

FRESH BLOOD SAMPLES VERSUS ARCHIVED SAMPLES IN HEPATITIS C VIRUS SCREENING: A COMPARATIVE STUDY

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Abstract

A low prevalence of Hepatitis C virus infection ranging from 0.1% to 0.9% has continuously been reported in the Kenyan population. Several studies have however concentrated on special groups like Intravenous Drug Users (IDUs) and blood bank samples, with no major study carried out in the general population. This study aimed at testing and comparing results of fresh and archived Hepatitis C infected samples obtained both from patients in Kenya and in Germany. Fresh and archived samples in Kenya were obtained from patients attending the liver clinic at the Kenyatta National Hospital and those stored at the Kenya Medical Research Institute (KEMRI), respectively. Fresh and archived samples from Germany were obtained from patients attending HCV treatment at the two main Ludwig Maximilian University hospitals in Germany and those stored at the Max von Pettenkofer Institute (MvPI)- Munich Germany, respectively.

Freshly obtained samples were subjected to serological assays by Enzyme Linked Immunosorbent assay platforms (Ortho HCV 3.0 ELISA test system with an enhanced SAvE and AxSYM ELISA test system, for German samples and Murex ELISA test system, for Kenyan Samples) commonly used in each individual country before subjecting all the samples to a similar nested PCR diagnosis. All the archived samples had also been subjected to PCR diagnosis and confirmed positive at least once in the course of their storage. A total of 25 and 50 samples from Kenya and Germany, respectively, were tested and compared. All the 50 (100%) ELISA positive German samples were again confirmed PCR positive in the standardized PCR diagnostic system, whereas Kenyan samples realized varied results. Despite 100% (4 out of 4) detection by PCR on fresh samples, no detection, 0% (0 out of 21), was realized on the archived samples. These archived Kenyan samples could not also be detected by the available antibody based rapid detection kits. Based on the results realized with archived samples, whose conditions are deemed similar to the blood bank conditions in Kenya, this study asserts that although stored blood bank samples have continuously been used to estimate the prevalence of Hepatitis C infection in Kenya, this parameter may not be appropriate in estimating the true prevalence of this infection in the general population. Therefore, the study concludes and recommends the need to screen and determine the true prevalence of the infection using samples from the general population, since together with Hepatitis B, Hepatitis C infection are emerging as a major point of focus in blood transfusion screening in Kenya. The study further recommends that together with serological assays, Nucleic Acid based Techniques (NAT) should be employed in screening all freshly obtained blood before storage.

Key words: Hepatitis C virus, Fresh and archived samples, Nucleic Acid based Techniques, blood transfusion, blood bank samples

1.0 Introduction

The discovery of transfusion-transmissible infections (TTIs) has brought in a new era in the practice of blood transfusion worldwide with emphasis on two fundamental objectives, safety and protection of human life (Klein, 1995). Blood safety still remains a major concern in transfusion medicine in Kenya where bacterial and viral infections, including HIV, hepatitis B virus, that can be transmitted through blood transfusion still record a significant prevalence, with uncertain prevalence of hepatitis C in the general population. Human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) are of great concern because of their prolonged viraemia and carrier or latent state. They also cause fatal, chronic and life-threatening disorders.

According to a UNAIDS report released in 2002, blood transfusion accounts for 5-10% of HIV infections in sub-Saharan Africa. Similarly, it is estimated that 12.5% of patients who receive blood transfusion in sub-Saharan Africa are at risk of post transfusion hepatitis (Fasola and Otegbayo, 2002). HCV plays an important role in the causation of chronic liver disease (Lesi *et al*, 2002). Muasya *et al.*, 2008, estimates a current prevalence rate of 0.2% to 0.9% among the general population in Kenya. This estimate seems to be supported by findings of Ilako *et al.*, 1995 who recorded a prevalence of 0.9% among volunteer blood donors. However, this may not reflect the true picture since no major survey on disease burden of HCV has been carried out in Kenya in recent times. It is also important to note that these estimates by Muasya and his group are contrary to his findings of a 22.2% prevalence rate among a cohort of drug users in Kenya.

Secondly, Kenya currently concentrates more on the screening for Hepatitis B, HIV and bacterial related sexually transmitted diseases, with little attention given to Hepatitis C virus in donated blood in blood banks. Further, HCV infection can proceed with no apparent clinical signs during the early phase of infection and is often diagnosed only by chance in asymptomatic population. Success of HCV treatment among those infected mainly depends on the infecting HCV genotype. Due to high costs involved in this type of treatment, many developing countries are yet to employ this treatment strategy, thus it is not assured whether all those who are diagnosed for HCV fully recover. Finally, Kenya borders Tanzania without any major cross-border movement health related restrictions. Tanzania has been shown to be among those countries with the highest HCV prevalence rate in the world (WHO, 1997) with Talatela *et al* (2007) recording a prevalence rate of 13.8% among children infected with HIV. These factors highlighted seem to suggest a higher prevalence rate of HCV in Kenya than the current rates, as assumed so far.

It is also clear that most studies on HCV prevalence use blood donors to report the frequency of HCV usually by anti-HCV antibodies and do not, normally, report follow-up HCV testing. It is important to note, however, that using blood donors as a prevalence source may underestimate the real prevalence of the virus because donors are generally from a healthier population stratum.

Following a 1994 study showing a high rate of transfusion-associated HIV (Moore *et al.*, 2001), Kenya implemented WHO blood safety recommendations including: organizing the Kenya National Blood Transfusion Service (NBTS), stringent blood donor selection, and universal screening with fourth-generation p24 antigen and HIV antibody assays, aimed at reducing HIV post transfusion transmission, a strategy which has realized great achievements in HIV reduction (Basavaraju *et al.*, 2010). These stringent measures have not been so in the case of HCV screening, which has largely depended only on antibody detection. This study thus compared the rate of detection of HCV in freshly obtained and archived blood from Kenya and results obtained compared with a similar study with the infected German samples.

2.0 Materials and Methods

2.1 Study Design

This was a cross-sectional comparative study.

2.2 Study Sites

Fresh Kenyan samples were obtained from patients attending the liver clinic at the Kenyatta National Hospital, which is a national referral hospital in Kenya, whereas archived samples were obtained from those stored at the Kenya Medical Research Institute (KEMRI), an institute accredited for medical research in Kenya.

In Germany, the study was done using samples from patients attending HCV treatment at the two main Ludwig Maximilian University hospitals in Germany and those stored at the Max von Pettenkofer Institute (MvPI)- Munich Germany.

