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RESEARCH ARTICLE OPEN ACCESS

# Evaluation of a Quantitative Taqman Real-Time PCR Assay to Measure Proviral load from Human Immunodeficiency Virus Type 1 individuals

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## Abstract

**Human Immunodeficiency Virus (HIV) is a virus belonging to the family Retroviridae. HIV – 1 is found to be predominant in India and many parts of Africa. The intention of this study was to quantify the HIV Proviral Deoxyribonucleic Acid (DNA) from newly infected HIV-1 individuals. Fifty patients who were tested positive for HIV were included in this study. Proviral Ribo Nucleic Acid (RNA) was extracted by QIAmp® RNA Mini Kit (QIAGEN, Germany) method. Complementary Deoxyribo Nucleic Acid (cDNA) was synthesized by using Invitrogen Superscript III cDNA synthesis Kit (USA). This cDNA was subjected to Polymerase Chain Reaction (PCR) and Gene cloning by transformation method. The quantification of Real time PCR was done by Applied Bio-System (ABI)-Prism 7700. A linear standard curve was obtained 10 copies to $10^6$ copies per reaction. The assay had good analytic sensitivity and linear dynamic range greater than 6 logs. From the results obtained in this study, It was concluded that Taqman Real-Time PCR Assay plays a major role in monitoring the HIV infected patients in routine diagnostics and clinical practice.**

**Keywords:** Viral load, HIV-1, Reverse transcriptase, Taqman probe assay, pGEMT-Vector ligation, Gene transformation

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## INTRODUCTION

Viral load measures the density of virus particles. Viral load is a quantitative measurement of HIV nucleic acid (RNA) describing the number of copies present in the blood.[1] It is one of the important measures of severity of HIV-1 infection. The viral amount directly expresses that the disease is existing and duplicating. The viral loads of untreated and uncontrolled HIV patients would be in higher range from one million or more copies/thousand microliter. Depending on the tests used, a low viral load frequently varies from 40 - 500 copies / thousand microliters and a huge viral amount may be between five thousand to ten thousand copies/ thousand microliters during treatment and monitoring of the cases.[2] An increasing viral load is usually suggestive of worsening of the infection or development of drug resistance. A decreasing viral load is suggestive of patient's improvement and effectiveness of the treatment.[3] Therefore, measuring the viral load is really helpful in monitoring of the HIV disease, effectiveness of treatment and prediction of future course of the infection.[4]

HIV-1 RNA is usually undetectable in the initial stage of the infection where in the virus may be present in the cells and tissues as "HIV-1 provirus". When the HIV-1 provirus moves into the cell and joined with the DNA of the host cell termed as "HIV-1 proviral DNA". One type of virus that can develop a provirus is a retrovirus. When a retrovirus penetrates a cell; the RNA of the retrovirus is converted into proviral DNA by reverse transcription, and then introduce into the host cell by an integration process.[5] Thus, it is important to adequately measure the proviral HIV-1 DNA and to examine its progressiveness, especially when plasma HIV-1 RNA level lies within undetectable limits. PCR method is one of the sensitive methods in detecting the HIV viral load. Various tests are available to measure the viral load. There are three main tests used to measure viral load. These are reverse transcription-polymerase chain reaction (RT-PCR) tests, branched DNA (bDNA) tests, and nucleic acid sequence-based amplification (NASBA) tests.[6,7]

However, the conventional assays measuring HIV viral load are costly for routing use. In this present study, a rapid assay was used to monitor the HIV-1 proviral load in real time Reverse transcriptase PCR (RT PCR) using Taqman quantification. The current study may help in evaluating the proviral load which can be used to monitor the prognosis of HIV patients.

## MATERIALS AND METHODS

### Specimen & Quantification of plasma HIV 1 RNA

Fifty adults who were tested positive for HIV-1 were added in the current study after obtaining consent and Institutional ethical approval. Viral Ribo Nucleic Acid was separated from plasma samples and tested by a quantitative RT-PCR assay (ABI)-Prism 7700) was carried out in patients with low viral load of either 200 or 50 copies per ml.

### Preparation of biological samples by Gene cloning method

### Sample preparation and DNA extraction

Peripheral blood specimen was collected in Vacutainer, and DNA was separated from plasma specimen using QIAamp® DNA Mini Kit (QIAGEN, Germany) according to the manufacture guidance and quantified spectrophotometrically.

