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Effect of Specimen Handling on Quantitative HIV-1 Viral Load Measurement at Saint Paul Hospital Millennium Medical College, Addis Ababa

By

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This is to certify that the thesis prepared by Gadissa Gutema, entitled:

The Effect of Sample Management on Quantitative HIV-1 Viral Load Measurement at St Paul Hospital Millennium Medical College, Addis Ababa and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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List of abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretro viral Therapy
CD	Cluster of Differentiation
CDC	Center for Disease Control
cDNA	Complementary Deoxyribose Nucleic Acid
EDTA	Ethylene Di amine Tetra Acetic acid
EPHI	Ethiopian Public Health Institute
FMOH	Federal Ministry of Health
HIV	Human Immunodeficiency Virus
IC	Internal Control
IRB	Institutional Review Board
NHIVRL	National HIV Reference laboratory
OWA	One World Accuracy
PBS	Phosphate Buffered Saline
PLWHIV	People Living with Human Immunodeficiency Virus
PPT	Plasma Preparation Tube
RANOVA	Repeated Analysis of Variance
RNA	Ribonucleic Acid
RT PCR	Real Time polymerase Chain Reaction
RVI	Retroviral Infection
SD	Standard Deviation
SOP	Standard Operating Procedure
SPHMMC	Saint Paul Hospital Millennium Medical College
SPSS	Statistical Package for the Social Sciences
Tth	Thermos Thermopiles
UNAIDS	United Nations Program HIA/AIDS
WHO	World Health Organization

Abstract

Background: Proper specimen handling prior to quantification of plasma HIV-1 RNA viral load is important, since some reports suggest that variations in specimen handling may affect detection and quantification of plasma RNA. However, there is limited evidence on the effect of time of plasma separation, storage, freeze-thawing and dilution on the HIV-1 RNA viral load.

Objective: To determine the effect of sample management on HIV-1 RNA viral load measurement in St. Paul Hospital Millennium Medical College from April to July 2019.

Methods: Experimental study design was conducted in St. Paul Hospital Millennium Medical College from April to July 2019 GC in people living with HIV. Whole blood sample was collected into two EDTA test tubes from 88 eligible participants. The viral load test was done by Abbott m2000sp/rt analyzer. Data was entered into Microsoft excel and analyzed by SPSS version 20. Repeated measure analysis of variance was used to compare HIV RNA viral load mean difference between different time of plasma separation, storage, freeze-thawing and dilution. Post-hock analysis was employed to locate the place of significant difference. Level of significance was set at 5%.

Results: There was significant HIV-1 RNA viral load log mean difference between plasma separation time at 6 hours (hrs) and 24hrs ($p<0.001$). Similarly, there was significant HIV-1 RNA viral load log mean difference between plasma tested within 6hrs, stored at 2-8^{0}c for 6 and 15 days ($p = 0.006$ *and* <0.001). HIV-1 RNA viral load log mean difference was not observed when plasma was stored at 2-8^{0}c for 6 days ($p= 0.999$) and at - 20^{0}c for either 30 ($p = 0.899$) or 60 days ($p = 0.999$). There was significant log mean difference between plasma that exposed to 4th cycle of freeze-thawing in -20^{0}c when compared with plasma tested within 6hrs ($p = 0.013$). For the three dilution proportions (1:2, 1:3 and 1:5) there was no significant difference on mean RNA copies when compared to each other and tested within 6hrs ($p = 0.999$).

Conclusion and recommendation: Only plasma separated at 24hrs, stored at 2-8^{o}c for 15days and freeze-thaw 4th cycle had statistically significant effect on HIV RNA viral load variation. Though the differences were not clinically significant at a cut-off viral load level of 0.5 $\log_{10}$, therefore focusing on these factors could improve the result quality and ultimately improve patient care.

Key words: *Human immunodeficiency virus, HIV-1 RNA viral load, quantitative RT PCR, Abbott m2000sp/rt analyzer, Ethiopia.*

1. Introduction

1.1. Background

The human Immunodeficiency virus/Acquired immunodeficiency syndrome (HIV/AIDS) disease progression and antiretroviral therapy (ART) success as well as failure are mostly monitored by immunological markers Cluster of Differentiation (CD4 T-cell quantification), clinical criteria, and viral load monitoring [1]. However, plasma viral load is the best and the gold standard predictor for detecting of HIV-1 disease progression and treatment failure among people receiving ART [2]. It also enables clinicians to assess the success of treatment and detect treatment failure prior to onset of clinical symptoms [3].

The reproducibility and accuracy of HIV-1 Ribonucleic Acid (RNA) viral load quantification are still a major concern, because HIV naked RNA is extremely unstable in whole blood or plasma [4]. Careful specimen handling prior to testing of plasma for HIV-1 RNA levels is important since way of handling may profoundly affect the detection and quantification of plasma HIV-1 RNA [5, 6]. Moreover, controversies exist as to whether values of HIV-1 RNA obtained under different conditions may not vary significantly [7].

According to World Health Organization (WHO) technical recommendations, plasma sample is recommended for HIV viral load [2] and it must be separated within 6 hours at room temperature after whole blood collection [2, 8]. Evidences indicate, plasma sample can stay at room temperature without viral degradation for up to 24 hours, 5 days at 2–8 ° C and for longer periods at -80^{O}C [2]. Without plasma separation from whole blood, there is a large amount of viral RNA degradation during the first 3 to 6 hours [6, 9]. Studies, however, have shown that viral RNA level found in plasma or whole blood is relatively remained stable after 6 hours of sample collection at 4°C for 24 to 48 hours [6, 9].

RNA is relatively stable in Ethylene Di amine Tetra Acetic acid (EDTA) tube, but the concentration could be increased due to the release of necrotic and apoptotic blood cells [10]. In contrast, the RNA could decrease due to degradation by the action of enzyme RNases [10] Thus, plasma prepared in EDTA tube is recommended to maintain RNA stability.

Another factor that can affect the stability of HIV quantification is frequency of freeze-thaw cycle [11]. It is indicated that there is direct and close association with HIV viral load quantification and three times freezing and thawing particularly after long term storage (12 months) at -70°C [11].

Plasma could be diluted by diluents when the volume is insufficient and the concentration of HIV-1 RNA is above the reading limit of a given instrument. However, plasma dilution by phosphate buffered saline (PBS) has been shown to have an effect on the HIV-1 RNA concentration [12]. In general proper handling of specimen is required and mandatory, because, any inconvenience happened from sample collection to amplification could affected the final result significantly [13].

Ethiopia is among high HIV prevalence countries with a recent national prevalence of 0.9% [14] and according to the latest Spectrum modeling, an estimated 610,335 people were living with HIV in 2018[15]. However, there are only 19 HIV viral load testing centers in the country, and not decentralized to point of care hence necessitating for specimen referrals which require proper handling.

1.2. Statement of the problem

Laboratory infrastructure and samples transportation are the main complications that affect HIV-1 RNA viral load quantification in developing countries [16]. Plasma sample is the first choice for RNA based viral load testing for HIV according to standard protocols [2]. To obtain reliable results specimen collection, preparation, storage and appropriate analysis are the basic requirement. That means the final results could be affected significantly if specimen management problems occur at any of the steps, starting from sample collection to amplification [13].

In developing countries only 20% of Retroviral Infected (RVI) patients get accesses to viral load testing because of lack of infrastructure and financial limitation [17]. Furthermore, immediate sample processing and testing in HIV viral load laboratory is difficult due to reagent stock out, backlogs, insufficient human resource, equipment downtime, centralization of instruments and lack of maintenance. Moreover, as a result of lack of storage space, specimens are stored in refrigerator which affects the quality of specimen and hence giving unreliable results [17, 18].

On the other hand, specificity, sensitivity and reproducibility of Real Time Polymerase Chain Reaction (RT-PCR) based assays of RNA quantification depend on stability of viral RNA in both whole blood and plasma. However, the stability of viral RNA in both whole blood and plasma in turn depends on sample management process. Poor specimen handling could result in viral RNA degradation which may leads to underestimation of viral RNA concentration and leads to unsuppressed HIV viral load result to be wrongly reported as suppressed [9]. It also leads to drug regimen shift delay from first line to second line treatment and hence resulting in the dissemination of resistant strain. In so doing, it misleads policy makers and other stakeholders by assuming viral suppression is good in the population [19].

There are 19 HIV viral load testing laboratories found in Ethiopia which are far from blood collection sites (hospitals and health centers). As a result, there is a need for a referral system to ensure accessibility of the service. This condition leads to sample preparation and management (sample storage and transportation) which in turn delay the plasma separation time, require long time storage until transported and frequent freeze-thawing. Although the aforementioned sample management related problems are documented in developed countries, there is limited information in developing countries where the burden of HIV is

high. As far as my literature search goes, there is no published study in our country addressing the effect of specimen handling on HIV viral load quantification which is widely utilized in the country for ART monitoring. Therefore, this study was aimed to determine the effect of plasma separation time, storage temperature variation, freeze-thaw cycle and dilution proportion on HIV-1 RNA concentration in Saint Paul Hospital Millennium Medical College (SPHMMC) from April, 2019 to July, 2019.

1.3. Significance of the study

The findings of this study will help in revealing the variation that occurs due to plasma separation time, storage conditions, freeze-thaw frequencies and dilution proportions so that medical laboratory professionals could take appropriate care. It also supports to indicate the problems that are related to delay in plasma separation time, storage temperature variation and freeze-thaw cycle and dilution proportions. Identifying the problems associated with improper specimen management will help laboratory managers and policy makers to design targeted intervention as some of the interventions may require resources as well as increasing the viral load testing sites. Finally, this study could help in improving quality of patient care through reliable laboratory result generation.