2.3 Ethical Considerations

The study in Kenya was done with the approval from the KEMRI ethical committee, whereas the study Germany was done with the permission from Max von Pettenkoffer – institute, an institute accredited for conducting medical research apart from routine diagnosis. In general, patients' confidentiality was completely maintained, with samples only identified with specially assigned identification numbers.

2.4 Laboratory Procedures

Kenyan samples

Antibody detection in samples

2.5 Rapid Kit Testing of HCV

All samples collected were detected for HCV antibody using a KEMPAC® rapid kit - a chromatographic immunoassay (CIA) for detection of HCV antibodies for hepatitis C in human serum. 60µl of the serum was added to the sample well and results read after 15 minutes. Two bands on the membrane indicated that the patient was positive for HCV-ab while one band indicated a negative result.

2.6 Screening for HCV Using ELISA Method

Samples were further analyzed by Murex (Abbott/ Murex Biotech Corp., Dartford, UK) ELISA kit as per the manufactures' instructions. Briefly; 180µl sample diluent was added to each well followed by 20µl of samples and the controls to the respective wells. The mixture was then incubated for one hour at 37°C thereafter it was washed using automated ELISA washer. Immediately after washing 100µl of conjugate was added into each well, covered the wells and incubated at 37°C for another one hour. After incubation the wells were washed and 100µl of the substrate solution added to each well. The plate was kept in a dark room for color development for 30 minutes at 37°C after which a stop solution (1M of H₂SO₄) added to each well prior to reading the absorbance at 450nm using ELISA plate reader.

2.7 German Samples

2.7.1 Ortho HCV 3.0 ELISA Test System with an Enhanced SAVE

Blood samples were first tested using Ortho HCV 3.0 ELISA test system with enhanced SAVE (Ortho Clinical diagnostics, Johnson and Johnson, United Kingdom) in an automated system as per the manufacturer's instructions.

2.7.2 AxSYM HCV version 3.0

After evaluation with Ortho ELISA system, blood samples were further evaluated with a more sensitive AxSYM ELISA system (AxSYM system HCV version 3.0, Abbott, USA), also as per the manufacturer's instruction.

2.7.3 Detection for RNA by PCR

Samples from both study sites in Kenya and Germany were subjected to similar extraction and amplification protocols as follows;

2.8 Sample Extraction

Sample extraction was done using the High Pure Viral Nucleic Acid kit, version 3 (Roche applied science; Cat no. 11858874001 - Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions.

Serum 200 µl was mixed with 200 µl of binding buffer supplemented with poly (A) and 50 µl proteinase K. These were mixed immediately and incubated for 10 minutes at 72°C. After incubation; further 100 µl of binding buffer was added to the mixture and separated using high pure filter tubes. During separation the mixture was washed with 500 µl of a special inhibitor removal buffer to get rid of PCR inhibitory contaminants. There were two wash steps using 450 µl wash buffer to either, remove bound nucleic acids, purification from salts, proteins and other cellular impurities, was

done two times using. The bound RNA was finally eluted using 50 µl of elution solution. The purified RNA was then stored at -20°C.

2.9 Reverse Transcription

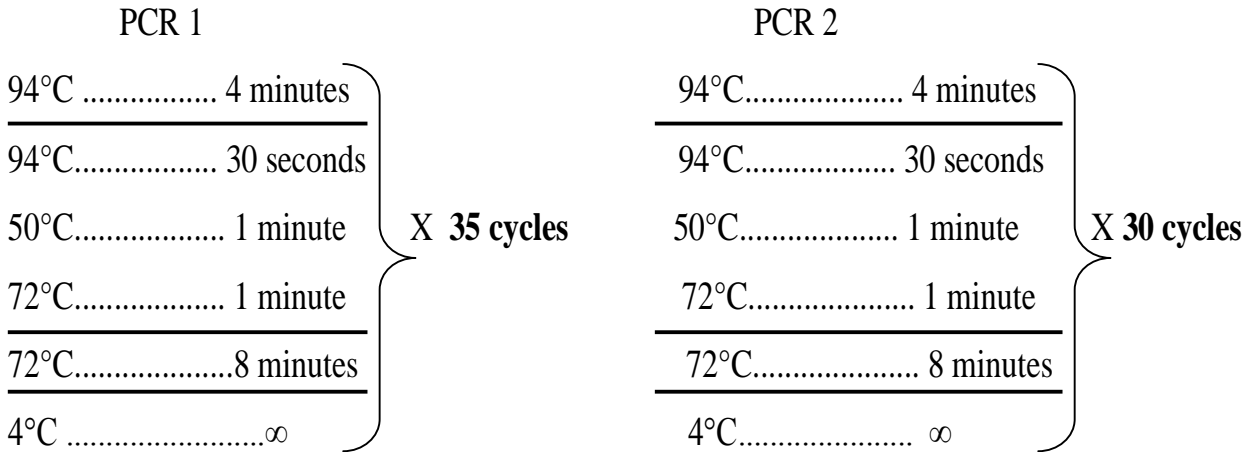
Purified RNA extracts were reverse transcribed to generate complementary DNA (cDNA). Reverse transcription was done using primers specific for 5'UTR (HCVN 02: 5' – gTg CAC ggT CTA CgA gAC C – 3' and HCVN 08: 5' –TAC TCA CCg gTT CCg CAg A – 3'). Reverse transcription was done using in-house protocol from the diagnostic virology department of MvPI. During this process, generated RNAs were linearized by incubating the RNA at 65°C for 10 minutes and then immediately subjecting the RNAs to a temperature of 4°C for a minimum of 10 minutes before further treatment.

The master mix used in reverse transcription contained 4 µl of 5x Buffer, 2µl DTT, 2.2 µl of 10mM dNTPs, 1.6 µl of random primer and 0.2 µl of superscript. These were mixed with 10 µl of linearized RNA products generated, and incubated at 45°C for 1 hour, in order to generate cDNA which was then used to generate PCR products in a nested PCR reaction.

2.10 Nested PCR

cDNA products generated were amplified by nested PCR using two sets of in- house designed primers, both forward and reverse primers, as described in the in-house protocol for MvPI. Primers used included outer primers for the first PCR reaction; HCVN 01: 5' – ggC gAC ACT CCA CCA TRR A – 3' (forward primer) and HCVN 02: 5' – gTg CAC ggT CTA CgA gAC C – 3' (reverse primer). Inner primers for the second PCR reaction; HCVN 03: 5' – CAC TCC CCT gTg Agg AAC T – 3' (forward primer) and HCVN 04: 5' - CCC ggg gCA CTC gCA AgC A – 3' (reverse primer). Generated PCR products were then run on 2% gels to asses the products.

