DETERMINATION OF THE OPTIMUM SAMPLE DILUTION OF COMMERCIAL TOTAL IMMUNOGLOBULIN G ELISA, THE TOTAL AND ANTI-MEASLES IMMUNOGLOBULIN G IN HIV INFECTED PREGNANT WOMEN

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this thesis to my family; heartfelt gratitude for their love, enthusiasm, moral and financial support throughout the study

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LIST OF ACRONYMNS AND ABBREVIATIONS

AIDS Acquired Immunodeficiency Syndrome

AMR Analytical Measurable Range

ART Antiretroviral Therapy

ARVs Anti Retrovirals

AZT Azidothymidine

CDC Center for Disease Control

CD4 Cluster of differentiation 4

CLIA Clinical Laboratory Improvement Amendments

DNA Deoxyribonucleic Acid

ELISA Enzyme Linked Immunosorbent Assay

HAART HighlyActive Antiretroviral Therapy

HIV Human Immunodeficiency Virus

Ig Immunoglobulin

ISO International Standards of Operation

IMR Immunofluorescence assay

KEMRI Kenya Medical Research Institute

LOD Limit of detection

LLOQ Lower Limit of Quantification

mIU/ml Millie-international units per milliliter

MMA Measles Maternal Antibody

MMR Measles, Mumps and Rubella vaccine

MOH Ministry of health

MTCT Mother To child Transmission

OD Optical Density

OD AG Optical density of antigen

OD COAG Optical density of control antigen

QC Quality Control

RCT Randomized Controlled Trial

RIA Radio-immuno-assay

RNA Ribonucleic Acid

RT Room temperature

SPSS Statistical Package for Social Science

TIgG Total Immunoglobulin

TTE Trans placental Transport Efficiency

UNSAIDS United Nations Aids

WHO World Health Organization

WT Wild type

ABSTRACT

Enzyme-linked immunosorbent assays (ELISAs) are widely used to quantify immunoglobulin levels induced by infection or vaccination. The first large-scale use of ELISA for antibody quantitation was in diagnosis and epidemiological viral screening to assess antibody levels after vaccination. Measles virus is transmitted through infected air droplets. Young infants are protected from measles infection by maternal antibodies. Maternal Human Immunodeficiency Virus (HIV) infection may reduce levels of measles antibodies in newborns due to reduced placental transfer. Total IgG has been reported to significantly increase in HIV infected individuals due to polyclonal activation hence mutagenic activation. ART exposure in HIV infection causes a significant boost to immunity of the mother which is subsequently transferred to the infant. The study was a nested prospective cohort study within Kesho Bora Study that was conducted between 2005 – 2009 in which HIV infected pregnant women were enrolled and exposed to different anti-retroviral drugs depending on the degree of immune suppression. This study aimed at comparing total and anti-measles IgG levels and determining changes in the level of IgG in HIV infected women before and after ART. The correct dilution for Total IgG needed to be determined and the failing measles assays quality controlled before performing the tests. Total IgG assays involved testing using the recommended dilution factor of $1:8x10^4$ which produced sample OD's 2.9-3.6 this were high and could not be interpolated from best fit standard calibration curves. Dilution used in this study, was$1:4x10^5$aftermultiple dilutions and calculation of standard error that produced tight error bars of +/-0.1 hence testing was done in singles. Cryopreserved maternal plasma was tested for TIgG and antimeasles IgG using commercial ELISA kits before and 4 weeks after exposure to ARVs .The data was analyzed using SPSS software version 12.0. Paired t-test was used to compare the changes for IgG levels. The findings were that in 164 HIV pregnant women the mean of TIgG was 22.7g/l before and 17.11g/l after ART meaning there was a significant drop p-value was 0.000.7% of samples done for antimeasles IgG tested equivocal which was significant compared to 5% negative and

was 2549.94 mIU/ml before and 2412.1mIU/ml after ART, p-value was 0.001hence there was a significant drop in the titres after ARVS administration. The findings will give insight on effects of exposure to ART on TIgG and antimeasles IgG which will be useful for revision of current infant measles immunization schedules on children born to HIV seropositive mothers.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Quantitative analytical methods have advanced significantly since the development of the enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) in 1971 (Engvall *et al*, 1971, Van weemen *et al.*, 1971). Before this, the only method for performing immunoassays was the radioimmunoassay (RIA) (Gosling, 1990)However, the RIA had several shortcomings including the potential health threat of radioactivity, short half-lives of radioisotopes, cumbersome radioactive waste disposal, and expensive counting equipment (Gosling, 1990). An important shift from radioisotope-labeled liquid-phase assays to solid-phase assays occurred in 1968 (Lequin , 2005)after development of an immuno-radiometric technique, which used radioactively labelled antibodies instead of labelled antigens for measuring insulin in human plasma (Lequin , 2005). Plastic tubes were subsequently coated with the antigen or antibody to create a solid-phase or immunosorbent platform (Gosling, 1990).

Modern commercial ELISA/EIA kits use 96-well microtitre plates, where either an antigen or an antibody is non-covalently bound to a solid-phase support. These methods are widely employed by laboratories and manufacturing companies for microbiological, virological and other serological diagnostic tests (Lequin, 2005). ELISA has been used with enthusiasm by workers on infectious diseases, where the need is often for measurement of antibody, and the indirect method of ELISA is particularly applicable (Jacobson, 1998).The main impact of ELISA has been in the measurement of antibodies because antibody titres in blood indicates previous infection and also if there is a recent infection (Karen *et al*., 2012).Antibodies measurement which are neither measures of disease directly or the cause of the disease but the patient's immune system poses a

problem because each person's immune system is unique (Jacobson, 1998). The immune response to an infectious agent is usually polyclonal in nature and the exact physiological properties of antibodies are unique for each antibody. The clonal makeup composition and the way an individual responds to an infectious agent depend not only on genetic background of the person but also on the individuals experience from former encounters with various infectious agents, this is usually the major drawback of serological tests (Lorenzo *et al*.,2005)

