



A simple and rapid DNA extraction method from whole blood for highly sensitive detection and quantitation of HIV-1 proviral DNA by real-time PCR



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Early diagnosis and access to treatment for infants with human immunodeficiency virus-1 (HIV-1) is critical to reduce infant mortality. The lack of simple point-of-care tests impedes the timely initiation of antiretroviral therapy. The development of FINA, filtration isolation of nucleic acids, a novel DNA extraction method that can be performed by clinic personnel in less than 2 min has been reported previously. In this report, significant improvements in the DNA extraction and amplification methods are detailed that allow sensitive quantitation of as little as 10 copies of HIV-1 proviral DNA and detection of 3 copies extracted from 100 μ l of whole blood. An internal control to detect PCR inhibition was also incorporated. In a preliminary field evaluation of 61 South African infants, the FINA test demonstrated 100% sensitivity and specificity. The proviral copy number of the infant specimens was quantified, and it was established that 100 microliters of whole blood is required for sensitive diagnosis of infants.

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1. Introduction

Infant HIV infection remains a significant problem. An estimated 390,000 new pediatric infections were reported in 2010, mostly in limited resource settings (WHO Progress Report, 2011). Early infant diagnosis of these cases would allow for prompt initiation of highly active antiretroviral therapy alleviating suffering and preventing premature death in these infants. WHO recommends that all infants born to HIV-1 positive mothers should be tested at 4–6 weeks of age, using a virological test (WHO, 2010); however, testing rates are too low. In 2011, only 35% of infants born to HIV-1 positive mothers were tested globally, with only 4 sub-Saharan African

countries responsible for more than 50% of HIV-1 exposed infants (WHO Global Update on HIV Treatment, 2013).

Access to virological infant testing is challenging in low resource settings. Persistence of maternal antibodies in an infant's blood limits the diagnostic utility of rapid HIV antibody tests in determining the status of HIV-exposed infants. PCR-based tests for the detection of proviral HIV-1 DNA are the current standard for infant diagnosis, as these tests are both sensitive and specific. However, many barriers exist in the implementation of PCR testing protocols in resource-limited areas. Current methods for nucleic acid purification are time consuming, require skilled processing, and use costly reagents and equipment (Roberts et al., 2012). There is an urgent need to improve access to early infant diagnosis particularly at peripheral sites where, increasingly, HIV clinical management is being provided. This is consistent with the need to decentralize treatment to improve access and retention in care (Kredo et al., 2013).

To improve access to PCR-based diagnosis in remote sites, a testing protocol of collection of dried blood spots (DBS) at the site of treatment, transportation to central laboratories for testing and the

Abbreviations: DBS, dried blood spot; FINA, filtration isolation of nucleic acids; POC, point-of-care; LOD, limit of detection; LOQ, limit of quantitation; HPR, hydroxypyruvate reductase; IC, internal control.

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communication of results back to the DBS collection site has been recommended by WHO ([WHO Global Update on HIV Treatment, 2013](#)). However, logistical problems associated with sample acquisition and transportation cause delays in returning results to the DBS collection site. In a recent review of early infant diagnosis programs, it was reported that many infants are lost from care due to time to results which can be as much as 5 months and that only half the test results are communicated to the infant's caregivers ([Ciaranello et al., 2011](#)). An early infant diagnostic that provides the results during the initial clinic visit may significantly increase the number of infants enrolled in antiretroviral therapy and should significantly decrease infant mortality.

The use of filtration isolation of nucleic acids (FINA) as a viable method for point-of-care extraction of leukocyte DNA from whole blood for detection of the HIV-1 provirus by PCR with high degrees of sensitivity and specificity has been demonstrated previously ([Jangam et al., 2009](#)). Processing by this method entraps DNA from lysed whole blood in a glass-fiber disk that can be washed to remove inhibitory components. The FINA process extracts cellular DNA from whole blood in less than 2 min. The disk containing the cellular DNA can then be put directly into a tube for PCR, either immediately or stored desiccated for more than a month. A qPCR prototype developed for use at the point-of-care (POC) that utilizes FINA extraction was reported recently ([Jangam et al., 2013](#).) This system uses a glass fiber membrane to extract the DNA that is inserted directly into a disposable PCR assay card that includes on-board reagent storage. The battery-operated analyzer rehydrates the lyophilized PCR mastermix, performs real-time PCR and reports the results to the user ([Jangam et al., 2013](#).)