Generally, the composition of the master mix for PCR included 31.7 µl and 33.7 µl PCR water for the first and the second PCR reactions respectively. Other components used for both PCR reactions included 2 µl of 5mM dNTPs, 5 µl of 10x buffer with Mgcl₂, 3µl of reverse and forward primers and 0.3 µl of Taq polymerase. Reactions for PCR 1 and PCR 2 were as follows;



3.0 Results

This study realized clear positive results with only 2 (8%) samples of all the 25 known positive Kenyan samples tested with the rapid antibody kits, commonly used for HCV screening in Kenya. Another 2 (8%) samples showed unclear results, indicated by the intensity of band formation (Plate 1a), whereas the remaining 21 (84%) could not be detected completely by these kits (Plate 1b).

When these samples were further subjected to Enzyme Linked Immunosorbent Assay, using Murex ELISA kit, the two positive and the two unclear samples by the rapid kits were detected making a total of 4 (16%) detections by this assay platforms. The remaining 21 (84%) samples that were not detected by the antibody rapid kits could not be detected by this ELISA kit. All the four samples positive by Murex ELISA kits were positive by RNA amplification (Plate 2). The other 21 samples that could not be detected by Murex ELISA and the rapid antibody kit could also not be

amplified by PCR system (Plate 3). All the samples detected within this group were all fresh samples obtained in the course of the study. Percentile detection within the two groups of samples varied across the different assay platforms (Figure 1) used.

All German samples were detected by AxSYM and Ortho antibody ELISA kits used in the study. Of the total 50 samples, 9 (18%) could not be amplified by PCR despite high optical density values realized with AxSYM ELISA kit. These samples were classified as PCR negative - Antibody positive samples (Figure 2).

The remaining 41 (82%) were both positive by the ELISA kits as well as by PCR, and categorized as PCR and Antibody positive (Plate 4a). They included 5 samples that had freshly been obtained during the study (Plate 4b).

It was also realized that all the archived samples had viral loads above the suggested 260,000 IU/ml theoretical threshold limit of detection for most HCV ELISA kits (Turke *et al.*, 2008). Except for only two samples, all other samples had the viral loads ranging from 300,000 to 7.5 million IU/ml, despite the fact that some samples had been in the archives for slightly more than 3 years, with the freshly obtained samples ranging from 8.9 to 29 million IU/ml. This study did not however realize any direct correlation between the viral loads and the optical signals generated for these samples, as some samples with viral loads of 40,000 IU/ml (which had stayed for 2 years in the archives) giving higher signals than those samples recently obtained and whose viral loads were high (Figure 3).

The study further assessed the differences in viral load measurements for four samples, immediately after thawing from -70°C and after exposure to room temperature for 24 hours. These samples were divided into 2 vials each. One vial for each was put back in the freezer at -70°C immediately while the next vial was exposed for 24 hours at room temperature, re-frozen for another 24 hours at -70°C before viral loads for all the vials, both frozen and exposed were measured. The study realized a reduction in viral load measurements for all the exposed vials across all the samples, with 2 samples undergoing 1 log reduction in viral loads as summarized in Table 1 and shown in Figures 4.

4.0 Discussion

All archived Kenyan samples could not be detected either by rapid, ELISA or even by PCR during this study, despite the fact that these samples had been categorized and archived as HCV positive samples. Despite the fact that freshly obtained samples were detected easily by antibody ELISA and by PCR amplification, two of these samples produced unclear results with rapid antibody test kits, which are deemed fast, affordable and easily available for HCV diagnosis. Most of these archived samples had previously been obtained from samples donated for blood bank from various regions in Kenya. These findings have major implications not only for HCV routine diagnosis but mainly for screening of blood intended for transfusion.

When compared to similar HCV positive samples obtained from archives in Germany, German samples could easily be detected by the ELISA systems commonly used in HCV diagnosis in Germany. These samples could also be amplified by PCR, which was not the case with archived samples from Kenya, despite the fact that a similar protocol for diagnosis was used for both samples. This finding in failure of detection for archived Kenyan samples generates various scientific arguments.

One argument could revolve around the possibility that after all, the samples could have been negative from the beginning and thus been a result of false positive samples mistakenly archived as HCV positive samples, leading to wastage of blood that could have easily been used to save lives. This argument may be supported by various observations based on the fact that most kits of HCV used in Kenya, are not evaluated using Kenyan isolated strains. This fact has resulted in continuous generation of conflicting data depending on the screening kit used with some kits reported to pick false positives (Tess *et al.*, 2000). In this study, however, a similar ELISA assay platform (Murex kit) that was used for detection of Kenyan samples has continuously been used for HCV detection, apart from the rapid kits. Similar protocols for HCV diagnosis, used here, have also been used in various studies involving Kenyan samples, non conflicting positive results generated (Karuru *et al.*, 2005; Muasya *et al.*, 2008; Muasya 2009). Further, the number of archived samples was also too large to warrant any false positive results by any assay platform.

Genotype variation within different areas of the world could be another argument for the different observation noted especially between the German and Kenyan samples. However, in this study, all German PCR positive samples were

genotyped and others sequenced. Genotypes 1 to 4 were identified (data not published), this was attributed to the fact that Munich is a cosmopolitan city, hence this possibility. Since other studies (Muasya *et al.*, 2008) identified mainly genotype 1a and 4 to be prevalent among Kenyan samples, the fact that these genotypes were identified among the German samples, is a reason enough to rule out this line of argument.

Another argument would be based on the fact that unstable temperatures lead to a reduction in HCV RNA detection (Busch, 1992). Frequent thawing and freezing could therefore have contributed to a reduction in the levels of RNA eventually reaching below the threshold levels of detection. During the study, it was realized that when some of the positive samples were subjected to a room temperature for 24 hours then frozen again at -70°C for another 24 hours, a reduction in the viral load was observed in all the samples. In two samples, there was up to one log reduction after the 24- hour exposure. This further supported this finding by Busch that a prolonged storage at room temperature resulted in a reduction in detectable HCV RNA concentration of more than 3 log, whereas freezing and thawing caused a half – log reduction (Busch, 1992). In their study Busch and colleagues (Busch *et al.*, 2006), also suggested ethnicity or race to be factors associated with HCV RNA negativity in sero-positive blood donors. This assertion could not fully be evaluated during our study.