1.2The statement of the Problem

Although commercial ELISAs have been used for more than two decades and are specific, highly sensitive for identifying and quantifying analytes, there are several laboratory issues. These issues include; failing assays, determination of correct dilution in a specific population and how to interpret equivocal results. These issues have significant effect on the results and final conclusions. Validation of assays and general quality control of commercial ELISA remains a key challenge. This is because the assays maybe developed and validated in the manufacturing countries without putting into consideration the uniqueness of the population in the application country (Westgard, 2008).Quality assessment, suitability and result interpretation are some of the key issues that need to be addressed. Quality assessment should be done on total IgG and anti-measles ELISAs for HIV infected women because young infants are protected from measles infection by maternal measles antibodies. The level of these antibodies at birth depends on the level of antibodies in the mother and the extent of placental transfer (Farquhar *et al.,* 2004). With the introduction of ARVs there is a potential boost of immunity of the mothers which is subsequently transferred to their infants (Bekker *et al.*, 2006).There is paucity of information on the specific effects of ARVS on maternal antibodies and subsequent transfer of the same which is important for infant protection.

1.3 Justification

Laboratory medicine is the backbone of medical treatment, diagnosis and prevention of diseases. Laboratory diagnostics influences 70-80% of hospital health care decisions and costs between 3-5% of total health care (Epner *et al*., 2013). Accurate, reproducible and reliable laboratory results are crucial for patient management and care, such as initiating treatment in patients with possible immunodeficiency and revaccination of children with insufficient protection following routine childhood vaccination. It is important for evaluation of study results or establishing reference ranges for specific populations or age groups. Determination of a population specific optimum dilution factor and internal and external quality control whenever an assay is performed is important to produce reliable, accurate and reproducible results. The findings will give insight on effects of exposure to ART on TIgG and antimeasles IgG which will be useful for revision of current infant measles immunization schedules on children born to HIV seropositive mothers.

1.4 Research questions

1. How is the optimal dilution factor for a quantitative TIgG assay determined?
2. What is the precision of the optimal dilution factor for TIgG ELISA?
3. What is the distribution of antimeasles antibody among the HIV pregnant women?
4. What are the actual TIgG and antimeasles IgG titers before and four weeks after ART?

1.5 Hypothesis

Validated commercial Total Immunoglobulin G ELISA kits do not need optimization of dilution before use.

1.6 Objectives

1.6.1General Objective

To determine the optimum sample dilution of commercial Total IgG ELISA, the total and anti-measles IgG in HIV infected pregnant women.

1.6.2 Specific Objectives

1. To determine the optimum dilution factor for a quantitative Total IgG ELISA.
2. To determine the precision of the optimal dilution factor forTIgG ELISA.
3. To assess the distribution of specific antimeasles IgG among HIV infected pregnant women.
4. To determine actual levels of Total IgG and the specific anti-measles IgG in HIV pregnant women before and after ART exposure.

CHAPTER TWO
LITERATURE REVIEW

2.1 Production of IgG

Antibodies are host glycoproteins produced in response to the presence of foreign molecules. The term immunoglobulin is used interchangeably with antibodies. They are synthesized primarily by plasma cells; a terminally differentiated cell of the B-lymphocyte lineage and circulate throughout the blood and lymph where they bind to foreign antigens (Harlow *et al.,* 1998). Functionally they can be characterized by their ability to bind both antigens and specialized cells or proteins of the immune system. Antibodies are divided into five classes, IgG, IgM, IgE IgA and IgD on the basis of the type of heavy chain polypeptide they contain (Harlow *et al.,* 1998).

The binding of the antigen to the surface antibody molecule of a virgin B cell is an absolute requirement for an antibody response. This binding determines the specificity of the resulting antibodies as the antigen binding site on the surface antibody molecule is complementary to the binding site on the secreted antibodies(Figure1)(Pier *et al*.,2004) The principal function of the immune system is to protect an individual from infectious organisms and from their toxic products. There is both adaptive and non-adaptive immunity. Innate immunity is mediated by cells that respond non-specifically to foreign molecules and includes such systems as phagocytosis by macrophages, secretion of lysozme by lacrimal cells and cell lysis by natural killer cells. Non adaptive immunity does not improve with repeated exposure to foreign molecules. In contrast, adaptive immunity is directed against specific molecules and is enhanced by re-exposure. Adaptive immunity is mediated by lymphocytes, which synthesize cell- surface receptors or secrete glycoproteins that bind specifically to foreign molecule. (Harlow *et al.,* 1998).

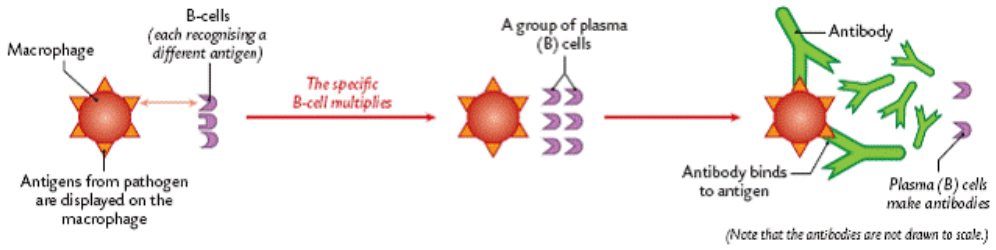

Figure 2.1: Antibody production(www.abcam.com).