2. Literature Review

Stability study of HIV-1 RNA is basic during quantification of viral load, which is determined by comparison of means difference between different conditions and should be within established thresholds. RNA stability was defined as less than or equal to 0.5 log degradation [11, 14]. Such studies are of paramount importance as specimen referral is unavoidable practice in developing countries where the burden of HIV is also high and where resource is limiting to expand viral load testing in all ART service provision areas. In this regard, stability studies revealed different results. For example, a study have shown that HIV RNA maintained stability in EDTA whole blood and plasma up to 168 hours (7 days) when stored at 4^{O}C. Moreover, stability was detected at 72 hours in whole blood stored at 25^{O}C, even though statistically insignificant variation was detected. In addition, plasma stored at 30^{O}C maintained stability up to 48 hours [14].

An earlier study investigated the effects of specimen handling on stability of HIV-1 RNA levels in plasma. Accordingly, blood specimens were collected, processed, and stored under a variety of conditions that might have affected HIV-1 RNA stability. The study found that when whole blood was processed within 2hrs of specimen collection the levels of HIV-1 RNA detected were comparable with plasma samples separated after 8 and 20hrs [5]. Moreover, there was no difference in viral load if the specimen was centrifuged and aspirated at 2hrs or 6hrs post phlebotomy [20]. In contrast, relatively higher rate of loss of detectable RNA was observed during whole blood storage at room temperature in the first 0 to 3hrs and then 3 to 6hrs post collection [6, 9].

Other studies also demonstrated decline of different degree of HIV-1 RNA concentration when analyzing samples after storage as whole blood for 24hrs, although, it was not statistically significant [18, 21]. Some evidence extend the stability time up to 30hrs [5]. In addition to these certain studies shows that HIV-1 RNA were stable up to 72hrs at room temperature as a whole blood, [11, 17, and 22]. A study which determined viral load stability of HIV-1 RNA in whole blood samples stored in EDTA tubes showed a decrease in viral load over time and a statistically significant decrease were observed at day 7 [23]. In contrast to these a decrease of 20% observed in a study which also shows that HIV-1 RNA viral load concentration decrease with rate 0.8% per hour [9].

Comparisons of plasma separated and analyzed with in 4hr versus plasma stored at room temperature for 24hrs as whole blood had good correlations (*0.923*) [5, 7]. It was also found that RNA copy numbers were maintained within 0.5 $\log_{10}$ (approximately threefold) in whole blood [11].

Viral loads in plasma after storage for 6hrs at either room temperature or 4^{o}c, followed by shipment at ambient temperature or on wet or dry ice, were not significantly different from baseline viral loads [18].This is contradicting to other studies which demonstrate great loss during the first 6 hours regardless of specimen storage condition, either room temperature or 4^{o}C. After 6hrs plasma viral RNA concentration remained relatively stable for both temperatures (RT) up to 24 to 48hrs. The decrease within these conditions was relatively less for plasma compared to whole blood [6, 9].

Evidence about stability of HIV RNA in plasma specimens stored at 4^{0}c indicates that keeping plasma at 4 ^{o}c for 1 week did not affect HIV RNA measurement when compared with HIV-1 RNA concentrations determined from fresh plasma [24, 25]. The correlation was significant for each of the three temperatures with no RNA decay [24]. Besides, plasma separated and analyzed within 4hrs and analyzed after 24hr storage of plasma at room temperature had good correlation (*0.951*) [7]. It was also found that HIV-1RNA copy numbers were maintained within 0.5 log10 (approximately threefold) in plasma samples held at room temperature for up to 3 days [11] and up to 28 days [26] . In contrast, HIV-1 RNA levels decreased significantly when plasma was stored at 37 ^{o}c [24].

An investigation of the effects of conditions often encountered during handling, transit, and storage of plasma on HIV-1RNA viral load concentration indicated that HIV-1 RNA levels remained stable for up to 30hrs after collection when stored at 4°C [5]. In contrast, evidence showed that the median HIV-1 RNA load concentration after storage at 4°C for 6hrs was statistically significantly lower. Although, the decline at 4°C were more pronounced after 24 and 72 hours of storage [22].

During viral load measurement one additional factor that could affect reliability of the results is freeze-thawing of the specimen due to various reasons including to overcome the problem related to work overload, instrument failure or reagent stock out. Handling condition showed no statistically significant difference in HIV viral load measurement because of freezing of plasma [20]. Moreover, plasma remained stable despite one cycle of freeze-thaw at -70°C [5] and limited freeze and thaw cycles, although insignificant

reduction of the level of HIV-1 RNA concentration was observed [11]. In addition plasma was stable despite frequent freeze-thaw for three [6] and four [27] cycles.

Specimen dilution is a mandatory practice in HIV viral load quantification when there is inadequate small volume or when the viral concentration is above detection limit of the instrument. In this regard, a study conducted to validate the dilution of samples with phosphate buffered saline showed that dilution of samples with 1× PBS produced comparable viral load measurements to undiluted samples [12].

Another factor that could affect HIV-1 RNA quantification is the level of the concentration of the virus in the specimen. Measurement result of plasma viral load <250 copies/ml and those close to the lower level of detection of each assay are interestingly over-represented than values 500 copies/ml [28].

Taken together, the studies reviewed above indicated that HIV-1 RNA level quantification could be affected by specimen type, storage temperature and duration of storage though some of the studies found no effect. Thus, there is a need to see the effect of specimen handling in the local context. Ethiopia has introduced routine HIV viral load testing for ART monitoring which is the preferred monitoring tool for diagnosing and confirming ART failure than clinical as well as immunologic monitoring [29], as per the WHO recommendation [30]. The country has also adopted the UNAIDS 90–90-90 by 2020 target [31]. To achieve this goal, particularly the third target which aims at achieving 90% virologic success, access to viral suppression monitoring by viral load testing is needed. To scale up the routine viral load monitoring service, the country follows a sample referral linkage system; thus, proper specimen handling is highly mandatory to ensure the quality of the test result. Studies regarding the effect of specimen management on the quantification of HIV viral load are lacking and hence this study tried to address this gap.

3. Objective

3.1. General objective

To determine the effect of sample management on HIV -1 RNA viral load measurement in Saint Paul Hospital Millennium Medical College from April to July, 2019.

3.2. Specific objectives

- To compare HIV VL plasma separation time at six and 24 hours.
- To determine the effect of plasma sample storage time duration (24hrs and 48hrs at room temperature; 6 days and 15 days at 2-8^{0}C; 30 days and 60days at -20^{0}C).
- To assess the effect of sample freezing and thawing cycle on viral load measurements
- To evaluate the effect of sample dilution on HIV viral load count
- To determine clinical significance for every specific objectives

4. Materials and Methods

4.1. Study design

Experimental study design was conducted to determine the effect of plasma separation time, storage temperature and duration of storage, freeze-thaw cycles and dilution proportion on HIV 1 RNA concentration in Saint Paul Hospital Millennium Medical College (SPHMMC) from April to July, 2019 GC.

4.2. Study area and period

The study was conducted at Saint Paul Hospital Millennium Medical College from April to July, 2019 GC. St Paul Hospital Millennium Medical College is selected for this study based on its closeness for Ethiopian Public Health Institutes (EPHI) to minimize the factor that can affect during transportation. It is found in Gulelle Sub-city, Arbegnoch Street, Addis Ababa, Ethiopia. It is a referral hospital that administrated under Ethiopian Federal Ministry of Health (FMOH). It is the second largest public hospital in the country established in 1961.The hospital has more than 2800 clinical, academic, administrative and support staffs. The hospital provides medical specialty services to patients who are referred from all over the country [32]. The hospital has 309 beds for inpatients and provides referral services for over 1.5 million populations. It also provides care and treatment for PLWHIV. Recently, there were 4,352 total active PLWHIV in the ART clinic of St Paul's hospital according to records of the ART clinic.

4.3. Study population

The source population was all known HIV positive patients who were receiving ART services at the ART clinic of St. Paul Hospital Millennium Medical College, during the study period.

4.4. Study sample

Study population was those who were on ART at ART clinic of St Paul's Hospital Millennium Medical College for at least six months; provide their sample for viral load testing during the study period and fulfill the eligibility criteria.

4.5 Eligibility criteria

4.5.1 Inclusion criteria

- Being on ART at least for six months
- Age greater than or equal to 18 years old
- Willingness to participate in the study

4.5.2 Exclusion criteria

- Severely ill and patients with co morbidity

4.6. Sampling method and sample size determination

4.6.1. Sample size determination

Sample size was determined by using comparison of two means with equal sample size and variance using Epi Info sample size calculation tool. X_1 and X_2 were assumed as two means in different conditions, 80% power and 95% confidence level. Table 1 depicts values of the two means used to calculate sample size for each main outcomes of the study (separation time, storage temperature, freezing-thaw and dilution). In addition, 0.2log [11] difference was used as variance in this sample size calculation.