From all these highlighted arguments, the only feasible explanation for our findings is based on instability in temperatures, coupled with constant freezing and thawing as factors that were responsible for our observations. This explanation is particularly important for blood transfusion practice in Kenya. Blood transfusion is an essential component of quality medical care. However, blood transfusion is a treatment that may also pose potential risks of transfusion related diseases to the patient. Before the introduction of the National Blood Transfusion Services (NBTS), whose aim was to reduce risks of transfusion associated transmission, the risk of transmission of HIV through infected blood was estimated to be 2% based on the fact that HIV prevalence among blood donors was 6.4% (Moore *et al.*, 2001). Although this risk has drastically reduced in HIV (Basavaraju *et al.*, 2010), no similar data show a reduction of this risk transfusion related transmission in HCV.

The findings in our study suggest that even with the introduction of NBTS risk of HCV post transfusion transmission would still be high depending on criteria of collection, blood screening methods and storage of blood bank samples. From our findings, it is clear that the discrepancy seen with archived German and Kenyan samples pointed mainly to the storage methods, hence the complete reduction of RNA below the threshold of detection.

Constant power interruptions, common in Kenya, are a major factor that immensely interferes with refrigeration temperatures. Longer periods of power interruptions will therefore lead to reduction in HCV viremia. Storage at the point of blood donation, distance from the regional blood transfusion centers, coupled with poor infrastructure in the country are a potential for significant reduction in viral load for HCV. This may lead to no detection by the commonly used antibody based assays available for HCV diagnosis. Nucleic Acid based Techniques (NAT) are currently not in use for regular screening or even HCV diagnosis due to high expenses associated with them.

One major limitation of this study was the fact that we were not able to determine the effect of temperature fluctuations on HCV specific antibodies. However, the results realized are suggestive of a significant effect of temperatures on antibody detection.

Just like other studies that have been done on HCV, this study asserts that HCV is an important blood borne viral infection, just like HIV and HBV infections. It concludes that the uncertainty surrounding the true prevalence of HCV in the general population in Kenya is a reason for increased concentration of this infection in blood transfusion services. Achievement in the reduction of HCV post transfusion will mainly depend on determining the true prevalence of the virus in the general population who are the main blood donors in Kenya. Furthermore, it was realized that all the 4 new cases in Kenya did not have any prior knowledge of infection, hence their diagnosis by chance. This may be an indicator on what exists among the general population.

Therefore, this study therefore recommends that a major survey to determine the true prevalence of HCV in the general population in Kenya should be carried out. It further recommends the use of Nucleic Acid based Techniques (NAT) in HCV screening for blood bank samples.

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PLATE 1A

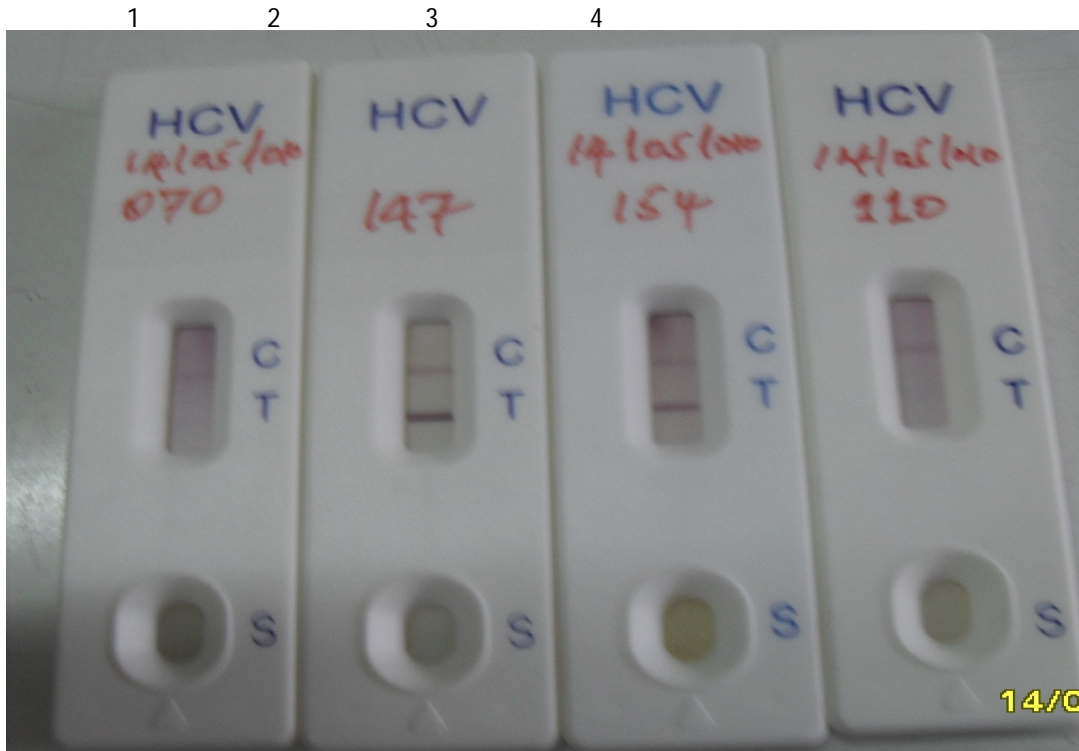


Plate 1a: Clear results shown in kits 2 and 3; kits 1 and 4 show faint bands indicating unclear results

PLATE 1B



Plate 1b: Samples not detected by HCV antibody rapid kit

PLATE 2

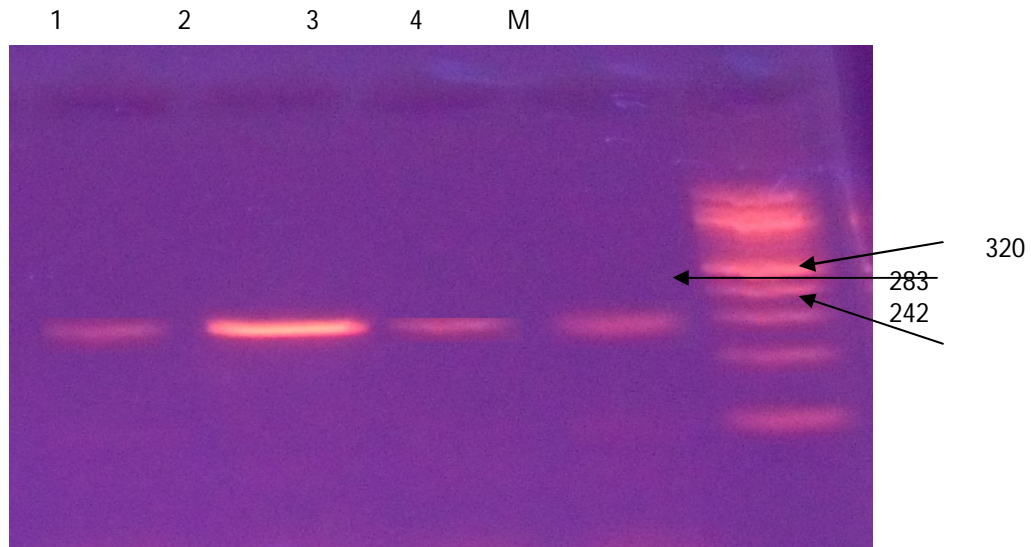


Plate 2: PCR Positive Kenyan samples. M: DNA molecular marker VIII; wells 1 to 4 HCV samples

