of rheumatoid factor-like Abs which bridge the capture and tracer Abs in immunoassays, resulting in false positive results (Schüpbach, 2002; Gutiérrez et al., 1995). Finally, as a protein target, HIV-1 p24 Ag is not quantified precisely (Summers et al., 1992; Layne et al., 1992; Vogt and Simon, 1999), especially as it relates to HIV-1 RNA copy number at very low concentrations (Prado et al., 2004); and therefore, can only be approximated within a numerical range of 1200–3000 molecules per HIV-1 virion (Summers et al., 1992; Layne et al., 1992; Vogt and Simon, 1999).

Concerning the relationship between free and virion-associated HIV-1 p24 Ag and RNA levels, it is rational to assert that the presence of both molecules parallel each other. That is, both molecules are viral components and are markers for particle-based assays. Detection of either HIV-1 RNA or HIV-1 p24 represents the presence of viral associated components, as well as viral components released from cell destruction. In fact, Tschochner et al. (2007) reported significant correlations between the particle-based quantification methods of HIV-1 RNA copy numbers and HIV-1 p24 Ag amounts ($p = 0.009$) in cell culture supernatants. Differences between the levels of both molecules in biological samples may arise due to the technical difficulties of detection of viral protein which may be damaged, destroyed, or present as aggregations at the plasma membrane (Schüpbach et al., 2006). Our studies used samples from patients infected with HIV-1 and thus we expect that the levels of HIV-1 RNA and HIV-1 p24 would be correlated, as supported by the studies of Tschochner et al. (2007).

Because of the limitations of protein detection methods mentioned above, the objectives of this study were: (1) to significantly reduce non-specific reactions by using magnetic bead technology as the solid support format in the qIPCR method; and (2) to improve the LOD of qIPCR using highly diluted samples and two sequential PCR amplification cycles (i.e., modifications to the method which reduce and/or eliminate sample matrix interference). The aim was to optimize numerous parameters from the conventional IPCR method to define test conditions that would enhance reproducibility using a simple and inexpensive testing platform.

To validate the performance of the immunomagnetic bead qIPCR method, samples were used that parallel the *in vivo* situation of ultra-low levels of HIV-1 p24 antigenemia in the presence of high concentrations of anti-p24 and heterogeneous Ab. Plasma samples of patients infected with HIV-1 with known HIV-1 RNA concentrations of 100–1000 copies/mL (determined using the Roche Amplicor Monitor Test) were selected and used to generate standard curves for the quantification of HIV-1 p24 Ag. Regression analyses were performed on 3–6 replicates of serial dilutions from 10 HIV-1 antibody positive patients with known HIV-1 RNA viral loads. These samples from patients infected with HIV-1 were diluted from 1:100 to 1:100,000 to approximate 100–1000, 10–100, 1–10, and 0.1–1.0 HIV-1 p24 Ag molecules/reaction. The specificity of this method was established by analyses of a total of 36 replicates (3–12 per run) using normal human plasma (NHP) as the negative control. Only samples exhibiting individual exponential amplification slopes ($\geq$2.5) with a correlation coefficient $\geq$0.9 over a 4-log dynamic range were considered positive. Fig. 1 shows the format and principle of the qIPCR method.

2. Materials and methods

2.1. Preparation of primers, probe, and 181 bp biotinylated DNA template (Biot-181)

For the synthesis of Biot-181, PCR was performed in a final 50 μL volume in a reaction mix of 1× Accuprime Buffer I with 1.5 mM $MgCl_2$, 0.20 μM of the 5′ biotinylated forward primer (5′-Biotin-GGA-TGA-ACC-TGT-GGC-ATT-TGT-GCT-3′), 0.20 μM reverse primer (5′-GCC-ATG-TAC-CCG -CGT-ATC-GTT-TCA-3′), 10 μg/mL λ DNA (for synthesis of Biot-181 template), and 1 μL (Accuprime, Invitrogen) Taq Polymerase. PCR amplification cycles for Biot-181 synthesis were 1× (95 °C for 3 min); 35× (95 °C for 15 s, and 62 °C for 1 min). The limit of detection, limit of quantification (LOQ), linearity, dynamic range, and correlation coefficient (CC) of the synthesized Biot-181 were determined. The Biot-181 can be used as a control DNA template for quality control of the qPCR reaction. The sequence of the Taqman probe used for qPCR was FAM46-CGC TCT GTC ATT TCG GCT GCC ACA CC-BHQ1.

2.2. HIV-1 anti-p24 Ab-coated (capture) magnetic bead preparation

100 μL (end use of 20 reactions) of stock magnetic beads (Dynabeads® M-280 Tosylactivated 2.8 μm diameter, stock concentration 30 mg/mL; Dynal Biotech, 142.03) in separate tubes with 1 mL 0.1 M Buffer B buffer (0.1 M borate buffer, pH 9.5) were used. The magnetic beads were captured using the magnetic plate (V&P Scientific, VP771E), and 200 μL 0.1 M Buffer B was added for a total volume of 300 μL. Capture antibodies (13B6 and 13G4: HIV-1 anti-p24 mouse monoclonal antibodies: Institute of Human Virology uQuant Core Facility) were used at a concentration of 30 μg/mL. Magnetic beads and coating antibodies were incubated for 12–18 h at 4 °C with constant rocking.

The magnetic beads were captured using a magnetic plate, supernatant was aspirated, and the beads were washed with 1× Buffer C (PBS, pH 7.4 with 0.1% BSA). Buffer C was removed and replaced with 1 mL Buffer D (0.2 M Tris, pH 8.5, 0.1% BSA). Incubation is continued for 2 h at room temperature with constant rocking. Buffer D blocks free tosyl groups and is required to prevent subsequent binding of other non-specific molecules to free tosyl groups present on bead surfaces. After incubation, the magnetic beads were captured using the magnetic plate, supernatant was removed, and 1 mL of Buffer D was added. Beads were captured, washed and stored at 4 °C for 2 weeks.