This report verifies the FINA method for the sensitive detection of proviral HIV DNA using a model system to establish the limit of detection (LOD) and quantitation (LOQ) of an improved version of the assay, and describes the introduction of an exogenous internal control to report PCR inhibition. Additionally, the clinical performance of the assay was evaluated with fresh blood samples obtained from HIV-exposed infants at Cape Town, South Africa. The proviral copy number in each of these clinical samples was calculated by comparison of the C_q of each sample to a standard curve run simultaneously. This copy number information is critical for determining the blood volume required to achieve the assay sensitivity necessary to diagnose HIV infection in infants using the POC PCR infant diagnostic in development ([Jangam et al., 2013](#)).

2. Materials and methods

2.1. Reference strains

8E5-LAV cells ([Folks et al., 1991](#)) harboring a single copy of the HIV-1 provirus were obtained from the Virology Quality Assurance Laboratory (VQA; Rush Presbyterian/St. Luke's Medical Center, Chicago, IL) as frozen cell pellets of 4000 cells/ μ l. Cell count was verified as described previously ([Jangam et al., 2009](#)). Cells were diluted in freezing medium (90% fetal bovine serum, 10% dimethyl sulfoxide) to concentrations ranging from 0.5 to 400 cells/ μ l to be spiked into 100 μ l of fresh HIV-1-negative EDTA-treated whole blood samples (Core Lab, NorthShore University HealthSystems, Evanston, IL) to create a standard curve of copy numbers 5–4000. These spiked samples were used during assay development and testing and to establish standard curves during testing of clinical samples. Standards of 8E5-LAV cells containing 20, 10, 5 and zero copies of the HIV-1 provirus, obtained from VQA, were used to assess the limit of detection and quantitation. The copy controls were prepared as described in the VQA HIV DNA Control Standard Operating Procedure (<https://www.hanc.info/labs/labresources/vqaResources/Pages/As>

[saySpecificSops.aspx](#); VQA SOP for DNA PCR assays v5 03-13-2012). To test for single copy sensitivity of the assay, 8e5-LAV cells were diluted to 0.1 cell/ μ l and spiked into blood samples at one proviral copy per sample and processed as described below.

2.2. Clinical samples

DNA was extracted using FINA modules from 61 EDTA-treated whole blood samples tested at the National Health Laboratory Service Virology Laboratory, Groote Schuur Hospital, Cape Town, South Africa. Blood samples were collected by heel stick into Microtainers with a scoop (Becton Dickinson; San Diego, CA) or MiniCollect tubes with a funnel (Greiner Bio-One; Monroe, NC) from infants less than 1 year of age. Total Nucleic Acid PCR (Roche AmpliPrep/COBAS Taqman HIV-1) was performed on 100 μ l whole blood precisely as described in the product insert. Samples identified as HIV positive in the Total Nucleic Acid assay which contained ≥ 100 μ l residual specimen were paired with an equal number of negative samples for blinded FINA analysis. The samples were processed up to the qPCR assay, and the filter disks containing the DNA were dried and shipped back to U.S. for qPCR testing. Before the study in Cape Town was initiated, a standard curve of 10, 40, 400 and 4000 8e5-LAV cells spiked into whole blood in triplicate were processed up to the qPCR analysis and the dried filters were stored until the Cape Town samples were ready to be tested by qPCR. After the all the samples were tested, the code was provided to Northwestern University and the assay sensitivity and specificity was determined.