### Preparation of HIV-1 DNA by PCR method

PCR Amplification of Long terminal repeats (LTR) gene was achieved by using primers as described in Damond F et al study.[8] The thermal cycler (Applied Bio-System) multiplication was achieved in a reaction of 25 µl consist of 2X master mix with 1.5mM Mgcl2, primers and diluted genomic DNA. PCR programme will be an initial denaturation at 94°C for 5 min followed by 36 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. The final elongation at 72°C for 10 min. The PCR product was visualized on a 2% agarose gel with ethidium bromide staining. The PCR products were purified by spin column method and quantified by A260 measurements.

### Gene Cloning & Standard curves preparation

The purified PCR product of the LTR gene region was cloned into pGEMT-T Easy plasmid vector (Promega, USA) according to the manufacture instructions and Transformation by Calcium Chloride, Heat shock method.[9,10] The positive clones were then processed for an overnight culture for subsequently plasmid isolation by using alkaline lysis method.[11] The plasmid concentration was checked by spectrometry at 260 nm. The copy number formula used was: Number of copies= (amount*6.022x$10^{23}$)/ (length*1x$10^{9}$*650) and

the standards were prepared by serial dilution method. ($10^{10}$ to 10 copies)

**Quantification of probe based real-time PCR**

The probe was amplified based on the chemistry of Real time PCR targeting the LTR region of HIV- 1 which was carried out by using primer sequences are shown in the table-1. The Real Time PCR was standardized using different probe concentrations-1Micro molar (µM), 0.6µM and 0.2µM and primer concentrations-1.5µM, 1µM and 0.4µM. Amplification was performed in a Real Time thermal cycler in a 10 Microliter (µl) reaction mixture containing 5µl of 2x Reaction mix, 0.4µM of primers, 0.2µM probe and 4µl of DNA and amplification profile shown in Table 1. Standard curve was created from clarified HIV-1 LTR plasmid ranging from 10 *$10^6$ to 10 copies.

**Table 1.** Primer sequences and amplification profile of LTR gene

| No. | Gene | Primer sequences | Amplification profile |
|---|---|---|---|
| 1. | LTR | LTR F 5'-GCC TCA ATA AAG CTT GCC TTG A-3'<br>LTR R 5'-GGG CGC CAC TGC TAG AGA-3'<br>LTR PROBE 5'-6-FAM-CCA GAG TCA CAC AAC AGA CGG GCA CA-TAMRA- | The cycling conditions were 15 minutes initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 15 sec at 95°C and 1 minute at 60°C. |

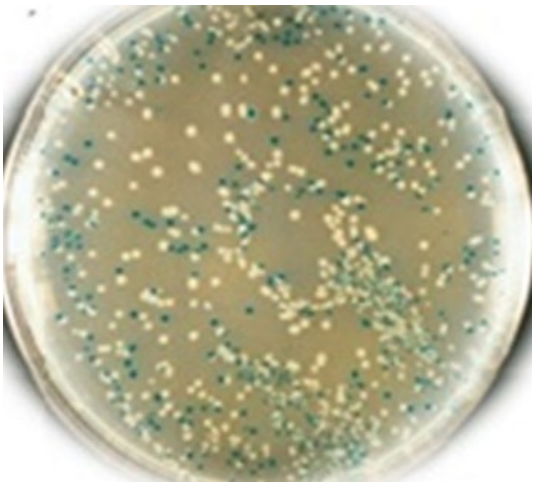

**Fig. 1.** Showing the transformed recombinant white colonies of LTR gene. Blue colours indicate non-recombinant colonies.

**Sub-typing of the HIV-1 individuals by Reverse Transcriptase (RT) and sequencing method**

HIV viral RNA was eluted from plasma samples using QIAamp® viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's information. Real-time quantitative PCR (ABI-Prism7700) was carried out using an EXPRESS superscript one step Quantitative Real time PCR (QRT PCR) kit according to the manufacture instructions, which has components for one step RT and q RT PCR. Both cDNA synthesis and PCR were achieved in a single tube using LTR