2.3. Pre-treatment (dilution and heating) of patient plasma samples

Patient plasma samples were diluted 1:100 in Sample Lysis Buffer (1×: 0.5% Triton X-100, 0.2% azide in PBS, pH 7.4) and heated at 100 °C for 5 min. Vials were kept on ice until serially diluted from 1:1000 to 1:100,000 in Sample Lysis Buffer. The negative control (normal human plasma) was diluted and heated in an identical manner as test samples. To reduce random and systematic error, a minimum of 10 μL was used for serial dilutions. All dilutions were

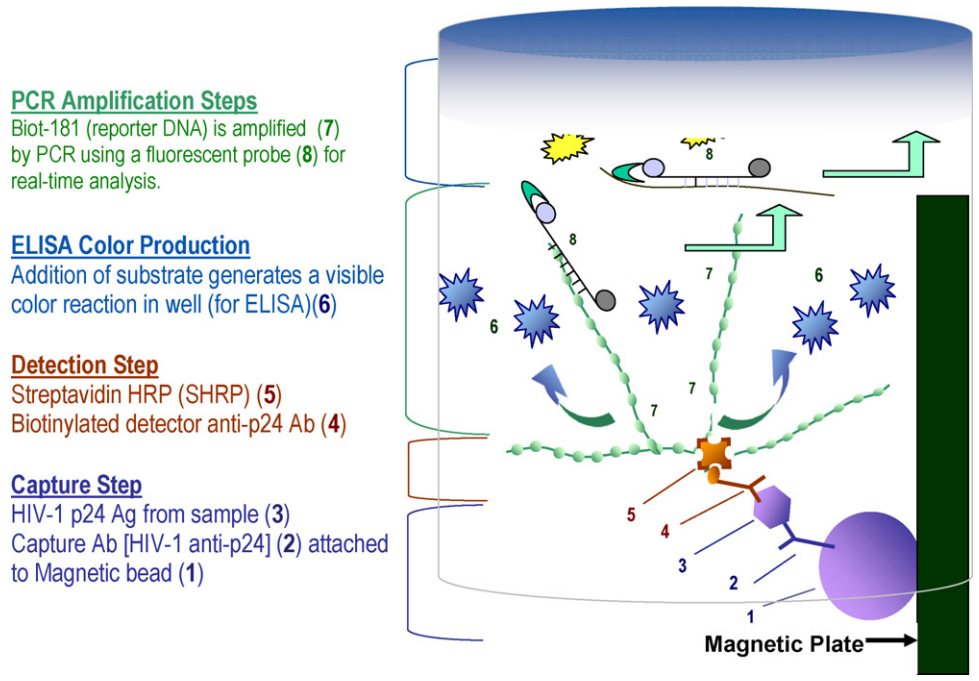


Fig. 1. The magnetic bead quantitative immuno-polymerase chain reaction method. Capture anti-HIV-1 p24 antibody (2), adsorbed to the magnetic bead (1), is used to capture HIV-1 p24 antigen (Ag) (3). Streptavidin-horseradish peroxidase (SHRP) (5) bridges between a biotinylated detector anti-HIV-1 p24 Ab (4) and biotinylated reporter DNA (Biot-181)(7). After addition of SHRP, the ELISA may be performed by addition of substrate (6). Biot-181 is amplified by PCR using a fluorescent probe (8) for real-time (quantitative) PCR analysis (qPCR).

mixed at least 30 s before aliquoting. Dilutions were used within a 4–6 h period, and kept at 4 °C. Plasma from patients infected with HIV-1 typically exhibits extremely high anti-HIV-1 Ab titers. The plasma sample was heated to cause dissociation and secondary structure destruction of patient HIV-1 anti-p24 Ab with patient HIV-1 p24 Ag (Schüpbach, 2002). Heating plasma samples at lower dilutions will result in coagulation of plasma proteins and sample clotting. Samples must be kept on ice to avoid re-association of dissociated patient HIV-1 anti-p24 Ab fragments with patient HIV-1 p24 Ag before addition to anti-p24 Ab-coated (capture) magnetic beads.

2.4. Magnetic bead qIPCR protocol

Capture plates (96-well 0.2 mL PCR Flat Plate: Genemate #%-1052-1) were pre-washed with Wash Buffer (ELISA Plate wash, Perkin Elmer, NEA107) to hydrate wells before the addition of magnetic beads. Although the selected capture plate is polypropylene and relatively hydrophobic which reduces adherence of ionically charged molecules, it is important to pre-wash the plate with Wash Buffer which contains detergent (Tween-20), to further reduce electrostatic adherence of charged molecules. 50 μL of the HIV-1 p24 Ab-coated magnetic bead suspension was added in Buffer D to each test well for each sample over a 3-log dilution range (3–6 replicates) with normal human plasma as the negative control (3–6 replicates). Additional sets of wells were set up for the ELISA assay over a 3-log dilution range with negative control (in duplicate). ODs of each well were read at 630 nm to determine an estimate of pre-wash bead concentration.

2.5. Manual washing procedure for magnetic beads

150 μL Wash Buffer was added to samples to resuspend the magnetic beads (which begin to settle during incubation); beads were captured using the magnetic plate. Supernatant was aspirated carefully so as not to disrupt and/or aspirate beads. Beads were rinsed by adding 150 μL Wash Buffer to samples without resuspension of beads and the supernatant was aspirated. At times, the beads must be resuspended by aligning the magnet on the capture plate in a different location. If the beads remain fixed to the side of the well and do not resuspend in wash solution when realigning magnet, they were gently resuspended using Wash Buffer.

2.6. Determine percent recovery of magnetic beads

It is important to be able to determine if a significant percentage of the beads were lost during the washing process. In our studies, even with careful manual washing, approximately 10 ± 0.05% of the original concentration of magnetic beads per well were consistently lost with washes. Optical density (OD) of each well was measured to determine if a significant percentage of beads had been washed away. After all washes, if the individual well OD was not within 10% of the average OD of beads recovered in all wells, that sample well was excluded from further analysis.

The magnetic beads were captured on the magnetic capture plate and the supernatant was aspirated. 50 μL heat-treated and diluted patient plasma samples or normal human plasma as the negative control were added. Incubation was with agitation on a rotator (30 min, RT), followed by washing. 50 μL biotinylated HIV-1 anti-p24 Ab were added to each sample well and incubated with agitation on a rotator (30 min, RT) before washing the beads as described above. Streptavidin-horseradish peroxidase (SHRP) (50 μL at 50 ng/mL in Pierce Starter Block with Tween-20: Pierce Co., 37543) was added to each sample well; incubation was done with agitation on a rotator (30 min, RT). Beads were washed as described above except they were rinsed 2× after streptavidin-HRP reagent incubation. TMB (50 μL) were added to the set of wells used for the ELISA. Beads in TMB (TMB 2-Component Microwell Peroxidase Substrate Kit: KPL, 50-76-00) were removed to a new well for color development at 450 nm (10 min, RT) as proteins adhering to the well may increase non-specific background in the OD reading. The magnetic beads were captured on the magnetic capture plate and the supernatants were aspirated, and transferred to new wells for color development and OD readings.

PCR was performed only if the ELISA ODs demonstrated positive reactions with a dose response and a signal:noise ratio of ≥1.5 for HIV-1 p24 Ag concentrations (3-logs higher than the ELISA assay method LOD). The ELISA is the read-out of the immunological reaction of the IPCR method. Thus, if ELISA signal development is less than optimal, the subsequent PCR amplification cannot compensate for the reduced efficiency of the immunocapture reaction.