2.3. DNA isolation

Cellular DNA was extracted from fresh whole blood by the FINA method, as described previously ([Jangam et al., 2009](#)) with some changes to the sizes of the FINA components. Briefly, a Fusion 5 membrane disk (diameter, 8.35; Whatman, Inc., Florham Park, NJ) was sandwiched between a square 707 blotter pad (25 by 25 mm; VWR International, West Chester, PA) and a thin sheet of Parafilm (Bemis Flexible Packaging; Neenah, WI) with a 7.14-mm-diameter hole in the center such that the hole of the Parafilm left the center of the Fusion 5 disk exposed ([Fig. 1](#)). Contact between the disk and the blotter pad was ensured by pressing the Parafilm firmly to stick it to the blotter pad around all edges of the disk. Blood that had been lysed by adding Triton-X (Sigma Chemicals; St. Louis MO) to a final concentration of 1% in the blood sample was added to the disk and allowed to soak in completely, followed by a single wash of 600–1000 μ l of 10 mM NaOH, until the disk appeared white, indicating clearance of the hemoglobin. The Parafilm was then peeled off and the disk was separated from the blotter pad with clean forceps, and placed into and stuck to the side of a 200 μ l PCR tube that had been prepared with a circular sticker made from PCR-safe, double-sided adhesive (3 M Double-Coated Polyester Diagnostic tape, product number 9965; diameter, 5.1 mm; 3 M Medical Specialties, St. Paul, MN). This filter was used as the template for subsequent qPCR amplification and detection. After the filter was placed in the PCR tube, the clinical samples from Cape Town were dried overnight in a box containing calcium sulfate desiccant, capped, and placed in a foil pouch with silica desiccant and sent to Northwestern University for qPCR testing. The stability of the dried filter had been previously established ([Jangam et al., 2009](#)).

2.4. qPCR amplification and detection

PCR amplification and detection were performed using the Abbott HIV-1 *RealTime* Assay (Abbott Molecular Inc., Des Plaines, IL) reagent kit including the primers & probes and their PCR

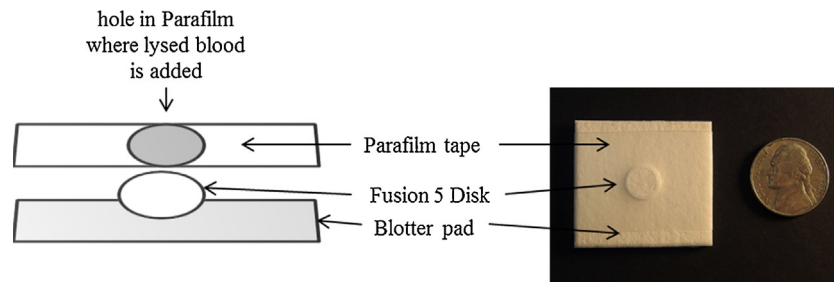


Fig. 1. Schematic of FINA in-house module production. An 8.35 mm diameter Fusion 5 membrane disk was sandwiched between a square 707 blotter pad and a thin sheet of Parafilm containing a 7.14-mm-diameter hole in the center such that the hole of the Parafilm was centered on the Fusion 5 disk for the direct application of lysed blood by pipet.

thermal cycling protocol. A number of modifications to Abbott's reagents & protocol were made to adapt the assay for eventual incorporation into the FINA POC instrument under development (Jangam et al., 2013). Z05 polymerase (Roche Molecular Systems, Inc., Branchburg, NJ) was substituted for *Tth* polymerase used in the Abbott assay because Z05 is available in a form that is more amenable to lyophilization which will be necessary for inclusion in a POC assay card. The reverse transcription step was omitted as described previously (Jangam et al., 2009), and the qPCR reactions were run on the Stratagene Mx3005p (Agilent Technologies, Santa Clara, CA) using the FAM and Cy5 channels for data collection rather than the Abbott's automated system, *m2000sp*TM and *m2000rt*TM (Tang et al., 2007). The HIV-1 amplicon was detected by a 5' fluorescein (FAM) conjugated probe, and the internal control amplicon (HPR, see below) was detected by a 5' conjugated Quasar 670 probe (Biosearch Technologies; Petaluma, CA.) Sequences of the forward and reverse primers, hybridization probes, and quenching oligonucleotide were described previously (Tang et al., 2007).

2.5. Exogenous internal control

The internal control was adapted from the Abbott HIV-1 *RealTime* Assay (Abbott Molecular Inc., Des Plaines, IL). The 136-bp amplicon derived from the hydroxypyruvate reductase (HPR) gene from the pumpkin plant (Tang et al., 2007) was cloned into pZerOTM-2 (Life Technologies, Carlsbad, CA). Plasmids containing the HPR target were spiked into the PCR mastermix containing the primers and probes for the HPR gene at 500 copies per reaction. Standard curves of 8E5-LAV cells were run with and without the primers, probe and HPR plasmid to demonstrate the consistency of the HIV assay. Samples that tested negative for the HIV assay and that had a failed IC were considered invalid. Samples that tested positive for HIV with a failed IC were considered positive.