PLATE 3

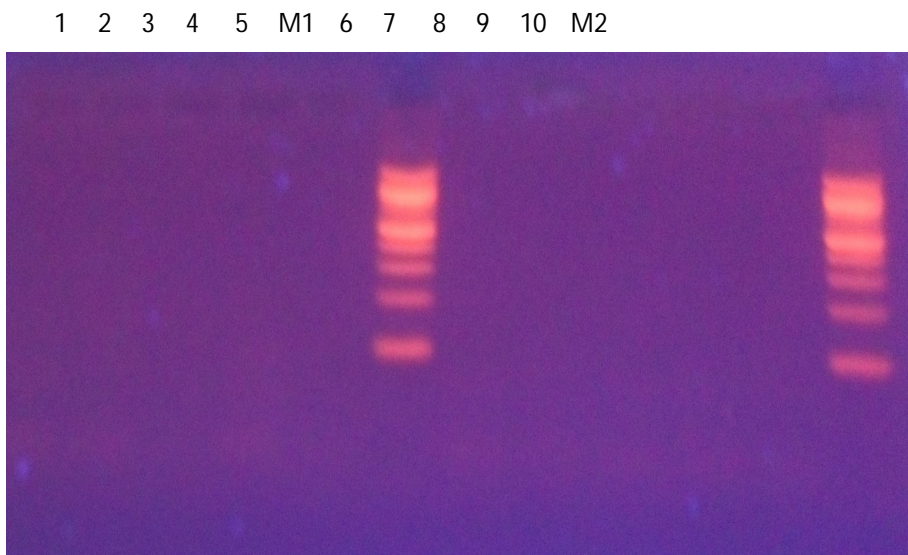


Plate 3: Non detected Kenyan samples by PCR. M1 and M2: DNA molecular weight marker VIII; wells 1 to 10 HCV samples

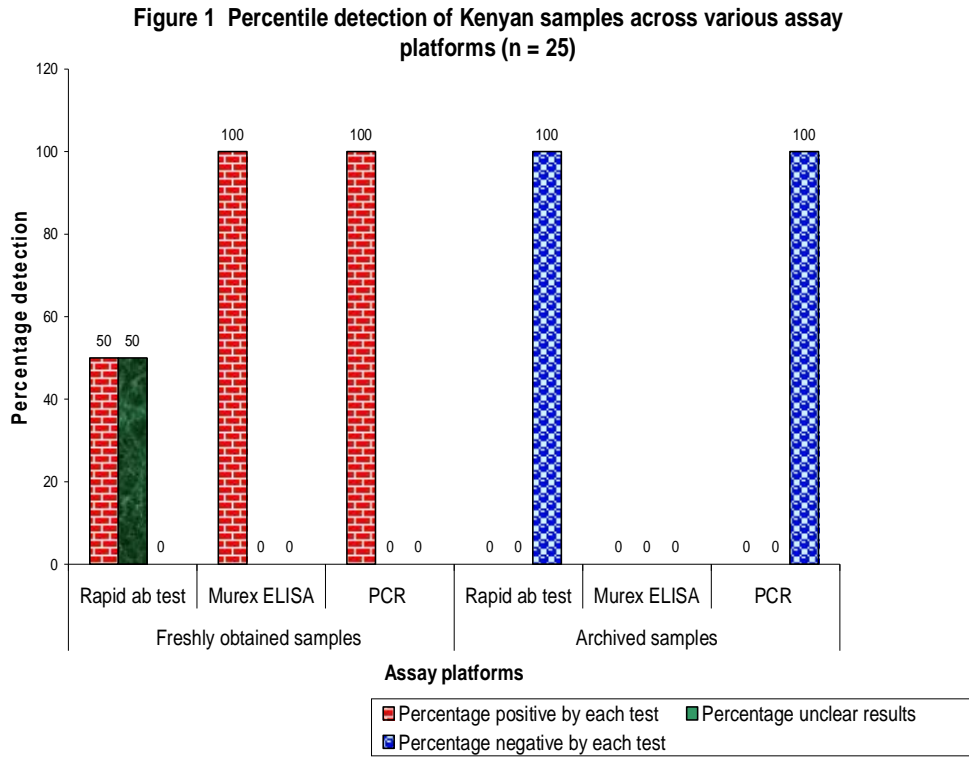


Figure 1: Percentile detection of Kenyan samples across various assay platforms (n = 25)

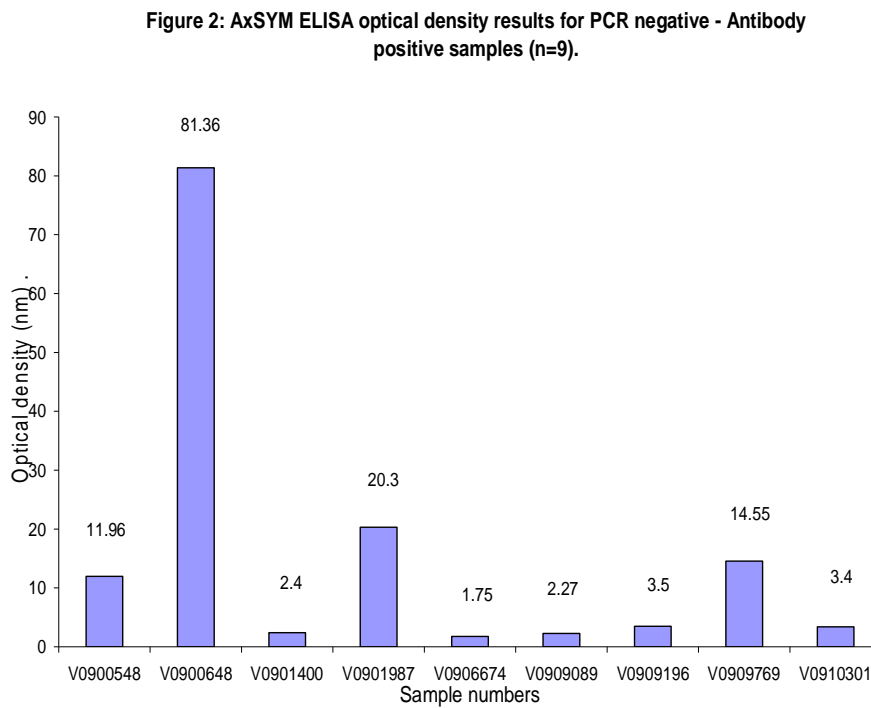


Figure 2: AxSYMELISA optical density results for PCR negative – antibody positive samples (n = 9)