Table 1: Sample size calculation

Condition	Separation time mean		Storage temperature		Free - Thaw		Dilution	
	X_1	X_2	X_1	X_2	X_1	X_2	X_1	X_2
6hrs Vs 24hrs	4.2	3.61						
Plasma 24hrs RT Vs 48hrs			4.1	3.9				
Plasma Within 6hrs Vs freeze – thaw					4.45	4.46		
Undiluted Vs diluted							0.4	0.49
Sample size	11		88		34,915		432	

RT-Room temperature, Vs-Verses

The values of each mean obtained from previous studies [5, 20]. Sample sizes were calculated for each study main outcomes based on the specific mean difference and the largest determined sample size was 34,915 which determined by using freeze-thaw cycle. However, a total of 88 participants which was determined by using plasma 24 hours at room

temperature and 48hours at room temperature were included to this study. This sample size was used based on the cost of test because viral load test is very expensive to run for several participants. We believe this sample size is enough because our experiment is within individual comparison.

4.6.2. Sampling Technique

St Paul Hospital Millennium Medical College was selected purposively for this study based on its closeness for EPHI to minimize the factor that can affect HIV viral load during transportation. Special sampling technique was not employed to enroll individual participants because all clients attending the hospital for HIV viral load monitoring during the study period were included consecutively.

4.7. Study variables

4.7.1 Dependent variable

HIV viral load count

4.7.2 Independent variable

Plasma separation time, storage temperature, storage period, freeze-thaw cycle and dilution were independent variables

4.8. Data Collection

4.8.1. Data and specimen collection

Fig 1 depicts the overall procedure of specimen collection procedure. Samples were collected by the hospital phlebotomists after training obtained on method of consent request, objective of the study, accident management method and data collection tools. The samples were transported from SPHMMC to EPHI by triple packaging and in temperature monitored condition at least within three hours of collection. The transportation was performed by EPHI NHIVRL staffs. Ten ml of whole blood samples were drawn from each participant into two different EDTA test tubes (each 5ml). Structured data extraction format was used (Annex 5) to follow the sample quality, storage temperature, time of sample collection, sample dilution and freeze-thaw cycle. Valid individual patient code was used to trace the viral load result of each participants to return their viral load results. Standard operational procedures (SOPs) and Job aids were prepared for each procedures of testing and employed during operation process.

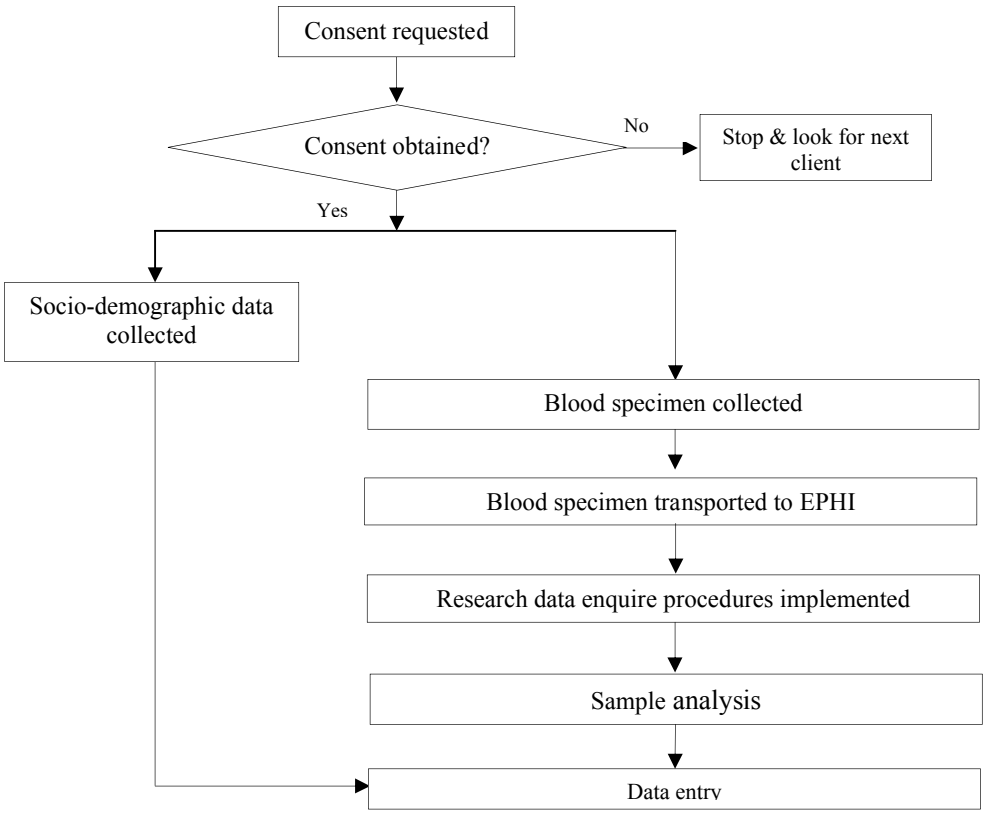

Figure 1: Specimen and data collection flow chart

Experiment

Ten ml whole blood was collected from all participants, transported within three hour of collection to EPHI NHIVRL. From the 10ml whole blood which was collected in to two 5ml EDTA tubes 2ml was subtracted in other EDTA tube and left at room temperature. The 8ml blood was centrifuged at 5000rpm for 5minutes and the plasma is aliquoted into six nunc tubes with the volume of 0.35ml. The plasma aliquots were stored as follows: two aliquots were stored at room temperature, two at 2-8^0 and two at -20^{0}c. One remaining aliquot with 0.2ml volume was diluted in the concentration of 1:2, one with 0.1ml diluted 1:3, one with 0.1ml diluted with 1:5 based on the actual dilution practice in different laboratories in Ethiopia. The remaining one with 1.5ml was used for freeze-thawing experiment. The dilutions and 1.5 ml plasma aliquots were tested within six hour of collection (taken as time zero) and the leftover of the 1.5ml plasma were used for freeze-thaw and analyzed for four cycles. The remained 2ml was stored as whole blood for 24hrs processed the next day. Mean viral load log and RNA copies used for comparison.

The samples were collected and aliquoted/distributed as follows:

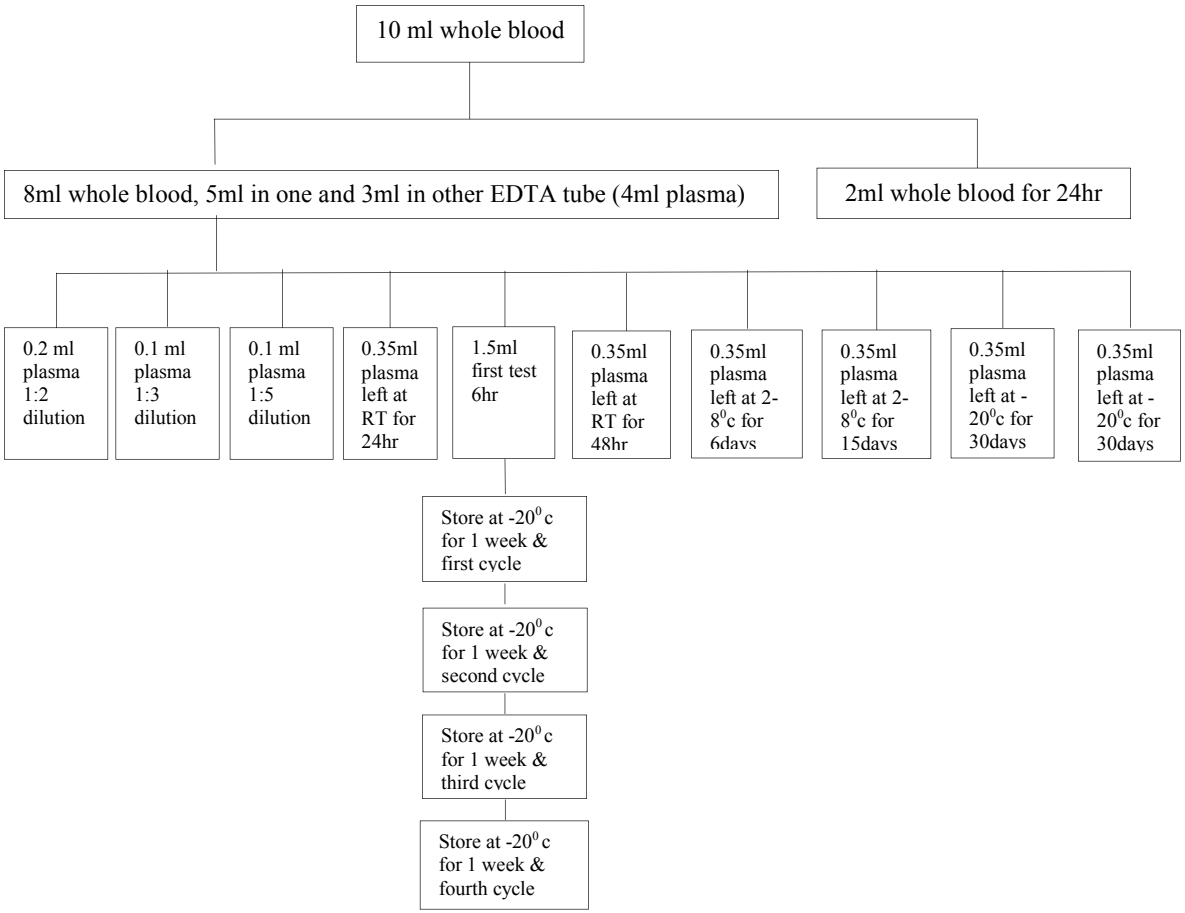

Figure 2: sample distribution or aliquot flow chart

4.8.2 Laboratory analysis

4.8.2.1 Sample collection

Depicts [Fig 1] show how whole blood specimens were collected by hospital phlebotomists and transported to EPHI NHIVRL. The collected and transported samples were separated in to plasma and required research procedures conducted [Fig 2].