2.6. Statistical analysis

C_q values were plotted against log (copy number) using Stratagene's MxPro Software to obtain standard curves. Slope parameters were estimated by linear regression of C_q values vs log (copy number). For the slope estimates, 95% Confidence Intervals were obtained using GraphPad Prism v 6.0 (GraphPad Software, La Jolla, CA). The value of the slope was used to calculate PCR efficiency (Lutfalla and Uze, 2006). To determine if the observed detection of proviral DNA in this single copy study was significantly different from the predicted number of samples with ≥ 1 a copy according to Poisson distribution, the two-tailed P value was calculated using Chi-square analysis using GraphPad QuickCalcs (<http://graphpad.com/quickcalcs/chisquared1.cfm>).

3. Results

3.1. Introduction of internal control

To monitor for the presence of amplification inhibitors and to verify that a negative sample is a true negative, the internal control used in the Abbott HIV-1 *RealTime* Assay, the hydroxypyruvate reductase (HPR) gene from the pumpkin plant, was adapted for use in a DNA assay. 500 copies of the HPR containing plasmid were spiked into the qPCR mastermix. Fig. 2 demonstrates that the presence of the IC did not compete for amplification reagents. The C_q values obtained from the amplification plots (Fig. 2a) were used to plot standard curves for determination of the efficiencies of the PCR. The efficiency with the internal control (+HPR) was calculated to be 103% (slope -3.23 ; 95% confidence interval -2.99 to -3.48) (Lutfalla and Uze, 2006). The efficiency without the internal control (-HPR) was calculated to be 104% (slope -3.24 ; 95% confidence interval -3.08 to -3.40), indicating very similar assay performance (Fig. 2b).

3.2. Determination of limits of detection and quantitation

Improvements to the assay including lysing the cells prior to adding the sample to the filter and attaching the filter to the side of the PCR tube were introduced. The LOD was reported previously to be 20 copies/100 μ l whole blood (200 copies/ml) (Jangam et al., 2009). In the current test format, the 5 copy standard was detected 9 out of 9 times (Table 1) indicating that the LOD is less than 5 copies per reaction or 50 copies/1 ml whole blood. The internal control C_q average 23.7 ± 0.3 ($N=52$) showed no inhibition in any of the samples.

It has been reported that 3 copies per qPCR reaction is theoretically the most sensitive LOD possible assuming a Poisson distribution so that there is a 95% chance of including at least 1 copy in the PCR, and that the assay can detect a single copy (Wittwer and Kusakawa, 2004, Bustin et al., 2009). In order to test if the assay can detect a single copy, 8e5-LAV cells were diluted such that 84 replicates contained a single copy. The Stratagene MxPro software determined that 24 of 84 (29%) single copy samples were positive. Upon inspection of the remaining curves, it

Table 1

Detection and Quantitation of VQA copy standards with FINA extraction and Abbott HIV-1 *RealTime* Assay primers and probes. NA = not applicable.

Detection			Quantitation		
Copy No.	N	% Detected	Input Log Copy No.	Measured Log Copy No.	% Relative Difference
0	10	0	NA	NA	NA
5	9	100	0.70	0.45	-35.90
10	10	100	1.00	0.93	-6.67
20	9	100	1.30	1.26	-3.48