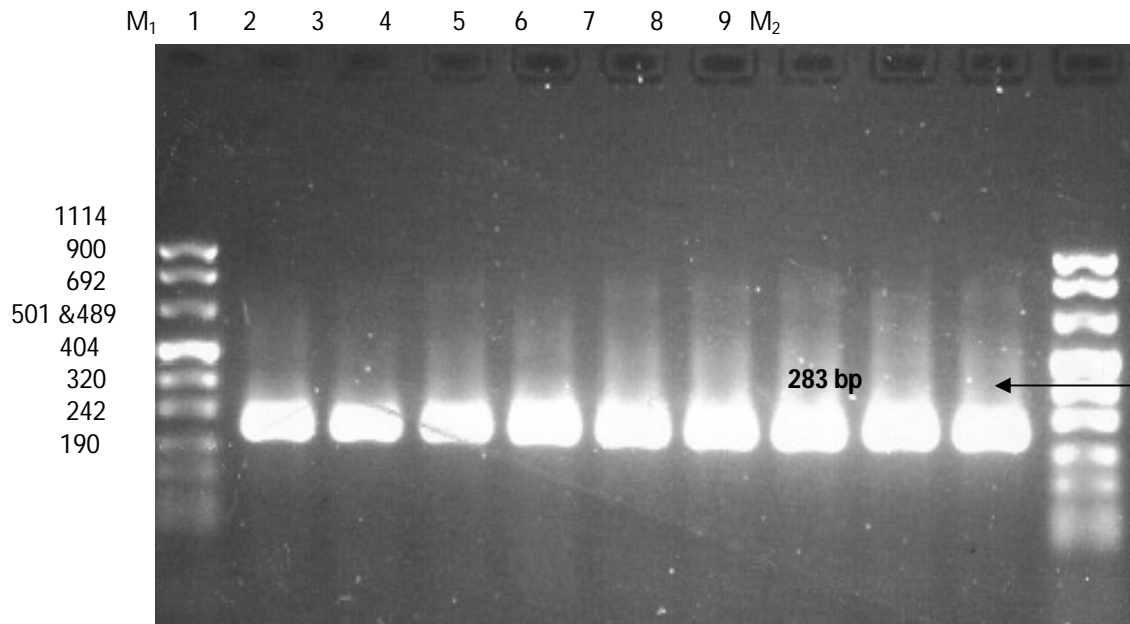


Plate 4a: Nine of the Thirty six archived samples amplified by PCR. M_1 and M_2 - DNA molecular weight marker VIII (0.019 – 1.11 kbp), wells 1 to 9 - bands formed at 283 bp indicating positive results

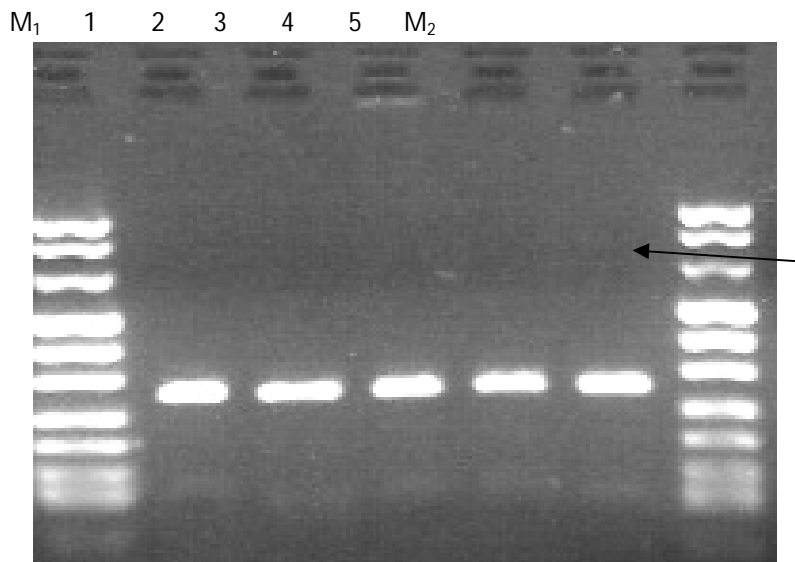


Plate 4b: Five fresh samples amplified by PCR M_1 and M_2 - DNA molecular weight marker VIII (0.019 – 1.11 kbp); wells 1 to 9 – positive bands