4.8.2.2 Principle of detection

The working principle of the Abbott instrument used to analyze the sample in this research was customized from the user manual of the instrument [30]. The Abbott Real Time HIV-1 assay uses RT-PCR to generate amplified product from the RNA genome of HIV-1 in clinical specimens. RNA sequence that is unrelated to the HIV-1 target sequence is introduced into each specimen at the beginning of sample preparation. This unrelated RNA sequence is simultaneously amplified by RT-PCR, and serves as an internal control (IC) to demonstrate that the process has proceeding correctly for each sample. The amount of HIV-1 target sequence that is present at each amplification cycle is measured through the use of fluorescent-labelled oligonucleotide probes on the Abbott *m*2000*rt* instrument. The probes do not generate signal unless they are specifically bound to the amplified product. The amplification cycle at which fluorescent signal is detected by the Abbott *m*2000*rt* is proportional to the *log* of the HIV-1 RNA concentration present in the original sample. In the presence of template RNA, a target-specific reverse primer and manganese ions, the Tth DNA polymerase, transcribes the RNA into a DNA strand. The product of reverse transcription is a hybrid, consisting of one strand RNA and the first molecule cDNA. Now, the PCR start, in the first step, the RNA/DNA hybrid is separated by heat denaturation. When the temperature is lowered, the forward and the reverse primers anneal to their target sequences. In the next step the Tth polymerase elongates the primers, thus synthesizing new, complementary DNA strands. These DNA copies again serve as templates in subsequently performed PCR cycles.

4.8.3. Sample rejection criteria

Test result with insufficient information, at least one invalid test result, clot, result below the lower limit of the instrument and insufficient sample volume were rejected.

4.9. Quality assurance

During data collection important variables such as plasma separation time, storage temperature, storage period, freeze-thaw cycle, and dilution proportions were controlled by recording on data and result extraction formats and temperature recording formats, to avoid any miss match and traceability issue. Standardized SOP were developed and used for every procedure. Every procedure was performed by competent laboratory personnel and internal quality control, and assay calibration was tested. Moreover, EPHI NHIVRL participates in external quality assurances with One World Accuracy (OWA) three times a year and two times with Center for Disease Control (CDC) Atlanta. The results are always 100%.

4.10. Data analysis

The data was entered into Microsoft office Excel and analyzed using SPSS version 20. Before main analysis data was checked for normality, linearity, univariate and multivariate outliers, and homogeneity of variance-covariance matrices with no serious violations noted [Fig 3]. Descriptive statistics was employed to explain socio-demographic characteristics distribution and HIV viral load copies. Repeated Measure Analysis of Variance (RANOVA) was done to determine the mean difference between different scenarios. RANOVA was used because the viral load of each experimental scenario did not seriously violate the normality and other assumptions. Post-hock analysis was employed to locate the place of significance between more than two categories. Level of significance was set at 5%. $P < 0.05$ was taken as statistically significant. The mean differences >0.5log or >±2SD were used as clinical significance [11, 17].

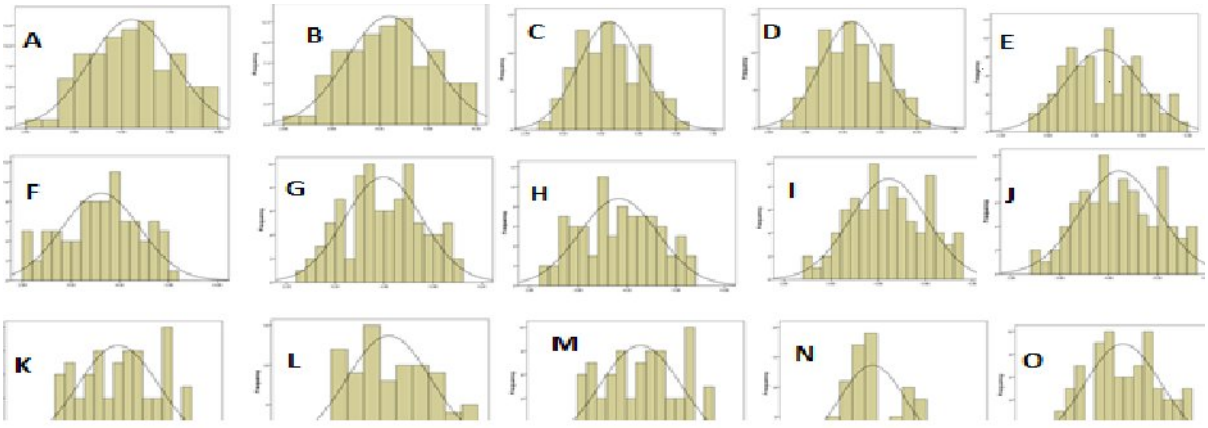

Figure 3: Normality curve to assess normality of the outcome variables
A- HIV viral load within 6hr, B- HIV viral load for one time Freeze at -20^{0}c for one week, C- HIV viral load refreeze for one week at -20^{0}c, D- HIV viral load refreeze for one week at -20^{0}c, E- HIV viral load refreeze for one week at -20^{0}c, F- 1:2 Dilution, G-1:3 Dilution, H-1:5 Dilution, I- HIV

Plasma viral load stored at room temperature for 24hr, J- HIV plasma viral load stored at room temperature for 48hr, K- Plasma HIV viral load separated from whole blood after 24hr, L- HIV viral load for plasma stored at 2-8^{0}c for 6 days, M- HIV viral load for plasma stored at 2-8^{0}c for 15 days, N- HIV viral load for plasma stored at - 20^{0}c for 30 days, and O- HIV viral load for plasma stored at 2-8^{0}c for 60 days.

4.11. Ethical clearance

Ethical clearances were obtained from Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University, Ethiopian Public Health Institute and St. Paul Millennium Medical College Scientific Ethical Review Committees. Data access was limited to data manager and the principal investigator through keys locked and password used for electronic files. All information that exposes the identity of each patient was removed from the result of this study and the codes and patient medical record number were used to return the viral load results. All participants were provided both oral and written informed consent after they thoroughly understood the objective and procedure of this study.

4.12 Dissemination of results

The study findings will be presented at the Department of Medical laboratory sciences of Addis Ababa University and copies shared to the university, EPHI and St. Paul Millennium Medical College. The findings will be presented in national and international conferences. Manuscript will also be published in peer reviewed journals.

4.13. Operational definitions

Freeze-thawing: Freeze-thaw cycle is the process where frozen plasma homogenized by letting it on the bench at room temperature for the maximum of 6hrs and freezing it again in deep freezer.

Clinically significant viral load level: is the practical importance of a treatment effect whether it has a real genuine, noticeable effect on patient management. Viral load less than $0.5\log_{10}$ or difference of ±2SD is considered as normal assay variation of plasma RNA levels, while changes greater than $0.5\log_{10}$ or ±2SD indicates significant clinical difference.

Dilution proportion: is the proportion of the concentration of plasma to the diluents (PBS)

5. Results

5.1 Ccharacteristics of study participants

A total of 88 PLWHIV were included from the ART clinic at St Paul's Hospital Millennium Medical College. The majority 60(68.2%) of study participants were female. The mean (± SD) age of the participants was 37.3 (±8.44) years with the age range of 18 to 53 years.

5.2 The mean viral load value of the participants

The mean viral load copies of the WHO recommendations (within 6hour/Gold standard/, 2-8^{0}c and -20 ^{0}c) were 4.24, 4.23, and 4.21 respectively [Table 2].

Table 2: Mean and median distribution of variables of participants at SPHMMC 2019

Variable	Mean (±SD)	Median (IQR)
HIV viral load at 6hr	4.24(0.82)	4.25(3.53 – 4.94)
HIV viral load 1 week freeze thaw first cycle	4.24(0.83)	4.25(3.56 – 4.93)
HIV viral load 1 week refreeze thaw second cycle	4.2(0.89)	4.13(3.5 – 4.94)
HIV viral load 1 week refreeze thaw for third cycle	4.22(0.86)	4.20(3.54 – 4.92)
HIV viral load 1 week refreeze thaw for fourth cycle	4.18(0.80)	4.2(3.51 – 4.84)
HIV viral load result of RT storage of plasma for 24hrs	4.22(0.81)	4.19(3.6 – 4.9)
HIV viral load result of RT storage of plasma 48hr	4.20(0.81)	4.15(3.6 – 4.91)
HIV viral load result of HB separated after 24hr	4.1(0.83)	4.06(3.42 – 4.8)
HIV viral load result of storage of plasma at 2-8 for 6days	4.23(0.84)	4.25(3.6 – 4.91)
HIV viral load result of storage of plasma at 2-8 for 15 days	4.18(0.85)	4.1(3.59 – 4.9)
HIV viral load result of storage of plasma at -20 for 30 days	4.21(0.83)	4.03(3.67 – 4.9)
HIV viral load result of storage of plasma at -20 for 60 days	4.20(0.86)	4.16(3.59 – 4.84)
HIV viral load result of 1 to 2 dilution(RNA copies)	3.98(0.79)	3.94(3.42 – 4.56)
HIV viral load result of 1 to 3 dilution (RNA copies)	3.83(0.8)	3.85(3.16 - 4.41)
HIV viral load result of 1 to 5 dilution (RNA copies)	3.61(0.79)	3.64(3.02 – 4.2)

RT-Room temperature, WB-Whole blood, HIV, SD-Standard deviation, IQR-Inter quartile range, HIV-Human Immunodeficiency syndrome and RNA-Ribonucleic Acid

5.3 Viral load level with different separation time

There was statistically significant difference between mean viral load tested within 6hrs and separated after 24hrs: *F (1, 87) =33.11, p < 0.001*. The mean (± SD) viral load was decreased when it stored as whole blood for 24hrs as compared to viral loads tested within 6hrs 4.1(0.83) versus 4.24(0.82) [Table 3]. With mean difference of 0.14 log/ml which was not clinically significant at <0.5log/ml.