The immune system is challenged constantly by an enormous number of antigens. One of the key features of the immune system is that it can synthesize a vast repertoire of antibodies and cell surface receptors, each with a different antigen binding site. The binding of the antibodies and T- cell receptors to foreign molecules provides the molecular basis for the specificity of the immune response. The primary exposure to an antigen is slow but a second exposure to the same antigen is stronger and rapid. This response termed as immunological memory. Immunological memory is specific; prior exposure to one antigen will not protect against an immunologically unrelated antigen. Immunological memory can last for as long as the animal lives. Various cytokines are involved in antibody production. IL-6 acts on proliferating B cells to promote differentiation into plasma cells and it stimulates antibody secretion while interleukin 4 (IL-4) which is released by T helper cells of the Th2 subtype and is particularly active on resting and active B cells. It was originally called B cell stimulating factor. IL-4 increases MHC II expression on resting B cells and on macrophages. On activated B cells, proliferation and differentiation is stimulated and an antibody class switch is induced (Pier *et al.*, 2004). Total immunoglobulin (tIgG) is the combination of all immunoglobulins in the blood. The increase of tIgG is called hypergammaglobulinaemia which may be caused by several conditions such as parasitic infections, viral infections it could also be congenital, it could be due to polyclonal activation due to repeated infection hence chronic mutagenic activation (Pier *et al.*, 2004).

2.2Total IgG in HIV infection

Total IgG has been reported to significantly increase in HIV infections due to B cell activation resulting in hypergammaglobinemia and nonspecific antibody production; hence patients are at risk of infections. All the antibody isotypes vary in their levels throughout the course of infection, and there is no known correlation between isotype appearance and stage of disease. In the case of HIV-1 infected pregnant women, the pattern of placental IgG transfer may change in case of hypergammaglobinemia, when total maternal IgG titers reach 15g/L then the cord total IgG titers tend to be lower than the corresponding maternal value above these concentration hence HIV infection and increased tIgG usually leads to decreased placental transfer (De Moraes *et al.,* 1998). Efficacy of transplacental transport is lower when maternal levels are high whether for total IgG or specific antibodies to measles (Goncalves *et al*., 2004).

2.3 Relationship between HIV and Measles infection

With almost two thirds of the estimated 33.2 million HIV infected individuals living in sub-Saharan Africa and 58% of them being women, the potential exists for HIV-1 infection to impact on measles control and elimination (UNAIDS 2007). Maternal HIV infection may reduce levels of measles antibodies in newborns because HIV infected mothers have lower levels of measles antibodies and have reduced placental transfer (De Moraes *et al.,* 1998).Reduced placental transfer has been observed consistently in various studies in HIV infected individuals in different populations.

In a study carried out in Malawi to determine the influence of maternal HIV infection and placental malaria on placental antibody transfer a significantly higher mean total serum IgG and measles antibody titres were found in HIV infected mothers compared to those not infected (De Moraes *et al.,* 1998). The study showed that there was no association between reduced placental transfer of measles antibody and HIV infection

but the reduction was due to maternal total IgG titres. In contrast to a study done in Brazil that showed that measles IgG antibody transfer was reduced by both maternal HIV infection and high total serum IgG(Christine *et al*.,1990).

Measles virus transmission in HIV infected children is enhanced by early decline in protective maternal antibody titers resulting in susceptibility to measles at a younger age, and high rates of primary and secondary measles vaccine failure. In a study carried out to compare the primary vaccine failure rates and antibody decline following administration of standard titer measles vaccine at 9 months of age to HIV infected and HIV uninfected Zambian children showed that children born to HIV infected women had lower levels of passively-acquired maternal antibodies to measles prior to the age of routine vaccination of 9 months, and HIV infected children were particularly susceptible to measles in infancy. HIV infected children develop adequate primary antibody responses to measles vaccine following vaccination but lose protective antibody titers by two to three years of age (Moss *et al.*, 2007).In a study carried out in Nairobi to determine relationship between maternal immune status and HIV-1 viral load measles antibody was detected in 40 (71%) maternal and 39 (70%) infant specimens. There was no significant association between detection of plasma IgG measles antibody and maternal CD4 count for mothers. However, transfer of measles antibodies was significantly less efficient in the setting of low CD4 count (Farquhar *et al.,* 2004). When specimens with positive maternal measles antibodies were evaluated, placental transfer among mothers with CD4 counts >500 was higher than among those with CD4 counts <200 (90% versus 51%). In addition, reduced maternal antibody transfer was associated with elevated maternal HIV-1 RNA. Each $\log_{10}$ increase in viral load was associated with a 37%reduction in the amount of antibody transferred. The conclusion was that high HIV-1 viral burden and advanced maternal immunosuppression were associated with reduced placental transfer of antibodies against measles virus (Farquhar *et al.,* 2005).

Studies among HIV-1 infected women in Nairobi demonstrated reduced placental transfer of IgG antibodies against measles and other pathogens and their infants had increased morbidity/mortality when compared to infants born to HIV un-infected women (Farquhar *et al.,* 2004). Due to reduced placental antibody transfer in HIV-1 infected women, children were born with lower measles antibody levels when compared with children from un -infected mothers. Among the 40 women(73%) with positive measles titers, 30(75%) were found to have abnormally low levels of materno-fetal IgG transfer (<95%). High maternal HIV-1 viral load at 32 weeks gestation and at delivery was associated with reductions in placental transfer. High maternal HIV-1 specific gp 41 antibody titer was also highly correlated with both decreased placental transfer and decreased infant IgG (Farquhar *et al.,* 2004). The results were consistent with the findings of another study carried out in Nairobi, Kenya which investigated whether the children from HIV infected mothers were more susceptible to measles at an earlier age. This study showed that children born to HIV-1 infected mothers have lower measles antibody levels at birth as compared to children born to un-infected mothers. However, at 6 months of age >86% of all children were susceptible to measles, and the figure increased to 95% at 9 months of age. Thus, infants born to women with HIV-1 infection appear not to acquire adequate passive immunity in utero. This could contribute to high infant mortality in this vulnerable population(Farquhar *et al.*, 2004).