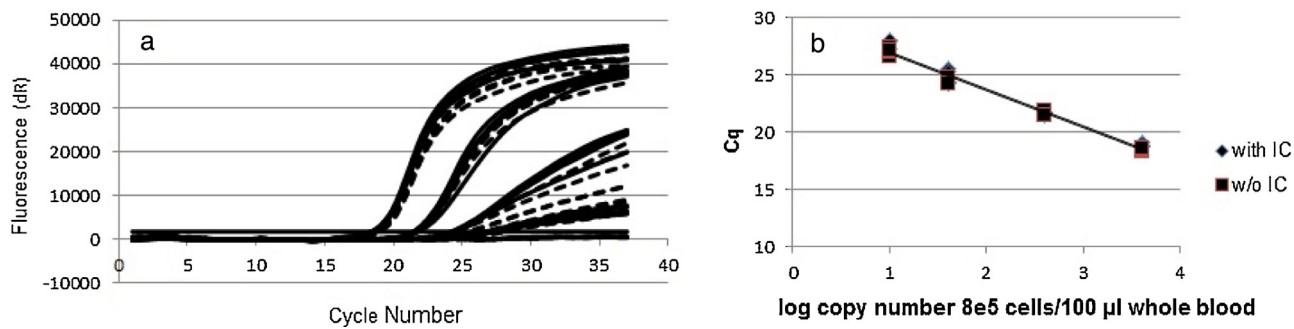


Fig. 2. (a) Amplification plots obtained from 100 µl of 5 replicates HIV-1 negative whole blood spiked with 4000, 400, 40 and 10 8e5/LAV cells with and without 500 copies/reaction of the internal control; dotted lines = +IC; solid lines = -IC. Y-axis is fluorescence units and X-axis is qPCR cycle number. (b) Standard curves of C_q values calculated from amplification plot versus log copy number of 8e5/LAV cells per 100 µl whole blood; diamond symbol represents samples with IC, $y = -3.23 + 30.27$; square symbols represent samples without IC, $y = -3.24 + 30.06$.

was clear that some of the samples had amplification that was not detected by the software because of early cycle baseline variation caused by the presence of the filter in the PCR tube (Fig. 3a). These remaining samples were analyzed by examining fluorescent data with a line fit program from cycles 15–29. If the signal deviated

positively from the straight line, then the sample was called positive (Fig. 3c). Combining this analysis with the conventionally called C_q s, 48 (57%) of the specimens were detected as positive. This value is close to that predicted by the Poisson distribution of 53 (63.2%) to have ≥ 1 copy. Assuming the proportion detected to be normally distributed, the two tailed P value was calculated to be 0.26 which indicates no significant difference between the observed and expected number of positives in the 84 single copy samples. Therefore, it was concluded that the FINA assay detects a single proviral DNA copy if present in the specimen and that the LOD is 3 copies per qPCR reaction when processing 100 µl of whole blood.

The copy standards were also quantified using a standard curve run on the same qPCR plate in order to estimate the LOQ. The LOQ is defined as the lowest concentration at which the analyte is not only reliably detected but that the relative difference in quantitation (input log copy number vs. measured log copy number) is less than 10%. A two tailed Student t-test was performed on the means of the C_q values of 5 and 10 copies (P value = 0.0071) and of 10 and 20 copies (P value = <0.0001) to determine the resolution of the assay at these low concentrations. Ten copies can be clearly distinguished from 5 copies; therefore, the estimated LOQ is 10 copies per reaction or 100 copies/1 ml whole blood.

3.3. Evaluation of FINA performance with clinical infant specimens

To determine the sensitivity and specificity of the FINA extraction method with whole blood collected from HIV exposed infants, the test was compared with the COBAS® Ampliprep/COBAS® Taqman System used at the National Health Laboratory Service Virology Laboratory, Groote Schuur Hospital, Cape Town, South Africa. Specimens had been collected via heel stick over a 3 week period, and 61 had more than 100 µl whole blood remaining after testing with the COBAS. The blood was processed with the FINA modules and the filter disk containing the DNA was dried and shipped back to U.S. for qPCR testing. One specimen did not give a valid amplification (the IC failed) and was excluded from the analysis. 29 true positives and 31 true negatives were detected yielding 100% sensitivity (95% CI: 88–100%) and 100% specificity (95% CI: 89–100%). The number of copies per reaction which corresponds to 100 µl whole blood was quantified using a standard curve performed on the same PCR plate (Table 2.) Two of the specimens gave C_q s that were detectable but below the LOQ of 10 copies/100 µl (100 copies/ml) whole blood. The remainder of the positive sample had quantifiable amounts of target. More than 40% of the specimens had less than 50 copies per reaction or 500 copies/1 ml whole blood.