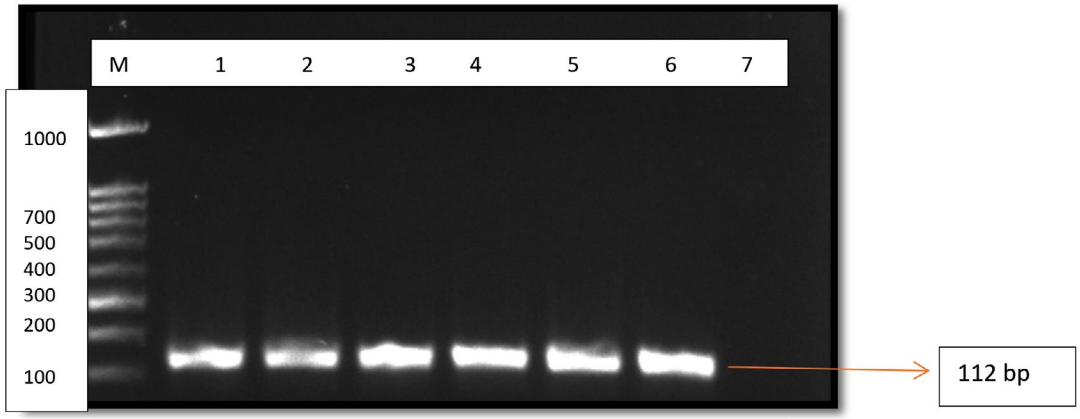


**Fig. 2.** Agarose Gel Electrophoresis showing the 112bp PCR Products of HIV-1 LTR gene. M-1000bp DNA ladder, 2 to 6 showing the PCR products, 1and 7 showing the positive control, negative control respectively.

**Table 2.** Experimental Report of standard curve and One Step QRT PCR Assay showing the ct values, mean values, viral load quantity measurements and cyclic conditions

| File name | LTR Stds | | | | | | |
|---|---|---|---|---|---|---|---|
| Plate type: | 7700 Reporter | Single Std Plate | | | | | |
| PCR volume | 10 | | | | | | |
| Thermal Cycler Conditions | | | | | | | |
| Cycle | Temp. | Time | Repeat | Ramp Time | Auto Increment | | |
| Hold | | 95 | 15:00 | Auto | | | |
| Cycle | 45 | 95 | 0:20 | Auto | | | |
| | | 60 | 0:40 | Auto | | | |
| Standard curve -3.048 | 39.83 | 0.988 | 0.12 | (3,15) | | | |
| Sample Information | | | | | | | |
| Well | Type | Sample name | Replicate | Ct | Quantity | Std. Dev | Mean |
| C3 | STND | C3 | | 18.39 | 1.20E+07 | 0 | 12000000 |
| C4 | STND | C4 | | 21.13 | 1.20E+06 | 0 | 1200000 |
| C5 | STND | C5 | | 23.89 | 1.20E+05 | 0 | 120000 |
| C6 | STND | C6 | | 28.26 | 1.20E+04 | 0 | 12000 |
| C7 | STND | C7 | | 30.07 | 1.20E+03 | 0 | 1200 |
| D3 | UNKN | D3 | | 27.22 | 1.20E+04 | 0 | 11500.51 |
| D4 | UNKN | D4 | | 30.26 | 1.20E+03 | 0 | 1204.55 |
| D5 | UNKN | D5 | | 31.12 | 6.40E+02 | 0 | 638.19 |
| D6 | UNKN | D6 | | 32.66 | 2.00E+02 | 0 | 203.44 |
| D7 | UNKN | D7 | | 31.99 | 3.30E+02 | 0 | 333.76 |
| D8 | UNKN | D8 | | 29.67 | 1.90E+03 | 0 | 1868.49 |
| D9 | UNKN | D9 | | 33.56 | 1.00E+02 | 0 | 103.91 |
| D10 | UNKN | D10 | | 33.77 | 8.90E+01 | 0 | 89.42 |
| D11 | UNKN | D11 | | 31.03 | 6.80E+02 | 0 | 681.09 |
| C8 | NTC | C8 | NTC | 0 | | 0 | 0 |

gene specific primers, probe and total RNA. Amplification of LTR gene was carried out by using primers as described in a previous study.[12] The amplified products were directly sequenced in Mega BACE 1000 system using Sanger's dideoxy chain termination method.[13] The sequences were aligned in NCBI database to find out the HIV-1 subtypes.