5.4 Viral load level of plasma storage time at different temperature

There was no statistically significant difference between mean viral load at 6hrs and stored at 24hrs and 48hrs at room temperature: *F (2, 86) =1.11, p=0.336.* There was slightly higher mean (± SD) viral load levels within 6hrs, plasma stored at room temperature for 24hrs and 48hrs at room temperature 4.24(0.82), 4.22(0.81), and 4.20(0.81), respectively [Table 3].

There was statistically significant difference between mean viral load at 6hrs and plasma stored in 2-8^{0}C for 6 and 15 days: *F (2, 86) =10.16, p < 0.001*. With Post-hock analysis still there were statistically significant difference between mean viral load at 6hrs and plasma stored in 2-8^{0}C for 15 days: *F (1, 87) = 10.17, p= 0,006.* Similarly, there was statistically significant difference on mean viral load of plasma stored in 2-8^{0}C for 6 days and for 15days: *F (1, 87) = 10.17, p< 0,001* [Table 3]. However, these differences were not clinically significant based on the mean difference 0.06log/ml for 6 days and 0.05log/ml for 15 days based on the < 0.5log clinical significance cut-off point.

There was no statistically significant difference between mean viral load at 6hrs and plasma stored in -20^{0}c for 30 and 60 days: *F (2, 86) =0.55, p = 0.576* [Table 3].

Table 3: Repeated measurement analysis of variance in log/ml for plasma separation time, storage at room temperature, 2-8^{0}c and -20^{0}c.

Variables	Measurement Indices			
	Mean(±SD) log/ml copies	Mean Difference	F- Statistics	P- value
HIV VL within 6hrs - HIV VL Separated after 24hrs (log copies/ml)	4.24(0.82) - 4.1(0 .83)	0.145	33.11	**<0.001**
Within group comparison			1.106	0.336
HIV VL within 6hrs - HIV VL 24hrs RT	4.24(0.82) -4.22(0.81)	0.025	1.106	0.999
HIV VL within 6hrs - HIV VL 48hrs RT	4.24(0.82) -4.20(0.81)	0.038	1.106	0.430
HIV VL 24hrs RT - HIV VL 48hrs RT	4.22(0.81)-4.20(0.81)	0.013	1.106	0.999
Within group comparison of (log copies/ml)			10.164	**<0.001**
HIV VL within 6hrs- HIV VL 2-8^{0}c for 6 days	4.24(0.82)- 4.23(0.84)	0.007	10.164	0.999
HIV VL within 6hrs- HIV VL 2-8^{0c} for 15 days	4.24(0.82)-4.18(0.85)	0.060	10.164	**0.006**
HIV VL 2-8^{0}c for 6 days- HIV VL 2-8^{0c} for 15 days	4.23(0.84) -4.18(0.85)	0.052	10.164	**<0.001**
Within group comparison of (log copies/ml)			0.554	0.576
HIV VL within 6hrs- HIV VL -20^{0}c for 30 days	4.24(0.82)-4.21(0.83)	0.029	0.554	0.899
HIV VL within 6hrs- HIV VL -20^{0c} for 60 days	4.24(0.82)- 4.20(0.86)	0.036	0.554	0.999
HIV VL -20^{0}c for 30 days- HIV VL -20^{0c} for 60 days	4.21(0.83)-4.20(0.86)	0.007	0.554	0.999

VL-Viral load, RT- Room temperature, WB-Whole blood, SD-Standard deviation, HIV-Human Immunodeficiency syndrome and RNA-Ribonucleic Acid

5.5 Viral load level of freeze-thaw cycle of plasma

The mean viral load within 6hrs and plasma freeze-thawed for four consecutive cycles in -20^0c for seven days duration between each cycle revealed a statistically significant difference: *F (4, 84) = 3.6, p = 0.009* [Fig 4]. Post-hock analysis indicated a statistically significant difference between mean viral load within 6hr and the fourth cycle of freeze-thawing *F (1, 87) = 3.6, p= 0.013* [Fig 4]. However, the mean difference was log 0.063 which was not clinically significant as it was < 0.5 log, which is the cut-off for clinical significance.

5.6 Viral load level of plasma dilution

There was no statistically significant difference between mean viral load for undiluted and diluted plasma: *F (3, 85) =0.47, p = 0.707* [Fig 4].

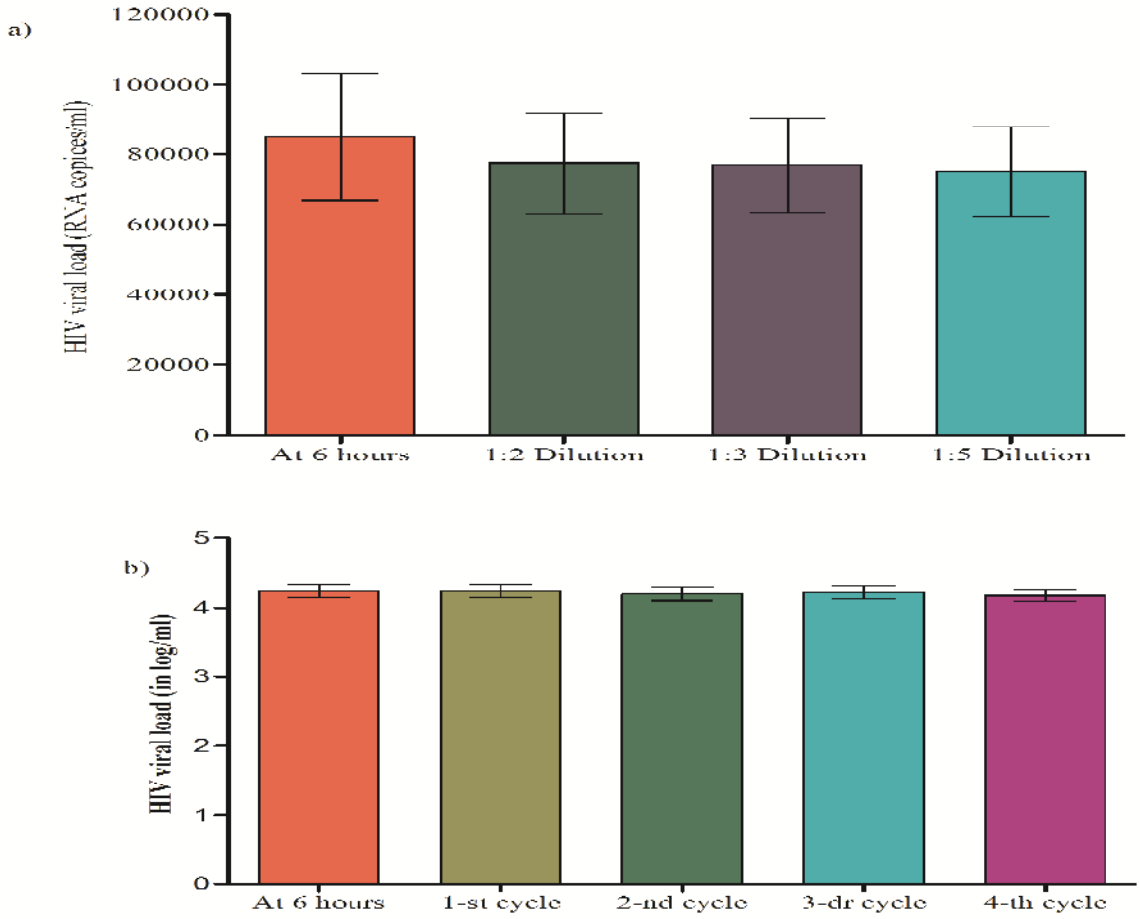

Figure 4: Graphic summary of freeze thaw cycle and Dilution of plasma with PBS

The lines of the box's indicates the mean value while the cross on the vertical lines shows the standard deviation, a) HIV viral load measurement after dilution of plasma With PBS b) HIV viral load measurement of four cycles of freeze thaw

6. Discussion

Several studies have focused on identifying problems related to blood collection and processing parameters that will affect accuracy and reproducibility of quantitative HIV-1 RNA viral load testing. However, there are few studies on the effects of plasma separation time, storage time and temperature, freeze-thawing cycle and dilution proportion for HIV-1 RNA viral load. Thus, this study aimed to investigate effects of sample management on HIV viral load measurement in a total of 88 participants from ART clinic at St Paul's Hospital Millennium Medical College.

This study revealed that mean difference in HIV-1 RNA viral load with plasma separation time at 24hrs post collection, plasma stored in 2-8^{0}c for 15 days and the fourth cycle of freeze-thawing were statistically significant compared to mean viral load tested at six hrs. However, these variables though they were statistically significant the changes were not clinically significant at the recommended cut-off point 0.5log$_{10}$ (two times SD). With regards to storage time, there was no significant mean difference in viral load of all scenarios as compared with the viral load tested within 6hrs of collection.

Evidence indicated that HIV viral load significantly decreases when stored at room temperature for 72hrs [11]. Moreover, mean HIV viral load decreases significantly when stored at room temperature for 30hrs when compared to viral load tested at 2hrs [21]. These studies findings were similar with the current finding in which the mean HIV viral load was significantly different between viral load tested within 6hrs of collection and after 24hrs.