Biot-181 template diluted to approximately 10–100 pg/mL in DNA dilution buffer {milliq-H_2O, 4.6 × SSC buffer, 0.5% Roche DNA blocking reagent (Roche Corp., 11096176001), 0.01 M EDTA (pH 8.0), 5 μL sodium heparin, 0.1% Tween-20} was added; followed by incubation with agitation (60 min, room temperature). The concentration of Biot-181 is critical to maintain a significant and specific amplification signal. If too high a concentration of Biot-181 is added, the negative control will be positive, and test samples will not exhibit a dose response (i.e., the majority of sample dilutions will have similar low Ct values). If too low a concentration of Biot-181 is added, the majority of test samples will be negative, and test samples will exhibit no (or a non-linear) dose response (i.e., the majority of sample dilutions will have similar high Ct values).

Beads were washed as described above except rinsed 2× after Biot-181 incubation. 50 μL of milliq-H_2O were added to each well. ODs were read at 630 nm to determine an estimate of post-wash bead concentration (recovery). After all washes, if the individual well OD was not within 10% of the average OD of beads recovered in all wells, that sample well was excluded from analysis.

2.7. PCR amplification round 1 (20 cycles)

Using an automated digital pipettor, 5 μL of the resuspended magnetic beads was transferred to each well in a conical PCR tube containing 20 μL of PCR Master Mix {1× Accuprime Buffer I with 2.0 mM $MgCl_2$ (final 3.5 mM $MgCl_2$), 0.40 μM of the 5′ biotinylated forward primer, 0.40 μM reverse primer, 0.05 μM Taqman probe, 1 mg/mL BSA, and 1 μL (Accuprime) Taq Polymerase} for PCR amplification round 1 (BioRad iCycler;BioRad Corp., Model IQ5). Amplification cycles for both 1 (20 cycles) and 2 (50 cycles) PCR amplifcation rounds were 1× (95 °C for 3 min); 35× (95 °C for 15 s; and 62 °C for 1 min). A Biot-181 control (at 5–50 ng/mL) was run for PCR amplification round 1 and should exhibit Ct = 16–19.

2.8. PCR amplification round 2: (50 cycles)

At 20 cycles, the PCR reaction was stopped, and the PCR plate was removed from the BioRad iCycler. Using an automated digital pipettor, 5 μL of the resuspended PCR reaction mix was transferred to each well in a conical PCR tube containing 20 μL of PCR Master Mix for PCR amplification round 2. After completion of the second PCR amplification, linearity, efficiency, and CC of patient samples were analyzed using the data analysis program of the BioRad iCycler. Only samples exhibiting slopes >2.5 (83.3% efficiency) were used for data interpretation.

3. Results

3.1. Standard curve of 5′ biotinylated-181 DNA template (Biot-181)

PCR amplification of the 5′-biotinylated 181-base-pair DNA reporter template (Biot-181) was optimized. The PCR standard curve of Biot-181 displayed a CC of 0.984, with a limit of quantification of 500 ag/mL (41.3 zeptoMolar (zM)), over a 7-log dynamic range. This LOQ approximates 12.5 molecules of Biot-181 per reaction (data not shown).

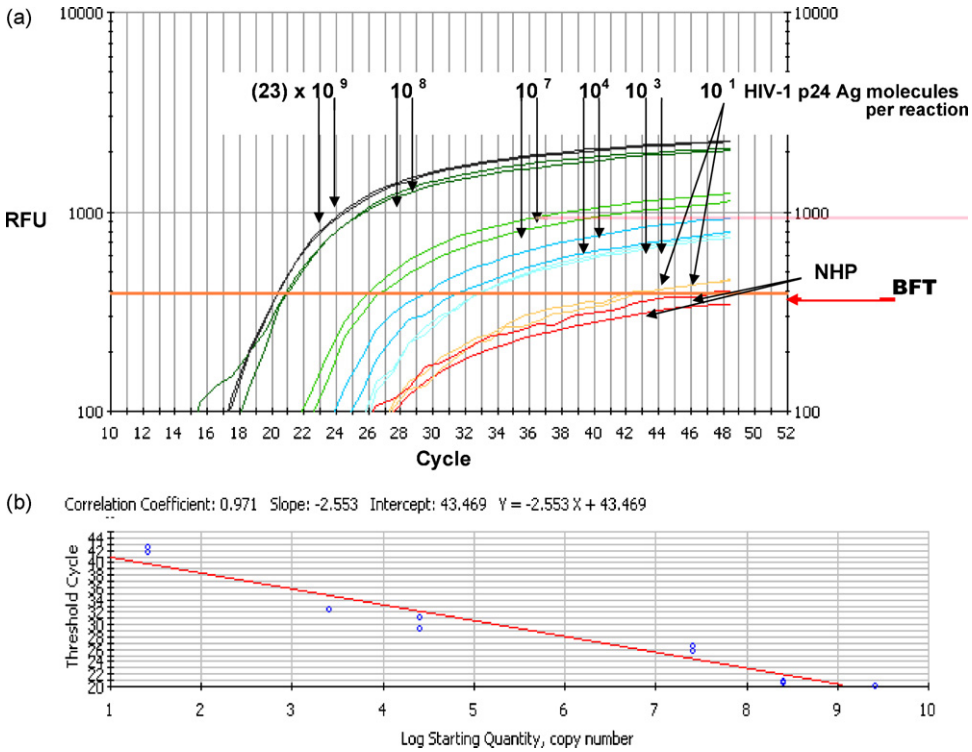


Fig. 2. qIPCR standard curves of HIV-1 p24 Ag from HIV-1 infected cell culture supernatent. qIPCR was performed on serial dilutions of quantified HIV-1 infected cell culture supernatants with known concentrations of HIV-1 p24 Ag. (a) A standard curve was plotted on log-fold dilutions from 23×10^9 to 23×10^1 HIV-1 p24 Ag molecules per reaction (rxn) and analyzed by linear regression. (b) Correlation coefficient of the qIPCR standard curve in (a) with the corresponding linear regression equation. The mean Ct for each sample dilution was determined from 2 replicates. NHP (normal human plasma), CC (correlation coefficient), BFT (background fluorescent threshold) and RFU (relative fluorescent units).

Table 1
HIV-1 virion copy number, HIV-1 p24 Ag concentration, molecular copy number, and molar concentration.