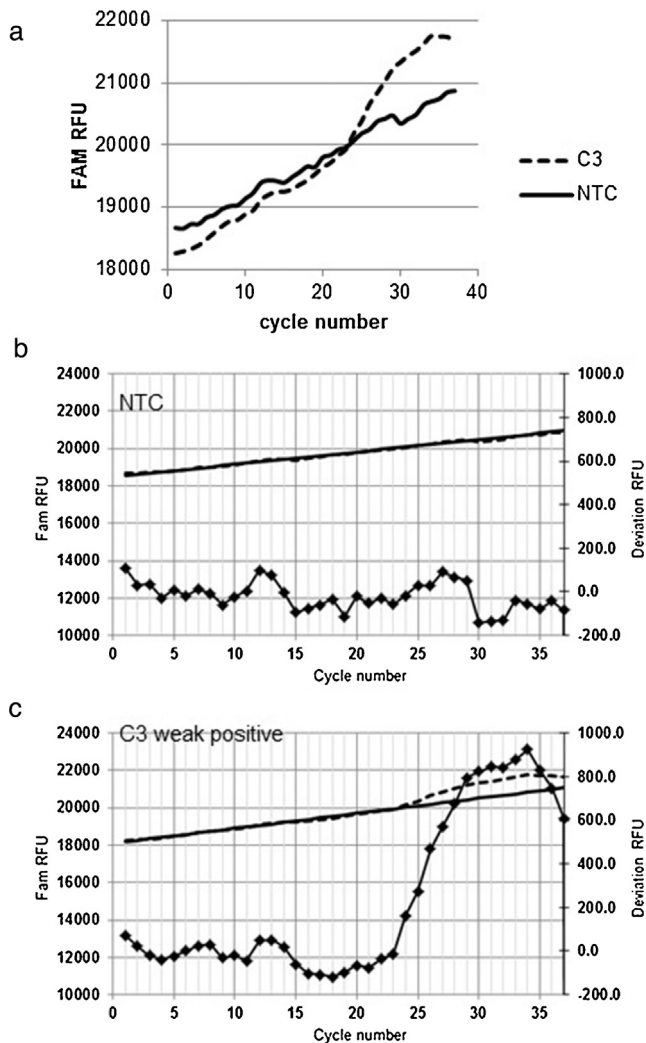


Fig. 3. (a) Raw data amplification plots of no template control (solid line; NTC) and a weak positive (dotted line; well C3). (b) NTC straight line fit analysis. --- raw data, — straight line fit from cycles 15–29 $R^2 = 1$, ◆ deviation from straight line fit; (c) weak positive well C3 straight line fit analysis. --- raw data, — straight line fit from cycles 15–29 $R^2 = 1$, ◆ deviation from straight line fit.

Table 2

Summary of quantitation of proviral DNA copy numbers from 29 HIV-1 positive infant specimens. The copy number was calculated from a standard curve of C_q values versus log copy number of 4000, 400, 40 and 10 8e5/LAV cells per 100 μ l whole blood in triplicate. The equation of the line was $y = -3.3 + 28.0$; $R^2 = 0.996$ (95% confidence interval for slope is $-3.4 - -3.1$).

Proviral Copy No./100 μ l WB	No. of Positives	% Positives (Cumulative)
≤ 10	2	7
11–25	3	17
26–50	7	41
51–100	8	69
101–200	5	86
201–300	3	97
>1000	1	100

4. Discussion

A comprehensive, simple to use, and highly sensitive assay for the detection and quantitation of HIV-1 proviral DNA in whole blood specimens by combining FINA extraction with the Abbott HIV-1 *RealTime* Assay primers and probes has been reported. The FINA method was developed for inclusion in a POC qPCR device which is under development for use in limited resource settings. The Abbott primers and probes were developed to equivalently quantify the HIV-1 group M subtypes A to H, group O and group N isolates through a combination of the selection of a highly conserved target region and a mismatch tolerant probe design (Tang et al., 2007). In this study, the introduction of an internal control and modifications to the FINA process are reported which greatly improve the analytical sensitivity of the assay to detect as few as 3 copies/qPCR reaction. In a small laboratory study performed on South African specimens, the FINA assay demonstrated excellent sensitivity and specificity with 100 μ l whole blood specimens. One of the goals of the study was to determine if the sample input volume could be reduced from 100 μ l whole blood in order to reduce the size of the DNA capture membrane. The quantitation of the South African specimens demonstrated that two of the positive samples (7%) had less than 10 copies per reaction; therefore to maintain the high level of sensitivity from this study, we need to maintain the input at 100 μ l.