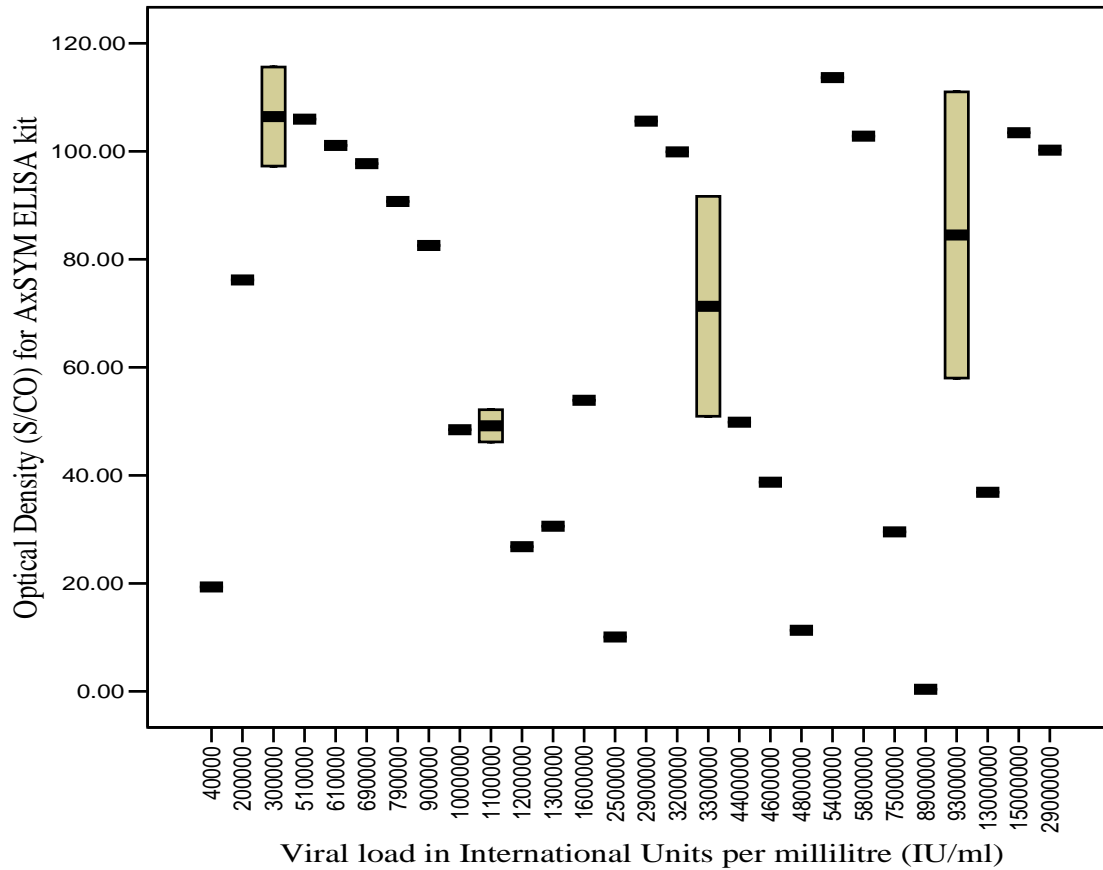
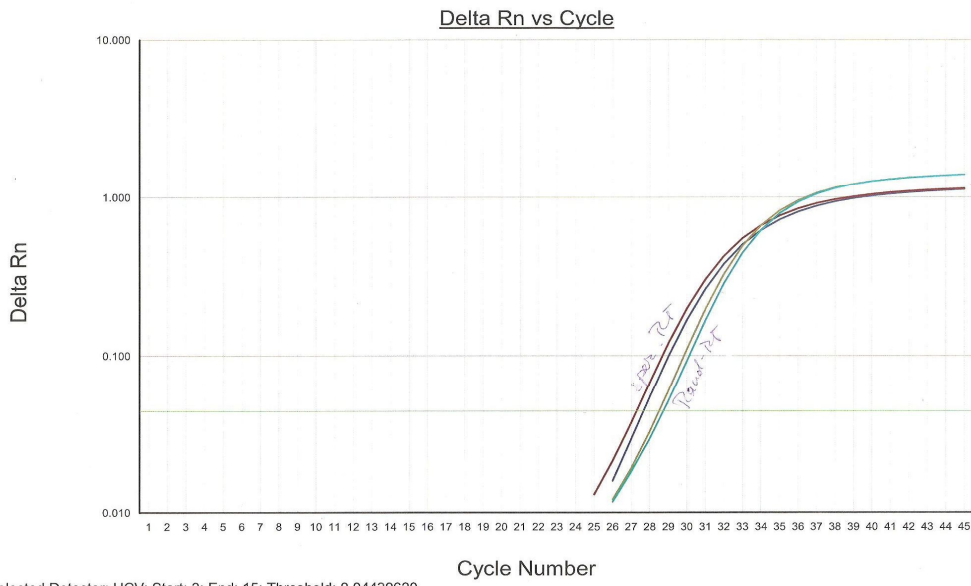
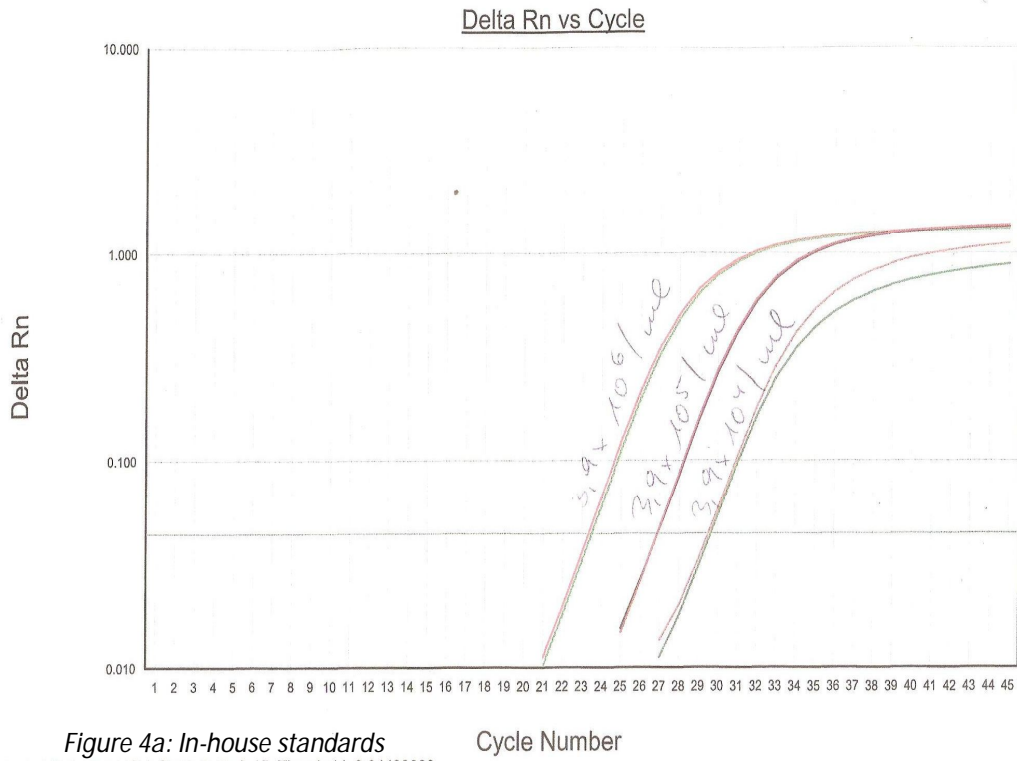


Figure 3: Optical Density versus viral load for some archived and fresh samples

Table 1: Viral load differences between Frozen and exposed HCV samples (n = 4)

Number	Viral Load (IU/ml)		
	Load before exposure	Load after exposure	Difference in VR
V0836795	2.1×10^5	9.0×10^4	1.2×10^5
V0836012	6.0×10^4	1.2×10^4	4.8×10^4
V0832282	2.0×10^6	8.9×10^5	1.11×10^6
V0831394	7.1×10^5	7.0×10^5	1.0×10^4