In contrast, a study demonstrated that the mean viral load was not significantly different between HIV viral load tested within 4hrs of collection and after 24hrs [7]. In addition, HIV RNA was found to be stable up to 48hrs in whole blood at room temperature [6, 9]. Even longer stability of HIV RNA until 3 days [17] and 7 days [4] at room temperature were reported. This difference may be due to HIV strains variation, the level of damaged HIV RNA in the plasma and the testing condition variation.

In this study, there was statistical significant difference between the HIV viral load test at 6hr and 24hrs. However, this significance was not clinically significant based on the cut-off point proposed by previous studies such as >±2SD or a variation of < 0.5log10 difference [11, 12, 17]. A previous study revealed a clinically significant difference in the mean HIV viral load when viral load was tested at 6hrs and 24hrs [11]. This finding was opposite to the current study finding in which the mean viral load was not clinically significantly different

between viral load tested at 6hrs and 24hrs. This difference most probably due to statistical test used, time duration of test and sample size difference. In the previous study they used paired t test which can increase type one error when used for more than two categories [34]. However, in the current study repeated measure analysis of variance was used which is free from the problem indicated above [34]. Furthermore, the study reported by Sebire *et al* [11] conducted the experiment on 20 participants. However, in the current study case, 88 participants were included which could increase the precision of comparison.

The difference in the mean HIV viral load quantified from plasma stored at 2-8^{0}c for 15 days was statistically significant when compared with viral load tested within 6hrs and that tested after 6 days in current study. This finding was in line with a previous study in which viral load was significantly different when tested within 2hrs and 72hrs for the plasma stored in 4^{0}c [11]. In contrast viral load remained stable at 4°c for up to 24 to 48hrs [6], 30hrs [5], 14 and 28 days [26]. This difference could be due to temperature fluctuation and poor temperature regulation in the different set ups where viral load testing took place. Although the difference in mean HIV viral load of plasma stored at 2-8^{0}c for 15 days was statistically significant in the current study, it was not clinically significant at the cut-off point of 0.5log$_{10}$. This difference might be due to strain variations and increased amount of damaged HIV RNA in the specimens.

This study also tried to see the effect of freeze-thawing. The mean HIV viral load had statistical significance different in fourth cycle of freeze-thawing after storage in -20^{0}C as compared with the mean HIV viral load within 6hrs of testing. However, the difference was not statistically significant up to three cycles of freeze-thaw by comparison with mean HIV viral load tested within 6hrs. In contrast, to this finding the plasma stored in -70^{0}C refrigerator and freeze-thaw for the 4th cycle was not statistically significantly different [6]. This difference is most probably because of temperature difference and might suggest lower storage temperature gives better result when freeze thawing is unavoidable. Of note, although the mean difference was statistically significant it was not clinically significant as the mean difference was <0.5 log$_{10}$, which is the cut-off for clinical significance. Thus, care has to be taken when interpreting statistical significance as it is demonstrated in the current study where the observed statistically significant mean differences were more likely to be clinically irrelevant.

7. Strength and Limitation of the Study

7.1 Strength

The strength of current study was inclusion of relatively large sample size compared to earlier studies which increases our estimation precision. In addition, this study was carried out at EPHI molecular laboratory which routinely practices internal quality control and participates in external quality assurance scheme delivered by One World Accuracy and CDC, USA. The temperature and storage equipment are also well controlled and regularly serviced.

7.2 Limitation

Even though standard operating procedure (SOP) was followed strictly, personal variation in test procedure and lower limit of detection of Abbott analyzer could affect the results of this study. Transportation condition in real situation is not studied. Electric power fluctuation could also impact the freeze-thaw test result of this study. However, since the laboratory has a backup generator power the impact of temperature fluctuation is minimal. In the current study -80^0C freezer was not used for freeze-thawing process, which could limit our study conclusion of freeze-thawing cycle at this storage temperature. Nevertheless, the study used storage temperature being used in the viral load testing sites which do not have -80^0C freezer, and hence studying the effect with the same storage condition as the testing sites is logical.

8. Conclusion and Recommendation

8.1. Conclusion

This study results was shown that the HIV RNA in plasma maintained stability and not degraded greater than 0.5 $\log_{10}$ for all scenarios of the experiment. The different storage, separation, freeze-thaw and dilution conditions tested in this study do not appear to have a large effect on viral load stability. However, there was statistically significant difference on the mean viral load between viral load tested at 24hrs, stored in 2-8^{0}C and 4th cycle of freeze-thaw when compared to viral load tested at 6hrs. All the statistically significant variables were not clinically significant.

8.2. Recommendation

Though it did not reach clinical significance level, statistical differences have been noted. Thus, to be on the safe side, plasma should be separated before 24hrs for the whole blood stayed at room temperature. In addition, plasma should not stay in 2-8^{0}C beyond 15 days and should not be freeze-thawed for more than three cycles. Moreover, future strong research which will address limitations of this study such as transportation is required.

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Annex-1: Information Sheet (English Version)

Addis Ababa University Medical Faculty, School of Medical Laboratory Sciences

Dear Participant,

My name is Gadissa Gutema, Post graduate student of Addis Ababa University, College of Health Sciences Department of Medical Laboratory Sciences; I am going to conduct study and collect data on effect of specimen handling on quantitative HIV-1 viral load measurement in St Paul Hospital Millennium Medical College. The objective of the study is to determine the effect of specimen handling on HIV -1 RNA viral load measurement. The information obtained will be used to improve specimen handling and improve quality of patient care. The study will identify gaps and challenges and provide recommendations for proper interventions laboratory professionals, laboratory managers and policy makers. If you decide to participate, I will guarantee that there is no any influence related to study but only request you that to provide 10ml blood. I can't guarantee, however, that you will receive any direct benefits from this study. However this information will be useful to manage that could improve result quality and ultimately patient care. Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission or as required by law. Your name will not be disclosed in any cases or be kept in any other records. Your participation is voluntary and you are free to withdraw and to discontinue participation at any time without consequence. Your participation or not, do not have any influence on the service you obtain from this Hospital Your signature below indicates that you have read the information above and have decided to participate in the study.

Thank you for your participation;

Gadissa Gutema

Contact address of PI, 0911 445383

Annex-2: Consent form

Part-1: Informed Consent for individuals participating in effect of sample management for quantitative HIV-1 viral load measurement in St Paul hospital millennium medical college. This informed consent will be read to you, please feel free to ask for further clarification in any issue that you may not understand. Your participation in this study is voluntary; you can withdraw from the study at any time and failure to participate in this study will not affect the services you receive.

The result of the control test will be given to your physician in a print form specially for participants having viral load result greater than 1000 copies/ml for better treatment or follow up; moreover this information will help in the future to prevent, identify, and manage effect of specimen handling on HIV viral load quantification in Ethiopia. There is no cost to you for participation in this study. There is no compensation for this study.

This study is being conducted by Ethiopian Public Health Institute and it will explore determinant factors for effect of sample management in quantitative HIV-1 viral load measurement. You are being asked to participate in this study because you are an adult on antiretroviral medications at this Hospital. We are providing information to you about this study and would like to invite you to be part of this study. This study is used to see the effect specimen handling on HIV viral load management. If you accept to participate, we are going to take about 10 ml (about two spoons) of blood. Because of your participation you may expose to shock because of fear of blood, and physical discomfort during injection of needle. If you feel such things you should tell to blood collectors as they are experienced and trained on how to manage such issues. Your participation is entirely voluntary, and your decision to participate or not to participate will not affect the services you receive there may be no direct benefit for you in participating in this study. Any information obtained will be kept confidential. Information collected from you will be stored securely and only the researchers will have access to it. Also we will not use your name on any part of the study; we will use an identification number that will be assigned to you. After data collection we will prepare a report which might be shared in conferences and publications but this report will not identify you in any way. Your confidentiality in participating in this research study is completely assured.

If you have any question about this study, you can contact:

Gadissa Gutema

Address: Ethiopian Public Health Institute, PO Box 1242, Addis Ababa, Ethiopia.

Mobile Phone: Tel: +251 911 445383; Office Phone: +251 112305037

For questions about your rights as a research participant, contact:

Dr. Aster Tsegaye (PhD)

Part 2: Certificate of consent

I have read the informed consent or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research.

_____	_____	_____
Name of participant	Date	Signature

If illiterate;

I have witnessed the accurate reading of the consent form to the potential participant and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely

_____	_____	_____
Name of participant	Date	Thumb print of participant

_____	_____	_____
Name of the witness	Date	Signature

Statement of the person taking consent

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked have been answered correctly and to the best of my ability. I also confirm that the individual has not been coerced into giving consent and the consent has been given freely and voluntarily.