## RESULTS

### Preparation of standard curves

HIV DNA was amplified using both LTR Forward, Reverse primers and the amplified fragments were cloned into pGEMT Easy vector. Blue-white screening is a rapid and efficient technique for the identification of recombinant bacteria. It relies on the activity of β-galactosidase,

an enzyme occurring in *E. coli,* which cleaves lactose into glucose and galactose and showing the transformed recombinant white colonies were shown in Fig. 1. Transformed positive clones were subjected to plasmid DNA isolation by alkaline lysis method from overnight cultures. The plasmid DNA was subjected to PCR and restriction digestion by restriction endonuclease EcoRI enzyme and gel purified. The amplified 112 bp of DNA PCR fragments exhibited in the Fig. 2.

**Taqman Real time PCR**

A standard curve was generated by serially diluting (10 fold) the LTR DNA cloned into a plasmid. A linear standard curve was obtained between 10 copies to 106 copies per reaction. The standard curve picture is shown in Fig. 3. The samples for the viral load were analyzed with one step QRT-PCR assay by using 400nm each of the primers and 200nm of the Taqman probe. The one step QRT PCR assay results were placed in the Table 2.

**DISCUSSION**

Viral load is one of the measures to assess the amount of the virus found in the blood of the person. It helps in measuring the severity of the disease.[14] in cases of undetectable HIV-1 RNA, Proviral DNA can serve as biomarker to monitor the antiretroviral management. Rutsaert S et al[15] studied the HIV-1 DNA quantification assays utilize real-time quantitative PCR (qPCR) to measure the abundance quantification and reproducibility.[15] This study used Taqman real time PCR assay to detect one copy of proviral HIV-1 DNA with 100% sensitivity.

Malatinkova E, et al[16] data implemented standard-curve free assay to quantify integrated HIV-1 DNA (*Alu*-5LTR) assay showed increased efficiency of detection in HIV-1 patients. *Alu*-LTR assay showed higher sensitivity in measuring integrated HIV-1 DNA as compared to *Alu*-gag assay.[16] In our study, we also measured the

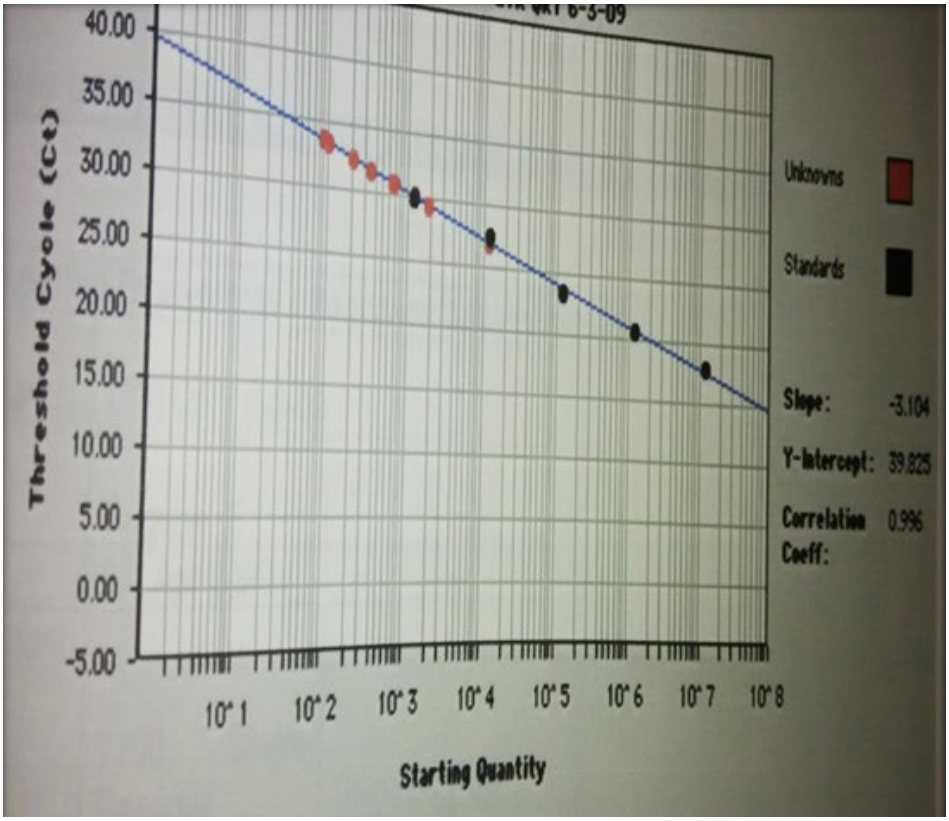


**Fig. 3.** Real time PCR analysis: Standard curve showing the log10 DNA amount plotted against cycle threshold (Ct) for different dilutions of HIV-1 proviral DNA of in PCR grade water.

sensitivity of HIV-1 subtype-C, LTR based Taqman PCR assay.