A prototype of the POC qPCR system utilizing the FINA extraction was reported recently (Jangam et al., 2013). The prototype used a larger DNA capture membrane diameter (11 mm) which required a much larger qPCR reaction volume (468 μ l) leading to reduced qPCR sensitivity and increased reagent cost. The laboratory study performed on South African samples demonstrated that a diameter of 8.35 mm which corresponds to a 100 μ l qPCR volume is sufficient to detect 100% of the infants, and thus a 100 μ l qPCR chamber can be used. In this study because the DNA capture membrane caused some optical interference in the Stratagene Mx3005p, the detection of very low copy numbers required visual inspection of each curve and a subsequent secondary analysis using a line fit program. In the POC qPCR system, the sample membrane is contained within a polypropylene injection molded holder called the sample introduction module which prevents the membrane from blocking the optical window (Jangam et al., 2013) thus eliminating the need for visual inspection of each curve.

Other assays such as the COBAS® Ampliprep/COBAS® Taqman System amplify both HIV-1 viral RNA and proviral DNA for enhanced sensitivity. Efforts to improve the sensitivity of the FINA assay by amplifying viral RNA in addition to proviral DNA were not successful. The FINA method is specific for isolating genomic DNA from whole blood; it does not capture viral RNA. This was determined by contriving HIV-1 positive samples by spiking whole blood with 750,000 copies/ml of HIV virus with and without 8e5/LAV cells to represent cells harboring proviral DNA, and

RT-qPCR was performed. Only the samples that contained proviral cells showed amplification which was equivalent to the 8e5/LAV cell control without added virus (data not shown.) The Fusion 5 membrane used in the FINA module may capture the genomic DNA by entangling the DNA on the filter as demonstrated by an electron microscopy image of the Whatman FTA™ card with entangled DNA (<http://www.whatman.com/FTANucleicAcidCollectionStorageandPurification.aspx>.) It is likely that the viral RNA molecules are either not trapped in the filter or are washed away during the 10 mM NaOH wash that removes qPCR inhibitors. Therefore, the sensitivity of the assay cannot be improved by including a reverse transcription step.

Detection and quantitation of proviral DNA is becoming an increasingly important biomarker for HIV-1 disease monitoring. Patients receiving highly active antiretroviral treatment typically achieve viral suppression rapidly. In recent reports from Guinea, Malawi and Mozambique showed that 85% of the people receiving antiretroviral treatment had viral suppression within six months (De Luca et al., 2012). Alternative methods are needed to allow further evaluation of therapy efficacy as well as compare different treatment regimens in clinical trials (Christopherson et al., 2000). In a preliminary study, the FINA extraction with the Abbott Real-Time PCR primers and probes was demonstrated to sensitively and specifically detect proviral DNA in adults as well as infants. Proviral DNA was detected in 72/75 HIV-1 positives specimens tested (96% sensitivity; 95% CI: 89–99%). This study was performed with the earlier version of FINA which had an LOD of 200 copies/ml rather than 30 copies/ml whole blood and did not allow accurate quantitation of the specimens. A high throughput version of this improved assay could prove to be useful in monitoring treatments aimed at HIV eradication. Additionally, the FINA extraction could be adapted for use with other inputs besides peripheral blood to look for reservoirs of HIV-1 infected cells. HIV proviral testing could also be used to screen vaccine trial subjects for true infection.

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- WHO Progress Report, 2011. http://www.who.int/hiv/pub/progress_report2011/en/. Fact Sheets, Key facts on global HIV epidemic and progress in 2010. <http://www.who.int/hiv/pub/progress.report2011/global.facts/en/index.html> (accessed 20.11.2014).
- WHO Global Update on HIV Treatment, 2013: Results, Impact and Opportunities. <http://www.who.int/hiv/pub/progressreports/update2013/en/> (accessed 20.11.2014).