HIV-1 virion number	RNA copies/mL	p24 protein/mL	p24 protein/rxn	#p24 molecules/mL	#p24 molecules/reaction	Molar concentration
25	50	3×10^{-15} equals 3 fg/mL	1.50×10^{-16} equals 150 ag/well	75,000	3750	1.25×10^{-16} equals 12.5 fM
20	40	2.4×10^{-15} equals 2.4 fg/mL	1.2×10^{-16} equals 120 ag/well	60,000	3000	1.0×10^{-16} equals 10 fM
1	2	1.2×10^{-16} equals 120 ag/mL	6.0×10^{-18} equals 6 ag/well	3000	150	0.05×10^{-16} equals 500 aM
0.1	0.2	1.2×10^{-17} equals 12 ag/mL	6.0×10^{-19} equals 600 zg/well	300	15	5.0×10^{-19} equals 50 aM
Immunomagnetic qIPCR						
Limit of Detection						
0.67–0.067	1.3–0.13	0.08–0.008	4–0.4	20–2000	10–100	33.3–333 aM
0.01	0.02	1.2×10^{-18} equals 1.2 ag/mL	6.0×10^{-20} equals 60 zg/well	30	1.5	5.0×10^{-20} equals 5 aM

3.2. qIPCR standard curves of HIV-1 p24 Ag from HIV-1 infected cell culture supernatant

A standard curve by IPCR for HIV-1 p24 Ag quantification was generated using an HIV-1 infected cell culture supernatant, previously quantified by a commercial ELISA. The qIPCR standard curve was performed for every qIPCR analysis. The background fluorescence threshold (BFT) which differentiates negative from positive reactions was set immediately above the negative controls (normal human plasma). Relative adjustment of the BFT sometimes resulted in slight variation in Cts from run to run. The mean Cts for 23×10^9, 23×10^8, 23×10^7, 23×10^4, 23×10^3, and 230 HIV-1 p24 molecules per reaction were 20.3 ± 0.1, 20.9 ± 0.0, 26.3 ± 0.4, 30.5 ± 0.3, 32.6 ± 0.1, and 42.3 ± 0.6, respectively. qIPCR standard curves for HIV-1 p24 Ag consistently demonstrated a limit of quantification below 500 molecules of HIV-1 p24 Ag per reaction. The qIPCR standard curve of HIV-1 p24 Ag displayed a correlation coefficient of 0.971, translating to a limit of quantitation of 184 ag/mL (767 attoMolar (aM)) over an 8-log dynamic range. This LOQ approximates 230 molecules of HIV-1 p24 Ag per reaction or 4600 molecules of HIV-1 p24 Ag per mL (Fig. 2).

3.3. Reduced mean Ct and increased percent detection of ultra-low concentrations of Biot-181 DNA using two PCR amplification rounds

One of the major modifications applied to the conventional IPCR method was to transfer product from the first round of PCR after 20 amplification cycles to a new reaction tube and continue PCR with an additional 50 cycles (in a second PCR amplification round). The rationale behind this approach was that with optimal efficiency, target molecules will have amplified to 2^{20} molecules in the first 20 cycles.

To validate this effect, we performed the second PCR amplification round using dilutions which approximated either a single molecule of Biot-181, or a single molecule of HIV-1 p24 Ag in samples from patients infected with HIV-1. Cts from sample dilutions of Biot-181 DNA at 500 pg, 50 ag, 5 ag, 500 zeptograms (zg)/mL (125×10^6 to 0.125 molecules/reaction), respectively, using either one or two PCR amplification rounds were analyzed. Since the analyses focused on the quantitation of ultra-low levels of target analyte after a second PCR amplification round, data from sample dilutions at relatively higher concentrations (greater than 50 fg/mL) are not shown.

A second round of PCR amplification decreased the sample mean Ct by 12.8–18.9 cycles (Fig. 3c vs. Fig. 3a). The mean Ct for the 50 ag/mL (Biot-181) sample amplified by two PCR amplification rounds was reduced by 18.9 cycles (e.g., from 30.3 ± 0.7 to 11.4 ± 0.3) (Fig. 3c vs. Fig. 3a). The mean Ct for the 5 ag/mL sample (Biot-181) amplified by two PCR amplification rounds was reduced by 12.8 cycles (e.g., from 35.3 ± 4.8 to 22.5 ± 2.2) (Fig. 3c vs. Fig. 3a).

Analysis of ultra-low copy numbers (12.5–0.125 molecules/reaction) Biot-181 demonstrated a dosimetric increase in Ct when subjecting a sample of increasing dilutions to two PCR amplification rounds. The mean Cts for 12.5, 1.25, and 0.125 Biot-181 molecules/reaction were 11.4 ± 0.3, 22.5 ± 2.2, and 27.7 ± 5.7, respectively. The CC of the linear regression line for these 3 sample dilutions was 0.91 (Fig. 3b). In contrast, when using one PCR amplification round, the mean Cts for 12.5, 1.25, and 0.125 Biot-181 molecules/reaction were 30.3 ± 0.7, 35.3 ± 4.8, and not detectable (i.e., no samples crossed the BFT), respectively. A CC could not be derived using one PCR amplification round at ultra-low sample concentrations (50 ag/mL to 500 zg/mL Biot-181) as only two of the three sample dilutions crossed BFT (three dilution data points are required for linear regression analysis and CC determination).

Table 1 is a reference conversion table which shows the calculated HIV-1 virion copy number and HIV-1 p24 Ag concentration, molecular copy number, and molar concentration in samples from patients infected with HIV-1.

Significant increases in the percent detection of ultra-low Biot-181 concentrations were noted when subjecting the sample dilutions to two PCR amplification rounds. For both one and two PCR amplification rounds, 6 of 6 replicates (100%) of 50 ag/mL Biot-181 concentration (12.5 copies/well) were detected. 4 of 6 replicates (67%) of the 5 ag/mL Biot-181 concentration (1.25 copies/reaction) were detected using two PCR amplification rounds, while only 2 of 6 replicates (33%) of this sample concentration were detected using one PCR amplification round. Finally, 2 of 6 replicates (33%) of the 500 zg/mL Biot-181 concentration (0.125 copies/reaction) were detected using two PCR amplification rounds, while 0 of 6 replicates (0%) of this sample concentration was detected using one PCR amplification round (Fig. 3c vs. Fig. 3a).

When using two vs. one PCR amplification rounds, the LOQ for Biot-181 copy number is reduced by 2-logs (from 10,000 down to 100 copies/reaction) while still exhibiting high CC of 0.98 and 0.99, respectively (Fig. 3d).

3.4. High correlation coefficients are derived from linear regression of qIPCR standard curves using samples from patients infected with HIV-1

Individual standard curves were derived for 10 samples from patients infected with HIV-1 which had been quantified previously for HIV-1 RNA viral copy numbers by RT-PCR (Roche Amplicor HIV-1 Monitor Test). It should be noted that samples used in these studies contain high concentrations of a heterogeneous population of anti-HIV-1 Abs. An additional complicating factor is that anti-HIV-1 p24 Abs typically represent a majority of anti-HIV-1 Abs in patient serum, and have nearly twice the titer of other antibodies (Constantine et al., 2005).