In another study in Kilifi Kenya, to assess maternally acquired immunity on 16 pairs of HIV-seropositive women and their newborns, the population of newborns were lacking in demonstrable measles antibodies compared to the HIV-seronegative control group, possibly because many of the HIV infected mothers lacked measles antibodies. This study showed that maternally acquired immunity may be lower in infants born to HIV infected women, either because of low maternal serum antibody levels or deficient transplacental transfer (Scott *et al.,* 2008). Results from a study in Kilifi (Susana *et al.,* 2005) on a total of 747 paired maternal cord serum samples (91 from HIV infected and

656 from HIV-1 un-infected) showed that maternal HIV infection may reduce levels of measles antibodies in newborns. Infants born to HIV infected mothers were more likely to be measles antibody sero-negative. The data suggested that almost one fifth of children born to HIV-1 infected mothers were sero-negative for measles antibodies at birth (Susana *et al.,* 2005).This suggests that maternal HIV infection is associated with increased risk of seronegativity for measles to the newborns and antibody reduction in cord serum. The reduction in the levels of measles antibodies in newborns arose from a combination of lower levels of measles antibodies in HIV infected mothers and reduced placental transfer (Susana *et al.,* 2008).

2.4 Effects of ART on HIV and Measles infection

A study conducted to determine the prevalence of measles protective antibody levels in HIV infected children with immune recovery after HAART in Chang Mai Thailand showed that out of 93 children, 39 (42%) had a measles-protective antibody level, defined as an anti-measles immunoglobulin G (IgG) level > or =320 mIU/ml. The study showed that despite a history of measles immunization and evidence of immune reconstitution after HAART, many healthy HIV infected children are still susceptible to measles (Bekker *et al.,* 2006).

A study in Netherlands a single-center cohort study of 59 children with HIV-1 infection were investigated for long term effect of HAART on titers and course of specific antibodies against measles, mumps, rubella (MMR) vaccine strains showed that age adjusted $CD4^+$ T cells and B cells increased, whereas total immunoglobulin levels decline. Although these children were pre-immunized before the start of HAART only 43% had antibodies against measles, mumps and rubella. Antibodies against MMR were lost in children who were seropositive at baseline. Humoral activity in children with HIV-1 infection remains abnormal during HAART, despite reconstitution, antibodies

against live-attenuated vaccine and wild-type natural virus strains disappear over time in up to 40% of children with HIV-1 infection (Hayley *et al.,* 2002).

In a study to determine the effects of loss of measles antibodies after MMR vaccine in HIV infected adults receiving HAART initial humoral immune response to measles vaccine was not different between HIV-infected adults and HIV-uninfected adults; however HIV infected adults have a rapid decline of measles antibodies despite their high CD4 + cell count and sustained cellular proliferative response (Aurpibul *et al.,* 2006).

A study in the USA showed that HAART improves the response to an additional MMR vaccination. This was demonstrated after comparing 28 HIV infected children who lacked protective antibody to measles virus and were undergoing HAART or non-HAART regimes. Nine (64.3%) of 14 children undergoing HAART, compared with 3(21.4%) of 14 in the non-HAART group, had antibody to measles virus after the additional vaccination with MMR (p=0.27).The study demonstrated the effect of HAART therapy on the serological response to additional measles vaccination of HIV infected children, in US the results showed that patient who receive HAART are more likely to respond serologically to repeat measles vaccination than those who receive non-HAART regimes (Belaunzaran *et al*., 2009)

2.5 Enzyme-linked immunosorbent assays (ELISA)

ELISAs are specific and highly sensitive procedures for identifying and quantifying analytes such as proteins in samples. This assay is based on the binding of a target molecule (analyte/antigen) to antibodies which recognize the compound. The presence of an antigen-antibody complex is detected using a secondary enzyme-conjugated antibody. Detection is obtained by addition of a substrate which yields a measurable

product. ELISAs are routinely used in many areas of biological research (Gosling, 1990).

2.6 The types of Enzyme-Linked Immunosorbant Assays

The ELISA involves a solid phase where the proposed antigen (or antibody) could be bound followed by the addition of sera for examination (Gosling, 1990). If the serum sample contained antibodies associated with the immobilized antigen or antibody, then they would bind the solid phase. This antigen/antibody surface binding is the basis of all immunoassays and occurs due to the 'lock and key' system, where the antibody contains a chemical 'lock' and the antigen contains a specific chemical group or 'key' that fits this lock. In an organism the attached antibody is a marker indicating to the body that the antigen it is bound to should be destroyed. (lequin, 2005). In an immunoassay, antibodies are bound in a 'well' to the antigen, allowing their concentration to be determined. However, what makes ELISA so superior to other techniques is the method by which the bound analyte is read. This more accessible read process makes ELISA a cheap, simple and direct process, whilst only losing negligible amounts of specificity and sensitivity (Gosling,1990). The most common types of ELISA techniques are, Direct ELISA, Indirect ELISA, Sandwich ELSIA, Competitive ELISA. Sandwich and competitive ELISA follow the same principle as direct and indirect ELISA, but methods are changed slightly to accommodate different types of antigen, or take different observations for experimental work (Gosling, 1990). When using direct ELISA the antibodies are bound to the solid phase and antigens are detected within sera, whereas indirect ELISA binds antigen to the solid phase so that antibody concentrations can be examined. In the four decades since the discovery of the ELISA technique, it has been adapted and tested as a viable bioassay for many hundreds of antigens and antibodies. Additionally, ELISA has been employed in the detection of the HIV (lequin, 2005). Despite the changes for which ELISA has been utilized, the general methodology has remained largely unchanged since it was first introduced. In its current form, the ELISA

technique uses a polystyrene microtitre well as a solid phase, arranged in to a 96-well plate, allowing for multiple assays to be carried out under the same conditions in a short space of time (Gosling, 1990).