_____	_____	_____
Name of the person taking consent	Date	Signature

Annex-3: የተሳትፎስምምነት(consent form)

ክፍል 1: በዚህጥናት(effect of sample management for quantitative HIV-1 viral load measurement in St Paul hospital Millennium medical College) ላይ ለመሳተፍ የስምምነት ፊርማ ነው፡ይህን የስምምነት ወረቀት በማበብ ወይም እንዲነበብሎ በሞረግ ያልገባዎት ነገር ካለ ማኛውንም ጥያቄ የማየቅ መብት አለዎት፡፡ በዚህ ጥናት ላይ ለመሳተፍ በሙሉ ፈቃደዎ ለይ የተመሰረተ ነው፡ በፈለጉት ሰዓት ጥናቱን የመቐረጥ መብቶ የተጠበቀ ነውበጥናቱ ላይ አለመሳተፎ ከሆስፒታሉ በሚገኙት አገልግሎት ላይ ተፅኖ የለወም ለመወዳደሪ የሚሰጠውናመኛ ወጫት ለሃኪም ይላካለ ይህ ጥናት በተለይ ወጪቃቸውከ 1000 በላይ ለሆኑት የተለየ ክትትል እና ህክምና እንዲያገኙ ይረዳለ እንዲሁምከዚህ ጥናት የሚገኘውወጫት ከናመኛ አያያዝ የሚመጣ አስቀደሞለማወቅ፡ ለማላከል እና ለመቆጣጠር ይረዳል፡፡ በጥናቱ ላይ በመሳተፍ የሚኝ ክፍያምሆነ እንዲከፍሉ የሚየቁት ክፍያ የለም፡፡

ይህ ጥናት የሚሄደዉበኢትዮጲያ ህብረተሰብ ጠፔ ኢንስቲቱት ሲሆን የተፈለገበት አላማየናመኛ አያያዝ በኤች አይቪ viral load ወጫት ላይ የሚደርሰወን ተፅዕኖ ለይቶ ለማወጫት ነው፡በጥናቱ ላይ እንዲሳተፉ የተፈለገበት ምክንያት እርስዎ ነፍስ ያወቁ (እድሜዎ ከ18 አመት በላይ ስለሆነ)እና ፀረ ኤች አይቪ መድሃኒት በዘህ ሆስፒታል እየተጠቀመስለሆነ ነው፡ በጥናቱ ላይ ለመሳተፍ የሚስፈልጉ መጀዎችን እየነገርኖ በጥናቱ ላይ እንዲሳተፉ እንጋብዝታለን፡፡ ፈቃደኛ ከሆኑ 10ml (ሀለት ማኪያ) የሚን ደምእንወሰዳለን፡፡ ደምበሚወሰደበት ወቅት ደም ለሚፈሩ ሰዎች ወቅጥ ለገቡ ይችላሉ የአወሳሰድ ቅደመተከተሉ መጠኛ የሆነ አለመመቸት ለፈጥር ይችላል ይህ ከተከሰተ ናመኛ ለሚሰዱት ያሳወቁዋቸውምክንያቱም በቂ የሆነ ልምድ እና ስልጠፔ ስላላቸውይህን መቆጣጠር የችላሉ፡፡ በጥናቱ ላይ የመሳተፍ እንዲሁም ያለመሳተፉ ሙሉ ፈቃዶ ላይ የተመሰረተ ሆኖ ሳለ በመሳተፎ የሚጠቅ ነገር እንደለለ ሀሉ የሚገኙት ቀጥተኛ ጥቅማም የለም የሚጠቅ መጀ ሀሉ ሚጥራዊ በሆነ ሀኔታ የሚዙ መሆናቸዉእንዲሁም አጥኚዎቹ ብቻ የሚወቁት ይሆናል፡፡በጥናቱ ላይ ስማ የሚገለጽ ሲሆን ለመለያ የሚን መለያ ቁጥር የሚጦይሆናል፡፡

ጥናቱ ከተጠፍቀቀ በሃላ ሪፖርት በተለያዩ ኮንፈረንሶች ላይ እንዲሁምበህትመት መልክ የሚሪፌጩሆን ማነኖን የሚልጽ ምንምአይነት መጀ አይኖርም፡ መጀወጣሚጥራዊነቱ የተጠበቀ ሲሆን በጥናቱ ላይ በመሳተፎ ምንም አይነት ክፍያ አይፈጸማም፡

ከጥናቱጋር ተያያዝ የሆነ ጥያቄ ካለት

ጋዲሰ ጉተማ

አደሪሻ: የህብረተሰብጠፔ ኢንስቲቱት, ፖስታሳ ቁ 1242, አዲስ አበባ ኢትዮጲያ

ስልክ ቁጥር: ሞባይል+251 911 445383; የቢሮ: +251 112305037

እንደጥናቱ ተሳታፊ ለሜሮት ማኛናወምጥያቄ

ዶ/ር አስቴር ጸጋዬ

ክፍል 2፡ <u>የ ተሳትፎ የ ምሽክር ፊርማ</u>

ይህን የ ተሳትፎ ስምማ ት ፎርም አን ብቤ/ተነ ቦልኝ ተረድቻለሁ፡ ፡ ጥያቄዎቸን የ መጠየ ቅ መብት ተሰቶኝ እንዲሠም የ ጠየ ከዋቸወጭ ያቄዎች በአጥጋቢ ሀኔታ ተመልሰወልኛል፡ ፡ በጥናቱ ላይ በፍላ ጎቴ ተስማሜ ተሳትፈያለሁ፡ ፡

_____ _____ _____

ስም ፊርማ ቀን

<u>ማበብለሚቸሉ ምሽክርነ ት</u>

መጠይቁ በአግባቡ ተነ ቦላቸው እንዲሠም የ መጠየ ቅ አጋጥሚ ተሰጥቶዋቸዋል፡ ፡ ይህ ሜደመ ምሽክር መሆኔን በፊርሜ አረጋግጣለሁ፡ ፡ ግለሰቡ የ ተሳትፎ ስምማ ቱን በነ ጻነ ት ነ ወየ ፈጸመት፡ ፡

_____ _____ _____

የ ተሳታፊ ስም ቀን የ አሻራ ፊርማ

_____ _____ _____

የ ምሽክር ስም ቀን ፊርማ

<u>የ ጠያቂውቃል</u>

ለጥናቱ ተሳታፊ ጥያቄ የ መጠየ ቂያ እደል ተፈጥሮላቸዋል በተፈጠሮም በምቸለው መጠን ጥያቄዎቹ በትክክል ተመልሶላቸዋል፡ ፡ ተጠያቂዉ ጥያቄዎቹን እንዲመልሱ አልተገ ደዱም መልሱን የ ሰጡት በራሳቸውፈቃድ እና ነ ጻነ ት ነ ው፡ ፡

_____ _____ _____

የ ጠያቂውስም ቀን ፊርማ

Annex-4 Waligaltte Hirmaana (consent form)

Kuta tokkoffaa: qoranoo kanarratti(effect of specimen handling for quantitative HIV-1 viral load measurement in St Paul hospital Millennium medical College) hirmaachuf waligalte mallatteessu ilaalata. Waraqaa ragaa kana dubbisuu yookin akka isiniif dubbiffamu gochuudhaan waan isinif hin gallin gaaffachuuf mirga qabdu.Qorannoo kana irratti hirmaachun fedha keesan irratti kan hunda'eedha.

Deebin Dhiiga qoratamee fi qabxiinsaa>1000 olta'ee xiyyeefannon akka itti laatamu jecha hakimota hordoffiii godhanif ni ergama.

Qorannoo kana irrati himachudhaf kanfaltin hin lataamu akkasumas hin gaafatamu.

Qoranoon Kun kan gaggeefamu institutii eegumsa fayyaa Ithiopiyaadhaan yommu ta'u faayidan qorannoo kana haala qabanadhiiga (HIV viral load) fudhaamuu dhiiba inni qabtiii HIV vira load irratti qabuu ilaaludhafi. Wanti qorannoo kana irratti aka hirmattaan sin tasiisu issin nama bekkaa wantatanifii (uumurin keesan 18 olwanta'eefi) fii qorricha HIV wanta hospitalla kana erraa fayddama jirttanifii. Haalumakanan raaggalee hirmachuuf barbaachisaan issini caaqassa qoorranoo kana irraati akka hirmaataan isin gaafana .Feedhii keessan yoota'ee dhiiga 10ml (kuudhan) fudhanaa.Yaamu dhignii fudhattamu soddadhan of wallalu yookin kufun nimala akksuma yaamu lilmoon namawarranu dhuukubin salpha ta'ee namatii dhaggahamu mala. Yoo kun isinitti dhaggahamee warra dhiga fudhati ihimu nidanddesu warrid higga fudhattan muxxanofii leenji gahaata'ee wan qabbanif. Hirmaachuffii hirmaachuu dhisun feedhi guutumma kessani irrati wanta huda'ee ta'eeottojiiru hirmaachuu dhisudhan wantii dhaabddan hin jiiru akkasummas hirmaachuu keesanin faydan adda wanti arggaatanis hin jjiru. Raggalen qoorranoo kanara arggamu hundi hiciitidhan wanta qabammuuta'aa. Raggaa arggamu kana wara qoorraanoo gaggeesan qoffaatu beekaa. Qooorraanoo kana irratii maqan hin caafamuu coodidhan fayyadamnaa.

Eergga qoorraanoon gaggefamee bodaa koonfarransii adadairrati akkasumas maxxaansudhan bassun niimala hatta'uumallee egnuuman nama hin beekamu. Qoorraano kana irratii hirmachuun kaffaltii hoom ahi nqaabu.

Qoorrano kana irrati yoggaafi qabaattan

Gaaddisaa Guutamaa

Bakkateeso: institutii eegumsa fayyaa Ithiopiyaadhaan**, Sanduqqa posta 1242, Addis Ababa**, Ithiopiyaa**.**

Lakk. Biilbilla +251 911 445383; kan bakka hojii +251 112305037

Gaaffi mirgga akka himattatii qaabdaanif nama armn gaddi walquunamu danddesu

Dr. Aster Tsegaye

Kuuta lamaffaa: Raggaa maallatto hirmaana

Raggaa maallatoo hirmaana kana dubbiisee/naaffdubbiifamee naf galleejirraa. Gaaffii akkan gaafadhuu caaran naf laattameeraa kkasuma gaaffiin gaffadhee haala qubssa ta'een nafd eebirra. Qoorraanicharrattii fedhii kottin hir maachuf maallateserra.