Standard curves were generated using samples from patients infected with HIV-1 diluted to ultra-low concentrations ranging from 10^3–10^0 HIV-1 p24 Ag molecules per reaction. The number of HIV-1 p24 Ag molecules were derived from quantified HIV-1 RNA determinations using the Roche Amplicor HIV-1 Monitor Test. The rationale behind this approach was that non-specific immunologic interactions, which may occur during the ELISA portion of the IPCR method and that are enhanced by the presence of large quantities of heterogeneous plasma proteins, have been reduced by high sample dilution. The range of HIV-1 p24 Ag molecules may vary as it is an estimation (1200–3000 per HIV-1 virion) based on physical, chemical, and biological assays (Summers et al., 1992; Layne et al., 1992; Vogt and Simon, 1999).

Fig. 4 shows a typical example of a standard curve generated from a patient sample diluted to ultra-low HIV-1 p24 Ag copy numbers, and analyzed after two rounds of PCR amplification. In Fig. 4a, 3/5 (60%) were detected as positive for 89 and 8.9 HIV-1 p24 Ag molecules/reaction with mean Cts of 25.4, and 27.8, respectively. 4/6 (67%) were detected as positive for 0.89 HIV-1 p24 Ag molecules/reaction with mean Cts of 32.7. 66% (8/12) of the negative control replicates were gated below the BFT (Fig. 4a). Although the qIPCR standard curve displayed a CC of 0.921 (Fig. 4b), the LOQ for this standard curve was determined to be 89 HIV-1 p24 molecules/reaction (296 aM; 1780 molecules/mL) because the sample dilutions of 8.9 and 0.89 HIV-1 p24 molecules/reaction exhibited amplification slopes <2.5 (i.e., 40 and 16% efficiency for the 8.9 and 0.89 HIV-1 p24 molecules/reaction samples, respectively).

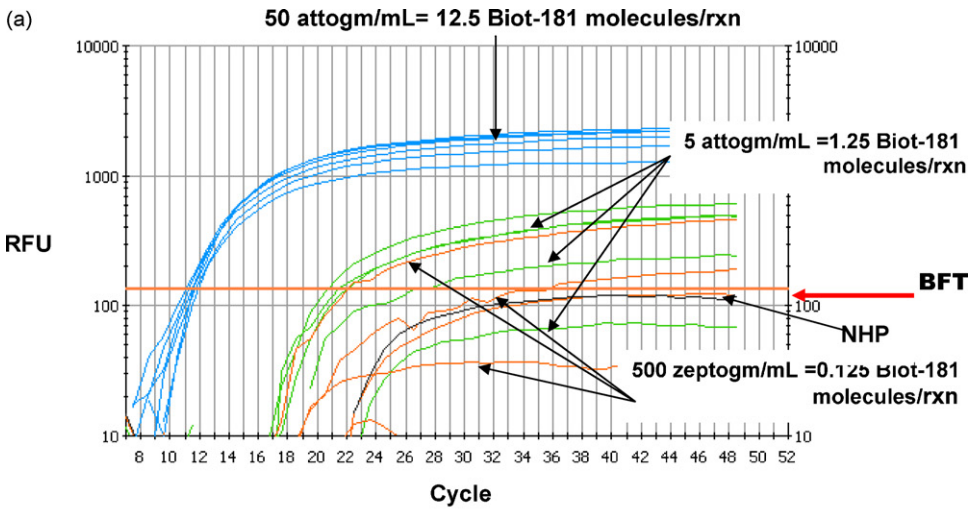
Table 2 summarizes the mean Ct, percent positive and negative samples, and CC for all samples from patients infected with HIV-1 analyzed in all qIPCR runs after two PCR amplification rounds. In summary, regression analyses were performed on 3–6 replicates of serial dilutions from 10 HIV-1 antibody positive patient samples with known HIV-1 RNA viral loads. These samples from patients infected with HIV-1 were diluted from 1:100 to 1:100,000 to approximate 100–1000, 10–100, 1–10, and 0.1–1.0 HIV-1 p24 Ag molecules/reaction. The specificity of this method was established by analyses of a total of 36 replicates (3–12 per run) using normal human plasma as the negative control. Only samples exhibiting exponential amplification slopes $\geq$2.5 (equal to $\geq$83.3% efficiency) were determined to be positive. The modified immunomagnetic qIPCR bead assay demonstrated a limit of quantification of (10–100) HIV-1 p24 molecules per reaction, with an average correlation coefficient of 0.948 ± 0.028 over a 4-log dynamic range.

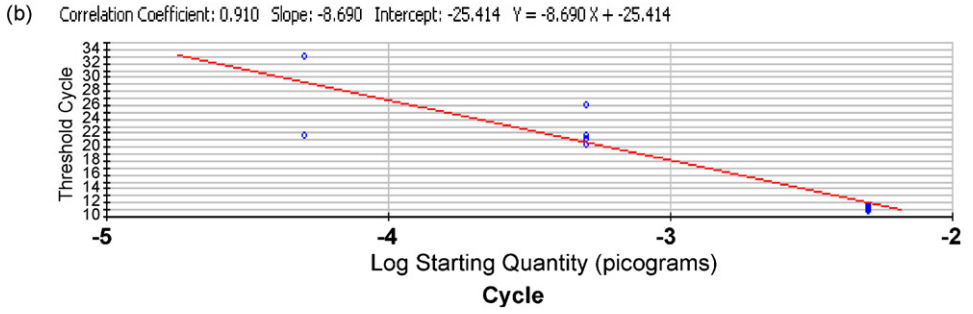
4. Discussion

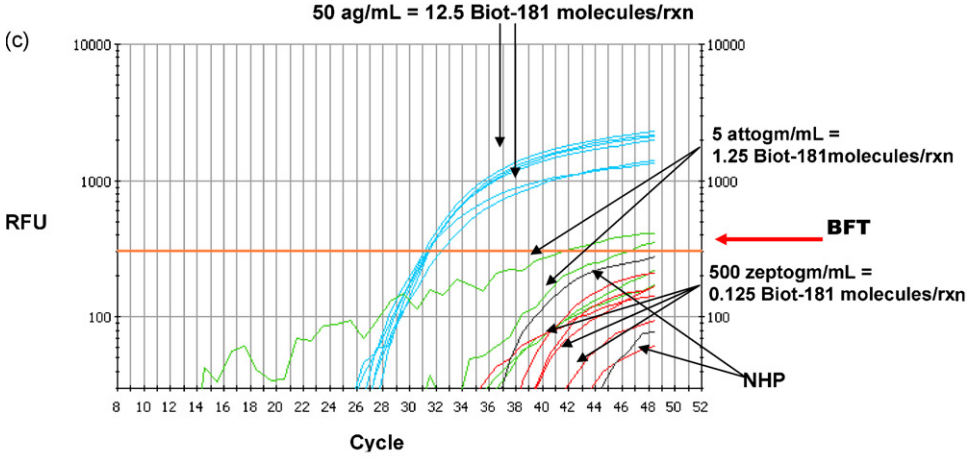
For analysis of human proteins to assess infection or disease, the sample medium which is most often used for analysis is human serum or plasma. Yet, analysis of proteins in these media is problematical because of the disproportionately large amount of proteins such as albumin (55%), the tremendous heterogeneity of glycoproteins, and the wide dynamic range of all other proteins. Potentially interfering substances include high concentrations of lipids, anticoagulants, antibodies, molecular degradation products, hemoglobin, etc. In fact, a detailed protocol for evaluation of interferences in sample analyses has been published by NCCLS (2002). In relation to immunoassays when detecting antigen, interference from specific antibodies present in the test sample must also be recognized. Several authors have documented 5–10% interference in the determination of immunoassay results in samples tested because of the presence of heterophilic Abs, or in patients presenting with autoimmune disease (Ismail et al., 2002; Marks, 2002). In the present study, we have applied the immunomagnetic qIPCR method to individual samples of a dilution series because reports in the literature document conditions where controls perform as expected by the assay, whereas patient samples (due to matrix interferences) do not (Linnet and Boyd, 2005, pp. 367).