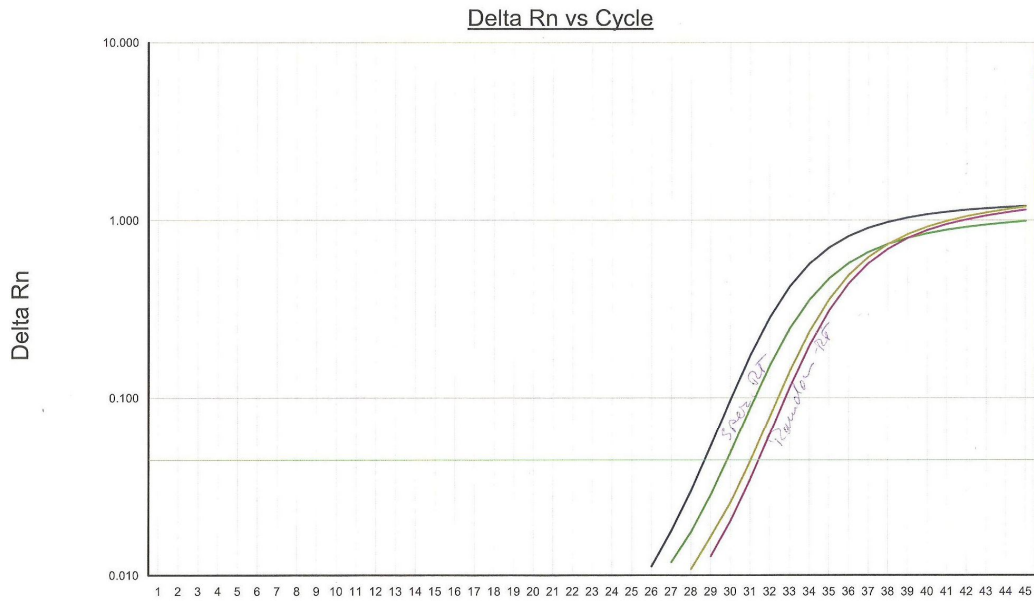


Figure 4c: Sample V0836012 Cycle Number

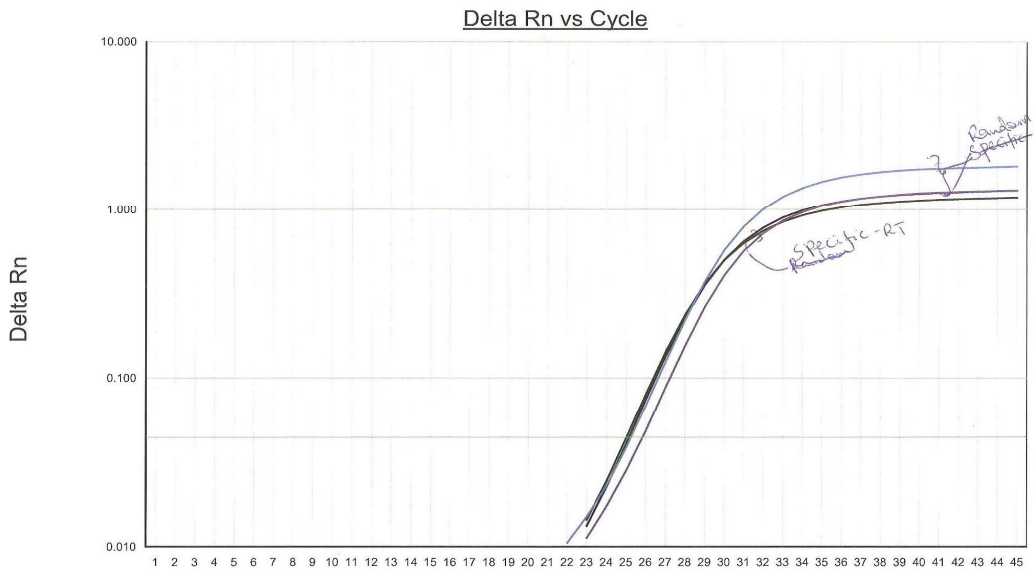


Figure 4d: Sample V0832282 Cycle Number

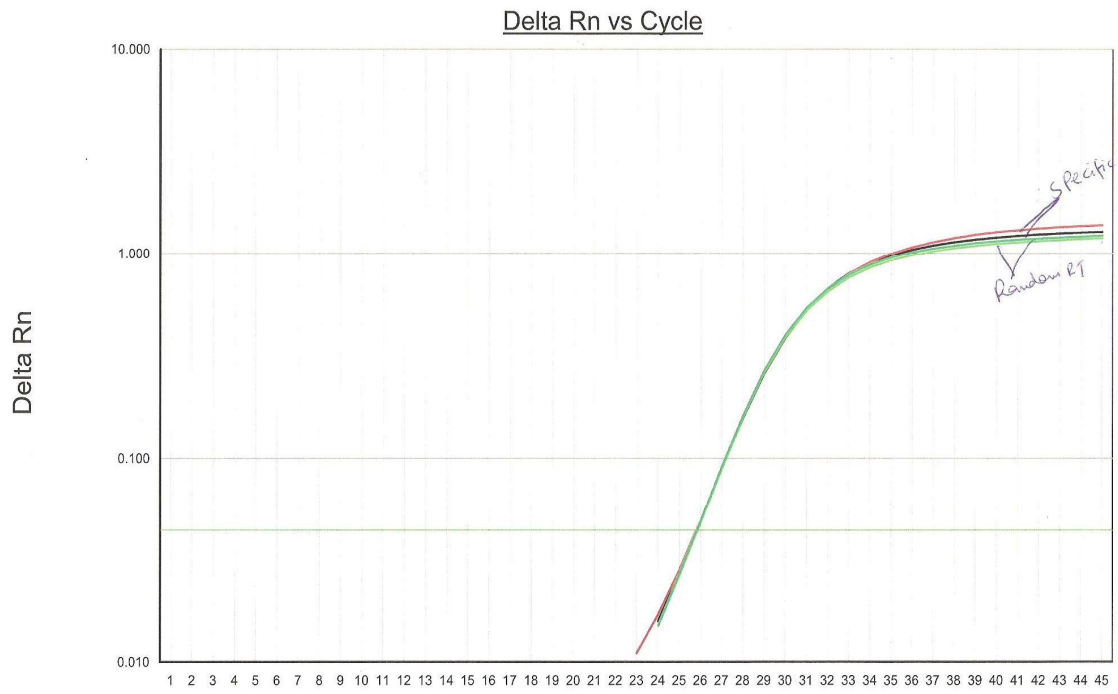


Figure 4 e: Sample V0831394

Cycle Number