2.7In-house verses commercial ELISA reagent kits

Most immunological tests used in clinical laboratories are commercially produced. Sometimes however, tests are developed, evaluated, and validated within one particular laboratory. These "laboratory-developed tests" are used only by the developing laboratory and are not distributed or sold to any other laboratories. Immunoassays are often created by a clinical laboratory because a commercial test for the analyte(s) of interest is not currently available. Tests may not be commercially available because the analyte is rare, and the market for such a product would be too small to be profitable (Karen *et al*.,2012).There are advantages and disadvantages of either of the two sources of the ELISA kits but one of the key disadvantage of commercial reagent kit is the cost and there could be false results (sensitivity, specificity and accuracy).This is because high serological criteria of an ELISA kit cannot be reproduced in another country (Mohammadi *et al*.,2008).

2.8Validationof laboratory Methods

Establishing performance specifications is a one-time process that must be completed prior to reporting patient results. The process includes determination of accuracy, precision, reportable range, reference interval, analytical sensitivity and analytical specificity. The laboratory then performs ongoing quality control and quality assessment as required in the Clinical Laboratory Improvement Amendments final rule (Thompson *et al.,* 2002).The International Standard of Operation definition of validation includes establishing performance specifications as well as quality assessment measures. According to the ISO definition, specific information should be documented as part of

the validation process (Federal regulation, 2010).Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use. Some test parameters that could be included in the validation protocol are accuracy, precision, linearity, and specificity. The protocol must also include predefined acceptance criteria for each of the assay parameters (Thompson *et al.,* 2002).

An analytical method is the series of procedures from receipt of a sample to the production of the final result. Typically, validation follows completion of the development of a method and it is assumed that requirements such as calibration, system suitability, analyte stability have been established satisfactorily. When validating using a method of analysis, measurements must be made within the calibrated range of the detection system used (Burd 2010).

2.9Reportable range of a test

CLIA uses the term "reportable range" to refer to the span of test result values over which the laboratory can establish or verify the accuracy of the instrument or test system measurement response. Laboratories may quantitatively report results that fall within the reportable range. Reportable range does not apply to qualitative clinical tests .Reportable range and other terms, such as measuring interval, analytical measurement range (AMR), and linear range, are used interchangeably to refer to the same performance characteristic of quantitative assays (CLSI/NCCLS, 2003).In practice, it is common to refer to the "linear range," and laboratories generally use a linearity experiment to determine the reportable range for a test. It is not mandatory that a method provide a linear response, but a linear response is desirable since test results that are in the linear range are considered to be directly proportional to the concentration of the analyte in the test samples (Westgand, 2008).The boundaries of the reportable range are the lowest and highest analyte concentrations that generate results that are reliably produced by a test method without dilution of the specimen. The lower limit must also be clinically

relevant and acceptable for clinical use. The lower limit of linearity is frequently referred to as the lower limit of quantification (LLOQ) and the upper limit of linearity as the upper limit of quantification (ULOQ). It is understood that poor precision will affect the linearity of an assay. Therefore, it is recommended to check for poor repeatability as part of the linearity study by testing two to four replicates at each concentration, depending on the expected imprecision of the assay. If values beyond the upper limit of linearity are of clinical interest, it may be desired to allow dilution of patient specimens to extend reporting of test values beyond the upper limit of the linear range. A dilution protocol to allow re-testing of out-of-range patient specimens must be validated and appropriate diluents documented (Westgand, 2008).

2.10 Analytical Sensitivity of an assay

The "analytical sensitivity" of an assay is defined as the ability of the assay to detect very low concentrations of a given substance in a biological specimen. Analytical sensitivity is often referred to as the "limit of detection" (LOD). LOD is the lowest actual concentration of analyte in a specimen that can be consistently detected with acceptable precision, but not necessarily quantified, under routine laboratory conditions and in a defined type of specimen (Saah *et al.,* 1997).

LOD is an important performance characteristic that must be determined for both quantitative and qualitative tests. LOD is expressed as a concentration such that the lower the detectable concentration of analyte, the greater the analytical sensitivity of the assay. Knowing the limit of detection is also important to determine the concentration to be used as a low positive control that will be monitored to ensure consistency of performance between runs at levels near the cutoff and to ensure that the LOD does not change when new reagent lots are used. For quantitative tests, it is possible for the LOD to equal the LLOQ if the observed bias and imprecision at the LOD meet the requirements for total error for the analyte. Most often, however, the LOD resides below

the linear range of an assay and is lower that the LLOQ (Burd, 2010). An assay with perfect analytical sensitivity may fail to give a positive result if the target substance is not present in the processed specimen because of vagaries in collecting or processing the specimen. The LOD is generally determined in one of two ways: (i) statistically, by calculating the point at which a signal can be distinguished from background, or (ii) empirically, by testing serial dilutions of samples with a known concentration of the target substance in the analytical range of the expected detection limit (Burd,2010).

2.11Precision of an assay

The "precision" refers to how well a given measurement can be reproduced when a test is applied repeatedly to multiple aliquots of a single homogeneous sample. When considering precision, it is important to remember that a measurement may be very precise (replicates have the same result) but not very accurate (the real value is much different). The ideal assay is both precise and accurate. Precision (also referred to as random analytical error) is related entirely to random error caused by factors that vary during normal operation of the assay. Precision does not have a numerical value but may be expressed qualitatively as high, medium, or low(Horowitz *et al*., 2010).For numerical expression, the term "imprecision" is used. Imprecision is defined as the "dispersion of results of measurements obtained under specified conditions" (Westgand, 2008). Different terms have been used to describe different components of precision (or imprecision). "Repeatability" and "reproducibility" are considered to be the extreme measures of precision, with repeatability (or within-run imprecision) being the smallest measure of precision and involving measurements carried out under the same conditions (same operator, reagent lots, instrument, laboratory, time) and reproducibility (run-to-run imprecision, day-to-day imprecision) being the largest measure of precision and involving results of measurements under changed conditions (different operators, reagent lots, time, laboratory) (Horowitz *et al*.,2010).