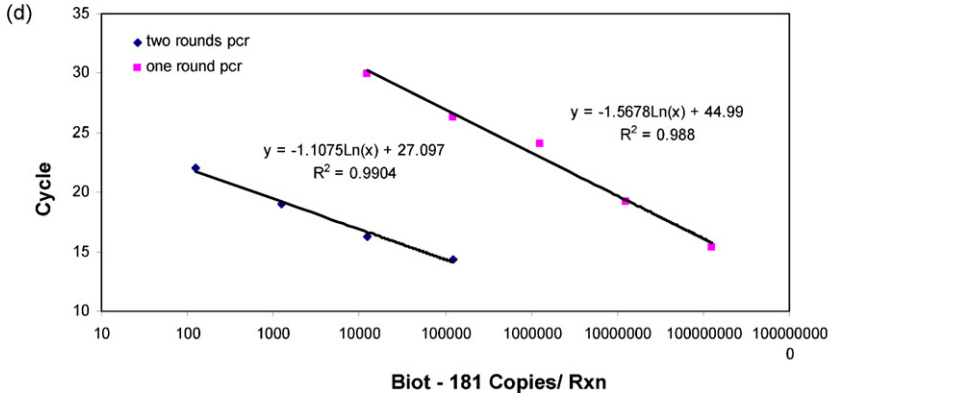
One problem with the iCycler data analyses is that the instrument calculates percent efficiency of the run as an average of all curves in a run. This analysis is an oversimplified approach, since the amplification efficiencies may vary considerably as the input concentrations change for individual samples (Tichopad et al., 2003), and has the effect of allowing specific samples to be considered 'positive' solely because they cross the flurorescent threshold, although the sample has not amplified with the expected efficiency of an optimized PCR reaction. Therefore, our determination of acceptable amplification efficiency used the reaction kinetics of a single sample.

As described by Liu and Saint (2002), determination of amplification efficiency is based on the absolute fluorescence increase in single reaction kinetics data. In this method, the portion of the data array believed to be exponentially behaving is taken, log-transformed and plotted. The authors consider the slope of the line as the individual sample's amplification efficiency. To counteract the averaging of sample efficiencies by the iCycler analysis {and as suggested by Liu and Saint (2002)}, we have separately determined the individual slopes of each PCR sample amplification curve in the run. The slope of each PCR sample amplification was calculated as the slope of the straight line that is tangent to the PCR sample amplification curve during exponential amplification (cycles 1–20 of the second PCR round). Our criterion for acceptance of the line as significantly different from the background fluorescent threshold was that the slope of each PCR sample amplification curve must exhibit a slope $\geq$2.5 (equal to an efficiency of $\geq$83.3%). The calculated effi-


(a)
50 attogm/mL= 12.5 Biot-181 molecules/rxn
5 attogm/mL =1.25 Biot-181 molecules/rxn
BFT
NHP
500 zeptogm/mL =0.125 Biot-181 molecules/rxn
RFU
Cycle


(b)
Correlation Coefficient: 0.910 Slope: -8.690 Intercept: -25.414 Y = -8.690 X + -25.414
Threshold Cycle
Log Starting Quantity (picograms)
Cycle


(c)
50 ag/mL = 12.5 Biot-181 molecules/rxn
5 attogm/mL = 1.25 Biot-181molecules/rxn
BFT
500 zeptogm/mL = 0.125 Biot-181 molecules/rxn
NHP
RFU
Cycle


(d)
two rounds pcr
one round pcr
y = -1.5678Ln(x) + 44.99
R2 = 0.988
y = -1.1075Ln(x) + 27.097
R2 = 0.9904
Cycle
Biot - 181 Copies/ Rxn