Maaqaa	Maallato	Guuyyaa

Waabii warra duubisuu hi dandeegneef

Gaaffiileen qoorraanicha hala gariita'een dubiifameffi wantta hingaliinif akka gaaffattan caaran lattaameeraf. Kuni rawatamu issa maallatoo kottiin nan mirkaneesa. Abban dhiima waligaltte kana feedhi sattiin maallateese.

Maaqaa hirmatta	Maallato	Guuyyaa
Maaqaa waabi	Maallato	Guuyyaa

Jeecha gaaffi geegesa

Hirmaatichi akka gaffii gafattan yeeron hund'eeraf akkasumas hama human kootti gaaffii issanii sirriti debisserra. Hirmaatichi gaaffiilee akka debissan yookan akka hirmattan hin dirqiisifamnee deebi kana kanlaatan feedhisanittini.

Maaqaa gaaffi gageesa	Maallato	Guuyyaa

Annex-5: structured data extraction form

SN	Age	Sex	MRN	CODE(four digit/18)	Type of test tube		Cross checked		Remark
					Tube 1	Tube 2	Yes	NO	

Annex 6 Result extraction form

SN	Code NO	Result of separation time		Result of Storage @ room T		Result of storage @ 2-8 o_c		Result of storage @ -20o_c		Result of Thawing effect		Result of Dilution effect	
		Con	Test	Con	Test	Con	Test	Con	Test	Con	Test	Con	Test

Annex 7 Standard Operating Procedure

Standard operating procedure (SOP) for HIV-1 Viral Load determination to see effect sample management using Abbott m2000s/ rt

Purpose: - This procedure provides instructions for quantification of HIV-1 RNA in in the provided sample

Principle: - The Abbott Real Time HIV-1 assay uses RT-PCR to generate amplified product from the RNA genome of HIV-1. An RNA sequence that is unrelated to the HIV-1 target sequence is introduced into each specimen at the beginning of sample preparation. This unrelated RNA sequence is simultaneously amplified by RT-PCR, and serves as an internal control (IC) to demonstrate that the process has proceeded correctly for each sample. The amount of HIV-1 target sequence that is present at each amplification cycle is measured through the use of fluorescent-labelled oligonucleotide probes on the Abbott m2000rt™ instrument. The probes do not generate signal unless they are specifically bound to the amplified product. The amplification cycle at which fluorescent signal is detected by the Abbott m2000rt is proportional to the log of the HIV-1 RNA concentration present in the original sample.

Materials

No	Reagents
1	HIV-1 Internal Control (List No. 2G31Y)
2	rTth Polymerase Enzyme
3	HIV-1 Oligonucleotide Reagent
4	HIV-1 Negative Control
5	Control L (HIV-1 Low Positive Control)
6	Control H (HIV-1 High Positive Control)
7	HIV-1 CAL A and CAL B
8	mLysis Buffer
9	mMicroparticles
10	mWash 1 Buffer
11	mWash 2 Buffer
12	mElution Buffer

No	Supplies
1	10.0x16 mm sample tubes
2	200 mL Reagent Vessels
3	5 mL Reaction Vessels
4	Abbott 96-Deep-Well Plate
5	Abbott 96-Well Optical Reaction Plate
6	Abbott Adhesive Cover Applicators
7	Abbott Optical Adhesive Covers

8 Abbott Splash-Free Support Base
9 Aerosol Barrier Pipette Tips (1000 µL)
10 Aerosol Barrier Pipette Tips (200 µL)
11 Disposable Tips (200 µL /1000 µL)

No Equipment
1 Abbott m Sample Preparation instrument
2 Abbott m2000rt instrument
3 Abbott Real-Time HIV-1 m2000 System Combined Application CD-ROM (List No. 1L68)
4 Calibrated Pipettes (20-1000 µL)
5 Wrench
6 Plate Centrifuge
7 STRATA COOLER 96 BENCH TOP COOLER
8 Vortex

Sample type: - whole blood or plasma which can be processed according to experiment protocol

Special Safety Precautions: - Lysis Buffer contains guanidine thiocynate. This is classified as a harmful chemical compound and should be used with the necessary precautions.

Step Action pre sample preparation

1. Collect about 88 participants10 ml whole blood in to two different test tubes each with 5 ml.
2. Store the plasma according to the require ments.
3. Perform viral load testing using Abbott m2000so/rt instrument
4. Clean centrifuge and vortex with 1%bleach followed; distil water and 70 % ethanol

Sample preparation

Step Action

1. Turn on Abbott m2000 System Control Center (SCC) and Abbott m2000 sp instrument
2. Thaw assay controls and IC at 15-30°C or at 2-8°C.

Thaw calibrators at 15-30°C or at 2-8°C only if performing a calibration run.

Once thawed, assay controls, IC, and calibrators can be stored at 2-8°C for up to 24 hours before use.

3. Gently mix each assay calibrator and control three times for ≈ 3 seconds.

Ensure bubbles or foam is not generated; if present, remove with a sterile pipette tip, using a new tip for each vial.

4. Thaw amplification reagents at 15-30°C or at 2-8°C and store at 2-8°C until required for the amplification master mix procedure.

Once thawed, the amplification reagents can be stored at 2-8°C for up to 24 hours if not used immediately.

NOTE: Use one bottle of mLysis Buffer, one vial of IC, and one Real Time HIV-1 Amplification Reagent Pack to support up to 24 reactions. Use a second set of reagents to support 25 to 48 reactions. A maximum of 96 reactions can be performed per run.

5. Invert gently the Abbott 2000 Sample Preparation bottles to ensure a homogeneous solution without generating any bubbles.

If crystals are observed in any of the reagent bottles upon opening, allow the reagent to equilibrate at room temperature until the crystals disappear.

Do not use the reagents until the crystals have dissolved.

Ensure bubbles or foam is not generated; if present, remove with a sterile pipette tip, using a new tip for each bottle.

6. Vortex each IC vial three times for 2-3 seconds before use.

Ensure bubbles or foam is not generated; if present, remove with a sterile pipette tip, using a new tip for each vial.

7. Use a calibrated precision PIPETTE DEDICATED FOR INTERNAL CONTROL USE ONLY to add 500 µL of IC to each bottle of mLysis Buffer.

Mix by gently inverting the container 5 to 10 times to minimize foaming.

8. Allow for a maximum of 96 samples for each run.

A negative control, a low positive control, and a high positive control are included in each run,

Therefore, allowing a maximum of 93 specimens to be processed per run.

9. Thaw specimens at 15-30°C or at 2-8°C. Once thawed, specimens can be stored at 2-8°C for up to 6 hours if not processed immediately.
10. Check sample volume
11. Aliquot each specimen into clean tubes or vials if necessary.

Avoid touching the inside of the cap when opening tubes.

Take care not to disturb contents of the tube while removing the tube from the centrifuge and that the bottom of the tube is not touched by the pipette tip.

Ensure that the newly aliquoted sample retains the minimum volume indicated in the preceding table

12. Place the low and high positive controls, the negative control, the calibrators, if applicable, and the patient specimens into the Abbott m2000sp sample rack.
13. Place the 5 mL Reaction Vessels into the m2000sp 1 mL subsystem carrier
14. Load the Abbott m2000 Sample Preparation System reagents and the Abbott 96 Deep-Well Plate on the Abbott m2000sp worktable as described in the Abbott m2000sp Operations Manual, Operating Instructions section
15. Select the appropriate application file from the Run Sample Extraction screen that corresponds to the sample volume being tested.

Initiate the sample extraction protocol as described in the Abbott m2000sp Operations Manual, Operating Instruction section

16. Enter calibrator (needed if a calibration curve has not been stored on the m2000rt) and control lot specific values in the Sample Extraction: Worktable Setup, Calibrator and Control fields.

Master Mix Addition

17. Load the amplification reagents and the master mix vial on the m2000sp worktable after sample preparation is completed. Each Amplification Reagent Pack supports up to 24 reactions.

18. Ensure that the contents are at the bottom of the vials prior to opening the amplification reagents by tapping the vials in an upright position on the bench.

19. Remove and discard the amplification vial caps.

20. Select the appropriate deep well plate from the Run Master Mix Addition screen that matches the corresponding sample preparation extraction.

Initiate the Abbott m2000sp Master Mix Addition protocol.

The m2000rt protocol must be started within 40 minutes of the initiation of the Master Mix Addition protocol.

21. Switch on and initialize the Abbott m2000rt instrument in the Amplification Area.

NOTE: The Abbott m2000rt requires 15 minutes to warm-up.

NOTE: Remove gloves before returning to the sample preparation area.

22. Seal the Abbott 96-Well Optical Reaction Plate

23. Place the sealed optical reaction plate into the Splash Free Support Base for transfer to the Abbott m2000rt instrument.

24. Place the Abbott 96-Well Optical Reaction Plate in the Abbott m2000rt instrument.

25. Start Amplification & Detection

26. Print the results and record it in to the prepared result extraction format

Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc. candidate: Gadissa Gutema (B.Sc.)

Signature:_____

Date of submission:_____

This thesis has been submitted with our approval as advisors.

Advisor: Dr Aster Tsegaye (MSc, PhD)

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: Habteyes Tola (MSc, PhD candidate)

Signature: _____

Date: 26/12/2019

Place: Addis Ababa, Ethiopia