2.12 Analytical Specificity of an assay

The analytical specificity of an assay is different from the diagnostic specificity of an assay. “Diagnostic specificity” refers to the percentage of individuals who do not have a given condition and are identified by the assay as negative for the condition. The two aspects of analytical specificity are cross-reactivity and interference substances (Saah *et al.,* 1997).

The term “interfering substances” refers to the effect that a compound other than the analyte in question has on the accuracy of measurement of an analyte. Specimens from any source may contain unpredictable amounts of interfering substances. These substances may originate from a variety of endogenous and exogenous sources (Horowitz *et al*., 2010)

2.13Accuracy (Trueness) of a test

Accuracy is a broad term that has generally been used to describe the extent to which a new test method is in agreement with a comparative or reference method (Westgard, 2008). The terminology has changed somewhat to align with that of the ISO. The current methodological use of the term “accuracy” refers to the closeness of the agreement between the results of a single measurement and the true value of the analyte (Federal regulation, 2010). What was previously considered to be “accuracy” is now termed “trueness.” “Trueness” has replaced the term “accuracy” when referring to the closeness of the agreement between the average value obtained from a large series of measurements and the true value (if there is an international standard) or accepted reference value (if there is not an international standard) of a measurement (Horowitz *et al*.,2010). Trueness is expressed numerically as bias (lack of agreement). Bias is inversely related to trueness and refers to the average deviation from the true value due

to nonrandom effects caused by a factor(s) unrelated by the independent variable (CLSI/NCCLS, 2005).

2.14 Reference Interval of an assay

The reference interval is usually the last performance characteristic to be studied, since it is used to decide whether a method is acceptable or not (Westgard, 2008). The reference interval ("normal range") of a test is simply defined as the range of values typically found in individuals who do not have the disease or condition that is being assayed by the test (the "normal" population) (Horowitz *et al*.,2010). To be clinically useful, the reference interval must be appropriate for the population being served.

To determine the reference interval, specimens from healthy subjects in the intended population are tested and the resultant values are used to determine the normal range (Burd, 2010). Reference interval studies are often conducted using residual specimens from tests done for other purposes as long as the specimens are representative of the population being served. The reference interval is usually computed as the interval between the lower reference limit (2.5th percentile) and the upper reference limit (97.5th percentile). If reference intervals of the assay being used have already been determined based on an adequate reference interval study, it may be possible to simply "transfer" the reference interval (Horowitz *et al*., 2010).

2.15Optimization of assays

The goal of assay optimization is establishing a plan for evaluating the factors that may affect an assay performance. The factors that may affect an assay are temperature, humidity, sample contaminants, and reagent composition (Saah *et al*., 1997). Assay developers should study these factors alone and in combination to assess their effects on the device's accuracy, precision, repeatability, and cross-reactivity. Whether for basic research or clinical purposes, an assay's intended use becomes the anchor to which all

optimization and validation activities are set. Optimizing an assay involves choosing its optimal format. With the intended use in mind, a new assay's appropriate performance characteristics are then defined. Although a number of performance characteristics, such as stability, accuracy, and precision, should be reviewed in all assays, other individual characteristics such as robustness and reproducibility may not be as important, depending on the assay intended use (OIE, 2013).

2.16Results of using linear fit and 4-parameter analysis on ELISA data

The determination of the analyte concentration on ELISA relies upon construction of a calibration curve (DeLean *et al*., 1998).The standard curve is prepared by performing a dilution series of a known concentration of the analyte across a range of concentrations near the expected unknown concentration. The calibration curves are then used to calculate the concentration of an unknown sample. For most analysis, a plot of response versus concentration will create a linear relationship, at least within a certain range of concentrations, and can be analyzed with linear regression. However, for those calibration plots which are sigmoid (a curve having an "S" shape), performing a linear fit can lead to errors in estimating sample concentration. These inaccuracies are most significant at the extremes of the standard curve, most often in the low end but sometimes in the high end as well (DeLean *et al*., 1998).Comparing the concentration values, four -parameter fit versus linear analysis, reveals disparities between the two methods. The largest discrepancies are noted at the lower concentrations. A number of linear fit techniques are available, although the least-squares approach is often sufficient (Georgiadis *et al.,* 2003). A simple transformation of the data (such as a log, log-log, and square root transformation) may also be required to obtain a linear fit. However, if an assay does not perform in a linear fashion throughout its analytical range (i.e., typical transformation methods are not adequate) and graphical plots show a sigmoidal relationship, the standard curve may be able to be modeled on the four-parameter logistic regression equation(Findlay *et al*., 2007).

2.17 Statistical practices in ELISA validation

Implementing proper statistical methods ensures the development of safe and effective assays. The coefficient of variation (CV) is the ratio of the standard deviation σ to the mean μ: This is expressed as a percentage of variance to the mean and therefore indicates any inconsistencies and inaccuracies in the results. The larger the variance, the more inconsistency and error there is. For example, some laboratories will set guidelines for ELISA results so that the coefficient of variation is no more than 2%. This will indicate that the results are not valid if they have a difference of more than 2% CV to the mean as there is too much variance and the results are considered to be inaccurate. This sets a standard for the quality of the validated results. Computer programs can be used to calculate the CV values from ELISA results (Ravindranath *et al*., 1994).