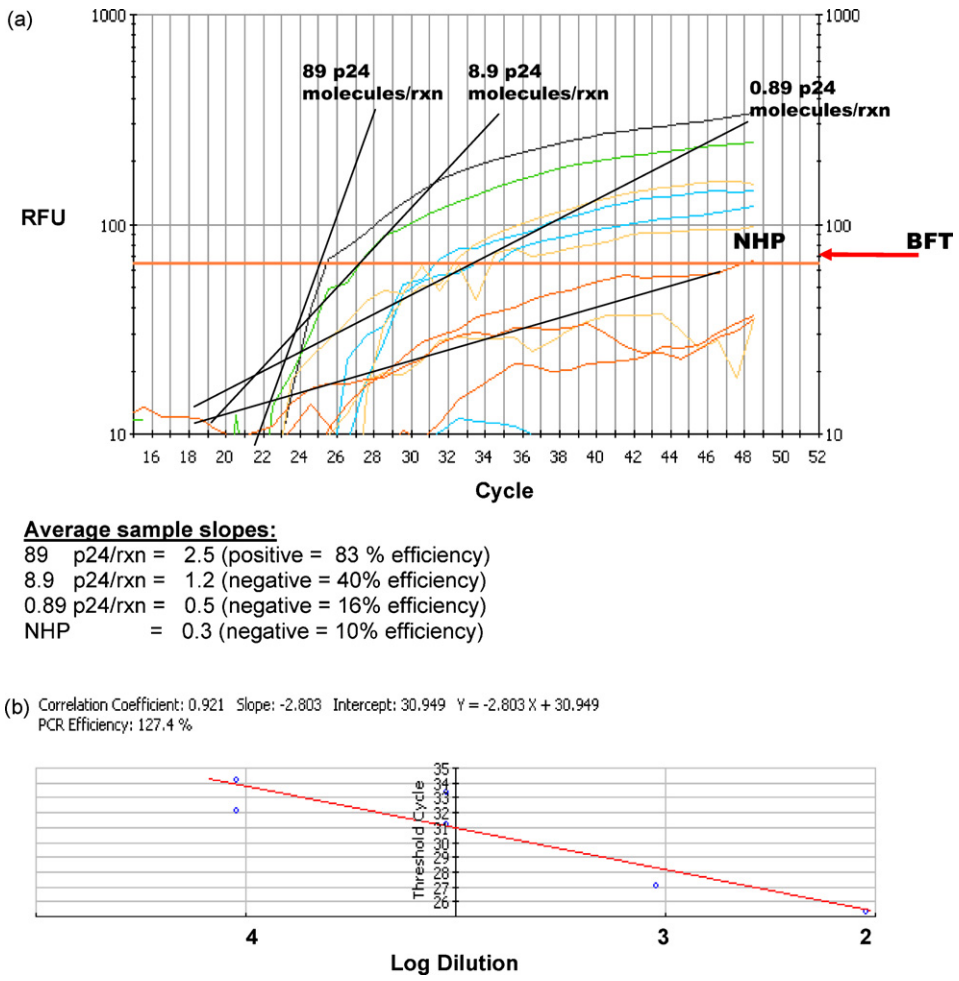


Fig. 4. qIPCR standard curves of HIV-1 p24 Ag from HIV-1 infected patient samples using two PCR amplification rounds. qIPCR analyses using two PCR amplification rounds was performed on serial dilutions of quantified HIV-1 infected patient samples with known concentrations of HIV-1 RNA copies/mL and analyzed by linear regression. The number of HIV-1 p24 Ag copy numbers was calculated based on an estimate of approximately 1200–3000 HIV-1 p24 Ag per virion (Layne et al., 1992; Summers et al., 1992; Vogt and Simon, 1999). Average sample slopes for each dilution series were determined. Only dilutions exhibiting slopes ≥2.5 were accepted as positive samples. (a) qIPCR analyses of log-fold dilutions from 89 to 0.89 HIV-1 p24 Ag molecules per rxn from one sample from a patient infected with HIV-1, amplified using two PCR amplification rounds (e.g., one PCR amplification round of 20 cycles followed by a second PCR amplification round of 50 cycles). (b) CC of the qICPR standard curve in (a) with the corresponding linear regression equation. NHP (normal human plasma), rxn (reaction), CC (correlation coefficient), BFT (background fluorescent threshold), RFU (relative fluorescent units).

ciency of each amplification line in the graph is described in the accompanying figure legend.

It has been shown in the literature that test sample dilution improves the efficiency of PCR by dilution of PCR inhibitors, while the sample target amplification is enhanced (Suslov and Steindler, 2005; Kontanis and Reed, 2006). In fact, the use of limiting dilution PCR (LDP) provides the best, statistically verifiable method of determining the amount of amplifiable template present in a preparation (Kontanis and Reed, 2006). It has also been established that a molecular interaction at the single-molecule level may occur during optimized reaction incubation conditions of ultra-low Ag concentrations with Abs of sufficiently high binding affinity (10^{-9} to 10^{-7}) (Tetin et al., 2002).

Accurate statistical measurements have been made of single protein molecules affixed to solid supports in heterogeneous immunoassays (Ferris et al., 2004) using fluorescence spectroscopy (Löscher et al., 1998; Nalefski et al., 2006) and atomic force microscopy (Li et al., 2002). Because it has been established that these intermolecular reactions do indeed occur and the effects are able to be quantified at ultra-low concentrations of the analyte, we focused our modification of the conventional IPCR method on optimization of PCR amplification of the DNA detector molecule for single-molecule binding events (which act as the surrogate for the indirect amplification of target).

We have modified the immunomagnetic bead qIPCR protocol with the intent to decrease matrix effects. Using serially diluted samples from patients infected with HIV-1 for p24 Ag quantification, we have shown that both higher dilutions of the sample, as well as a second PCR amplification, enhances true positive detection while reducing false positive background signal of the target

Fig. 3. qIPCR standard curves of Biot-181 using two vs. one PCR amplification rounds. qIPCR using two vs. one PCR amplification rounds was performed on serial dilutions of Biot-181 and analyzed by linear regression. (a) qIPCR analyses of log-fold dilutions (12.5–0.125 Biot-181 molecules per rxn) after two PCR amplification rounds (e.g., one PCR amplification round of 20 cycles followed by a second PCR amplification round of 50 cycles). (b) CC of the qICPR standard curve in (a) with the corresponding linear regression equation. The mean Ct for each sample dilution was determined from 4 to 6 replicates. (c) qIPCR analyses of log-fold dilutions (12.5–0.125 Biot-181 molecules per rxn) after one PCR amplification round (e.g., one PCR amplification round of 50 cycles BFT (background fluorescent threshold)). (d) CC of the qIPCR standard curves using two vs. one PCR amplification rounds on serial dilutions of Biot-181 and analyzed by linear regression. NHP (normal human plasma), rxn (reaction), CC (correlation coefficient), BFT (background fluorescent threshold), RFU (relative fluorescent units).

Table 2
Mean Ct, percent positive and negative samples, and correlation coefficient for all sample (Ct) data points.

HIV-1 p24 Ag copy number/reaction	100–1000	10–100	1–10	<1–1	Negative control
HIV-1 p24 Ag concentration/reaction (Molarity)	500 aM–5 fM	50–500 aM	5–50 aM	0.5–5 aM	N/A
Mean Ct	22.2	25.9	27.6	31.9	N/A
Percent of positive samples detected as positive	80% (8/10)	70% (14/20)	86% (18/21)	50% (17/34)	N/A
Percent of positive samples detected as negative	20% (2/10)	30% (6/20)	14% (3/21)	50% (17/34)	N/A
Mean sample slope (Efficiency)	3.5 (116%)	3.2 (104%)	1.7 (56%)	0.9 (29%)	0.4(12%)
Correlation coefficient for the mean of all data points	0.948				
Immunomagnetic qIPCR limit of detection		10–100			

All data were derived from seven runs of 2–5 replicates each for every sample dilution.
The best-fit line for the series of 3–4 sample serial dilutions was selected for determination of threshold and regression analysis.
Note that at <1–1 HIV-1 p24 Ag copy/reaction, false negative reactions increase to 50%, indicating there is no significant difference between positive and negative sample results.

analyte. Inherent in the second PCR amplification round is a dilution of the sample after 20 cycles of amplification. 20 PCR amplification cycles is approximately midpoint of the range at which exponential amplification of the target template occurs in the first PCR reaction (i.e., the cycle at which one target template initially present at the beginning of the cycling reaction will have replicated to 1 million $\{2^{20}\}$ copies at 100% efficiency).