Suzuki K, et al[17] demonstrated that HIV-1 DNA detection with the novel "πCode end-point PCR assay" is 100% sensitive, while the real-time PCR showed 92.3% in a head-to-head comparison in samples from HIV-1 infected individuals.[17] We have also focused the real time RT PCR assay to detect one copy of proviral HIV-1 DNA with 100% sensitivity.

Kibirige CN, et al[18] assay provides a convenient, sensitive, specific and reproducible measure of HIV-1 viral RNA patients under anti-HIV therapy. This assay is suitable for monitoring the efficacy of therapeutic strategies.[18] similarly, undetectable viral load infected patients were enrolled in this study and we have measured the sensitivity with a detection limit of 10 copies was developed by using RT PCR assay.

Jagodzinski LL, et al[19] studies show that the qRT-PCR assay provides a convenient, sensitive, specific, and reproducible measure of HIV-2 viral RNA in plasma and is suitable for use in guiding treatment, monitoring of therapy and following disease progression in HIV-2 infected individuals. In the current study, We developed a Quantitative Taqman RT PCR assay, specifically for HIV-1 Indian Subtype C viruses and compared to the HIV-2 infected patients similar to Jagodzinski et al study.[19] Palvinder Kaur, et al.[20] emphasized the use of quantitation of HIV 1 viral load in treatment monitoring. But their assay had a limited range of detection. We have found RNA of HIV-1 at low detectable concentrations as 10 copies per reaction and essential for management of HIV medication.

Viral load measurement plays a significant role during therapy, as most treatment guidelines propose that suppression of HIV replication is one of the major therapeutic goals and the viral load measurement also helps in monitoring of the treatment. Routine measuring of viral loads during the treatment phase with an interval of 3-6 months is recommended.[21] Active infection: - several billion particles milliliter of blood. Dormant, or inactive, infection: - a million viral particles per milliliter of blood.

In this study, rapid real time PCR (Taqman) assay chemistry was developed, which would be very helpful in evaluating HIV-1 subtype-C, targeting the LTR region. This examination is extremely precise, with a diagnostic sensitivity of 10 viral DNA copies. The current assay projects a narrow dynamic range of more than 6 logs. The probes and the primers used in this study were designed to the LTR region of HIV genome and the method was standardized using different reaction conditions, probe primer concentrations and the real time assay was carried out on ABI-Prism 7700. A linear standard curve was obtained 10 copies to $10^6$ copies per reaction. From these results it can be concluded that this assay is highly useful for monitoring of HIV infected patients in routine diagnostics and clinical practice. Our investigation concludes that LTR is an extremely relevant gene to be targetted for quantifying the HIV-1 subtype-C genotypes.

## CONCLUSION

This is an EXPRESS Superscript one step QRT PCR technique to detect the concentrations of HIV-1 RNA as minimum as 10 copies per reaction. This technique is convenient to detect, quantify, and check the presence of viremia in individuals on antiviral medications. Sample volumes of 10µl and 20µl can be used and the assay has a greater sensitivity compared to various other techniques. The good analytical sensitivity of our analysis gives an extensive quantification of viral loads in both medicated and non-medicated patients. The primary advantage of this method is its specificity, cost effectiveness and sensitivity with a detection limit of 10 copies. This assay which is mainly created for Indian Subtype C viruses, would be useful, economical and reliable for conventional monitoring of viral load in HIV-1 patients.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors have made a substantial, direct, and intellectual contribution to the research work, and approved it for publication.

## FUNDING

None.

## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

This study was approved by the Institutional Human Ethics Committee, Sree Balaji Medical College and Hospital, Chennai, India with Ref. No. 002/SBMC/IHEC/2017/60.

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