2.18Evaluation of ELISA results

Evaluation of ELISA results often presents some problems to the novice user. With highly specific antisera and samples with good antigen titer, results are normally very clear. When the antisera used are weak or contain some antibodies to host proteins and/or the samples have a very low antigen titer, determination of a positive result can be more difficult. Reactions can be evaluated visually with some precision if background readings for healthy controls are low. Normally, the eye can discern differences in OD_{405} of 0.05 to 0.1 above a low background. A graded scale with three to five levels is useful to report the relative degree of reaction. Where greater accuracy is required, the degree of reaction can be measured by testing a diluted sample in a spectrophotometer or by reading the plate in an ELISA plate reader. A wide variety of plate readers are available, from simple manual models suitable for modest numbers of plates to highly automated models capable of various levels of data analysis and storage. Recently, emphasis has increased on measuring reaction rate rather than a single final optical density value. This eliminates some sources of error where accurate quantitative data are needed and also

allows more accurate comparison of samples with large differences in antigen concentration. Rate calculation requires several measurements of the same plate at a measured time interval, and a plate reader is essential. Some plate readers do rate calculations automatically. Expensive plate readers are not necessary until a definite need for them is identified. (Karen *et al*., 2012)

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Design

This was laboratory based study conducted as a sub study of a study carried out at the University of Nairobi, Department of Pediatrics laboratory. The parent study was a prospective cohort study that was conducted between 2005 and 2009 (Kesho Bora) in which HIV infected pregnant women were enrolled and exposed to different ARVs depending on their degree of immunosuppression for prevention of mother to child transmission of HIV-1. They were followed up to delivery and thereafter for 12 months together with their infants to establish the effect of ARV's in prevention of mother to child infection.

3.2Kesho Bora Parent Study

The Kesho Bora project was a multi-site prospective cohort study of HIV-1-infected mothers and their infants to study the effect of ARV's in prevention of mother to child transmission. HIV-1-infected pregnant women identified prior to 32 weeks gestation in antenatal clinics in Nairobi and Mombasa, Kenya and Bobo-Dioulasso, Burkina Faso and their children were recruited into the study and followed for 1 year. The cohort included two groups:

1. Women who met the WHO criteria for HAART (having a CD4 count less than 200 cells/mm^3 or symptomatic HIV disease) were offered and accepted HAART begun at 34-36 weeks gestation and continued as long as clinically necessary.
2. Women who did not meet WHO criteria for HAART or, although meeting criteria, had contraindication(s) to or refuse HAART; these women were offered short-course MTCT prophylaxis. Within the second group, women with CD4 counts between 200 and 500 cells/mm^3 with no contraindications to HAART and willing to participate in

an RCT were randomized to one of two MTCT prophylaxis regimens: (1) HAART (300 mg ZDV, 150 mg lamivudine (3TC), and 400 mg/100 mg lopinavir/ritonavir (Kaletra) bid) beginning at 34-36 weeks gestation until six months postpartum, or (2) a short-course regimen ZDV (300 mg bid) from 34-36 weeks gestation to the onset of labor, when one dose each of ZDV (600 mg) and NVP (200 mg) were administered.

In this study the samples were from the two groups (1 and 2).**3.3 Sample Size determination**

Sample size was determined using Epi Info software according to Kelsey *et al.*, 1996

$$\frac{n\ddagger \{z_{\alpha}\text{ر}_{2pq}+z_{\beta}\text{ٲ}p_1(1+R-p_1(1+R^{2)}\}}{[p_1(1-R)]^2}$$

Where:

ṕ=Incidence of disease among the Non- Exposed

A_1 =1- $\acute{p}_1$

$\acute{p}=\acute{p}_1\frac{(1+R)}{}$

R=relative error

z_a=Z value corresponding to alpha error

$z_{\beta=}$ value corresponding to beta error

Confidence level (1-α): 95%

Power of study (1-β): 80%

Ratio of exposure I to exposure II: 1:1

Expected frequency of effect on unexposed group: 50% (used since there isn't any documented information on Total IgG levels in HIV infected women)

Risk ratio/relative risk: 0.5

Sample size was therefore 164 Samples

3.4Sampling of serum from Kesho bora study

Sampling of plasma samples was done randomly from University of Nairobi laboratory. Systemic random sampling was carried out by first having a list of the whole population of serum specimen numbers, secondly a random number was chosen at the start of the list. Thereafter every third number from the whole population was sampled. A skip interval had been computed using Ms Excel. Eight samples were randomly selected from these samples for dilution determination and quality control

3.5 Study approval/Ethical consideration

The study was cleared by KEMRI Scientific Steering Committee (SSC) and KEMRI Ethical Review committee (ERC) (AppendixA,Appendix B).Privacy and confidentiality was safeguarded for those whose demographic information was used for this study. There was no access to the link log between participant study codes to information that would identify them. Results obtained from the study were to be used for the intended purposes and not disclosed in public settings using participant names. Since the study was nested within Kesho Bora study that was approved by Kenyatta National Hospital ERC and the ethical issues that would arise were addressed which included voluntary participation, informed consent of adults/minors, potential risks/harm the study may present, and potential benefits that would be accrued from participation, there were no

ethical issues arose (Appendix B).In this study, there was no risk to the participants because archived samples were used.

3.6 Determination Total and Anti-Measles Immunoglobulin G

3.6.1.1 Construction of a calibration curve

Concentration (logarithmic scale) was plotted against optical density (linear scale) to produce the calibration curve. The quantification range for TIgG concentration has 0.11 mg/L – 9.0 mg/L. The R^2 value of a standard curve was calculated by SOFT max PRO. Results were calculated from the linear portion of a four-parameter fit logistic standard curve constructed with each assay.

3.6.1.2 Sample dilution for TIgG Assay

Immunology Consultants' Laboratory ELISA kit recommended dilution of $1{:}8x10^4$ was first tested and found to be concentrated hence produced high ODs. The optimum dilution for the samples needed to be determined so that the OD was in the linear part of the standard curve. A serial dilution was conducted at$1{:}16x10^4$,$1{:}8x10^4$,$1{:}6x10^4$,$1{:}4x10^4$,$1{:}2x10^4$ for both serum and plasma, HIV positive and negative samples, Nairobi cohort and Mombasa cohort. A dilution of $1{:}1x10^5$-$1{:}8x10^5$ were conducted six times on the eight selected samples. All assays were run in duplicates at the dilution of $1{:}4x10^5$and the standard error was calculated and error bars constructed by SOFT max PRO.