These modifications included:

(1) a solid format (paramagnetic beads) which is freely suspended in solution allowing continuous agitation in the reagent solution, in contrast to the microwell plate used as a fixed solid format in the conventional IPCR method (Barletta et al., 2004);
(2) use of digital, automated multi-pipettors for low volume transfers ($\leq$10 μL) to reduce manual and operator pipettor variability (the most influential source of variation in standard ELISA procedures) (Hayashi et al., 2004; Choi et al., 2007);
(3) low reaction volumes (30–50 μL) to increase molecular interactions;
(4) high (3–6 log) sample dilutions to reduce the matrix interference of samples from patients infected with HIV-1 (e.g., excessively high concentrations of HIV-1 anti-p24 Abs and other heterogeneous Abs present in the same sample) with heating to dissociate Ag–Ab complexes (Schüpbach and Böni, 1993; Schüpbach, 2002);
(5) linear regression of serial dilutions of patient samples of HIV-1 RNA copy numbers to validate linearity, precision, and demonstrate high linear correlations at high dilutions (10–100 molecules of HIV-1 p24 Ag) in the diluted patient sample; and
(6) a second round of PCR amplification (50 cycles) started at the exponential phase (20 cycles) of the first PCR round to remove target from inhibitor effects of the sample matrix present during the first PCR amplification round. In other studies, a significant increase in detection of positive samples (from 57% to 100%) was noted after a second round of PCR amplification (Miyaoto et al., 1997).

In the qIPCR method, the negative control constitutes the sample which sets the background fluorescence threshold for determination of samples that are considered to be true positives (significantly higher than background fluorescence of the negative control). Because there are inconsistent amounts of various proteins in the molecular matrix of human plasma (particularly HIV-1 infected plasma, which contains extraordinarily high concentrations of anti-HIV-1 Abs and other heterogeneous Abs), the BFT may vary per run resulting in Ct determinations for positive samples to vary coordinately. For example, and as shown in Fig. 2a, the BFT = 400, whereas the BFT = 65 in Fig. 4a. In some studies (Niemeyer et al., 2007), the negative control amplifies exponentially and crosses the BFT 3 cycles (equivalent to 1 – log difference in quantification) after the positive sample at the LOQ. We find this approach to be problematical, particularly in samples where the LOQ is expected to be extremely low (i.e., ag/mL or 1–10 copies/reaction), as the fluorescent signal of these samples may be obscured by the negative control which may cross the BFT earlier than the lowest positive sample. Thus, the acceptance criteria for the qIPCR runs are critical and include the following:

(1) The BFT was set either at the lowest fluorescent point of the samples, or by the iCycler default setting (defined as 10 times the mean S.D. of fluorescence in all wells over the baseline cycles). Cycle threshold of a positive signal was determined as the cycle at which the sample's fluorescence intersected the BFT;
(2) samples were determined to be positive when the relative fluorescence units (RFUs) of 50–100% or more of the test replicates were above the BFT set for 75–100% replicates of the negative control. Additionally, a minimum slope of 2.5 must be exhibited during the exponential amplification phase for the reaction to be considered positive;
(3) for run analyses, a minimum of 2 replicates of each of 3 data points from serial log dilutions of the samples must exhibit a dosimetric response (i.e., increasing Ct with increasing sample dilution or lower target concentration);
(4) a minimum of 3 data points from serial log dilutions of the samples must display a CC of $\geq$0.90.

Adherence to these criteria for the determination of BFT, and thus the designation of positive signal in samples, insures that samples exhibiting extremely low LOQ are identifiable, are significantly different from background fluorescence, and show the expected dosimetric response for increasing sample dilutions with good reproducibility and relatively high precision.

We have shown an improvement in the LOD (over previous formats, Barletta et al., 2004) of the qIPCR method for HIV p24 Ag using a magnetic bead protocol. The advantages of the magnetic bead solid format include:

(1) Reduced washing and incubation times, and reduced non-specific reactions when compared to the standard qIPCR method.
(2) A relatively large surface area (60–90 cm^2 using 5 μL beads for one reaction) compared to a single microwell (2.0–2.7 cm^2) (Olsvik et al., 1994; Yu, 1998; Yu et al., 1998) allows increased capture and physical concentration of low levels of analytes from a dilute sample volume.
(3) reaction conditions where microspheres (beads) are freely suspended in the reagent mix so that molecular interaction is facilitated and enhanced by constant agitation, resulting in more rapid reaction rates and reduced assay time;

(4) rapid isolation of the magnetic beads using a simple magnetic plate (or automated magnetic bead washer and capture system) to allow easier and more efficient washing and rapid removal of the heterogeneous sample matrix from the target analyte;

A relatively inexpensive cost per test, at an approximate magnetic bead cost per test = 0.58 USD, where 400 {50 μL} reactions can be performed with a 2 mL reagent volume at a cost of $230. A magnetic plate for bead capture costs $200–300 USD.

The modified immunomagnetic bead qIPCR assay demonstrated a limit of quantification of less than 100 HIV-1 p24 molecules/reaction with an average CC of 0.948 ± 0.028 over a 4-log dynamic range, and represents the detection of less than one HIV-1 virion (which contains two copies of RNA and an estimated maximum range of 1200–3000 molecules of HIV-1 p24 antigen). This LOD is up to 30,000 times more sensitive than commercial ELISA p24 assays which exhibit a LOD of 1–10 pg/mL ($3 \times 10^{6-7}$ HIV-1 p24 molecules/mL). Although viral RNA assessment by reverse-transcriptase PCR (rt PCR) can potentially detect one-half of a virion (a virion contains two RNA genomic copies), it cannot detect less. This is in contrast to HIV-1 p24 antigen testing where a small fraction of a virion can be detected due to the large number of HIV-1 p24 molecules per virion (i.e., 1200–3000).

This ultra-low level of detection is not merely an academic or experimental feat, but may have implications in HIV-1 pathogenesis research and treatment strategies. Questions often asked in retroviral treatment are "does HIV-1 replication occur at very low levels, particularly under the influence of anti-retroviral therapy?" That is, does therapy suppress the viral load to zero or only to levels below the detection limits of RT-PCR (i.e., less than 50 copies/mL?). If viral replication is still occurring at less than 50 virion copies, is the actual number of virion copies/mL actually 49 or 1 or zero? Other questions address the clinical implications (if any) relating to these differences in extremely low HIV-1 virion copy numbers. The immunomagnetic qIPCR method is able to address these questions. Obviously, the maximum suppression of HIV-1 replication is desired, and to determine the actual efficacy of an anti-retroviral treatment, an ultra-sensitive detection method is required. Accurate detection of HIV-1 p24 at quantities less than one HIV-1 virion may assist in determining if (and when) viral replication has completely ceased. The immunomagnetic qIPCR method is able to address these questions.

In summary, a simple, inexpensive, ultra-sensitive immunomagnetic qIPCR protein detection method was developed that is able to identify the presence of less than one HIV-1 virion in a complex sample matrix (plasma). Standardization of this method is now needed to move the method to a platform that will make it a candidate for use in clinical laboratories.

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