3.6.1.3Total IgG determination

Cryopreserved maternal plasma was tested for levels of total immunoglobulin using Immunology Consultants' Laboratory commercial ELISA kits before ARVs exposure and 4 weeks after exposure to ARVs for the 2 arms of exposure.

3.6.1.4 Assay forTIgG determination

A double antibody sandwich ELISA was used to measure the titers of Total IgG at enrolment and at delivery using Immunology Consultants' Laboratory ELISA kit.(Immunology Consultants Laboratory, Inc: www.icllab.com). In this assay the IgG present in samples reacts with the anti-IgG antibodies adsorbed on the surface of polystyrene microtitre wells. After removal of unbound proteins by washing with phosphate buffered saline (PBS) anti-IgG antibodies conjugated with horseradish peroxidase (HRP) was added. These enzyme labeled antibodies form complexes with previously bound IgG. Following another washing step, the enzyme bound to the immnosorbent was assayed by addition of chromogenic substrate, 3,3`,5,5` - tetra methyl Benzedrine (TMB). The quantity of bound enzyme corresponds directly with the concentration of IgG in the sample tested, thus the absorbance at 450nm, is a measure of the concentration of IgG in the test sample **(Figure2).** The quantity of IgG is the test sample was interpolated from the standard curve constructed from standards and corrected for sample dilution. Determination of the absorbance (450nm) of the contents was determined with a calibrated plate reader (Molecular Devices – Emax – precision micro plate reader).

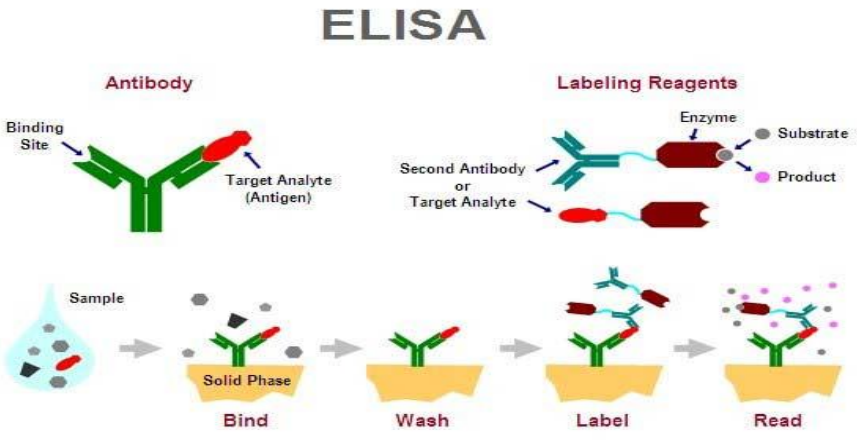

Figure 3.1Sandwich ELISA (www.abcam.com/technical)

3.6.2Laboratory Assay for measles Antibodies

Enzygnost measles indirect ELISA kit from Siemens (Marburg/Germany) was used to detect measles IgG with the appropriate conjugate. The kit contained one measles virus antigen conjugated well and one control antigen-coated well for each sample (figure3.2).

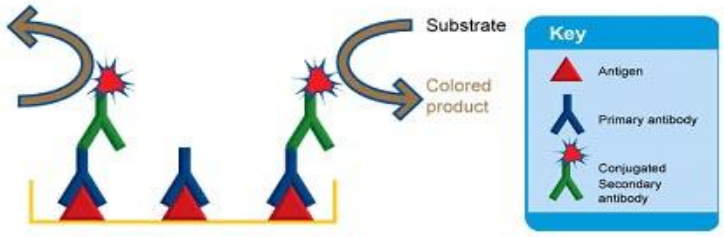

Figure 3.2: Indirect ELISA (www.abcam.com/technical).

Indirect ELISA was conducted according to the Enzygnost anti-measles IgG protocol. Validation criteria which is the absorbance value obtained from the measurements with measles virus minus the absorbance value of the same sample with measles virus control antigen (OD AG-ODCOAG) must pass and individual ΔA values (anti-measles virus Reference P/N at the start and end of series of measurement or test plate) must not differ by more than+-20%from the mean calculated from the values(Appendix C).Performance of QC for the failing assays was done the variables included incubation time, temperature and pipetting and use of different reagent kits. OD reading at 450nm using ELISA (molecular devices –Emax – precision micro plate reader) was done within 30 minutes.

Table 3.1 Interpretation of qualitative analysis of results on antibody index

Optical Density	**Interpretation/Remark**
<0.100	Negative(no detectable antibody to measles IgG by ELISA)
0.100–0.200	Equivocal (repeated)
>0.200	Positive (detectable antibody to measles IgG by ELISA)

Quantitative analysis α-method was used $\log_{10}$MIU/ml= $\alpha \times \Delta A^{\beta.}\alpha$ and β were lot dependent taken from enclosed barcode table of values. The results were then multiplied by the dilution factor

3.7 Data Management

Data was entered into Ms Access database and edited using consistency and range checks after data entry. However data was analyzed using a statistical package, SPSS version 12.01.

CHAPTER FOUR
RESULTS

4.1Determination of the Optimum dilution factor for a quantitative TIgG ELISA

4.1.1 Standard curve obtained using manufacturer standards

Based on this standard curve obtained using the manufacurers' standards the only optical density(ODs) that can be used to determine the concentration of the samples are the ones that fall into the linear part of the standard curve (approxiamtely between 0.5 and 2.5 OD).The ODs standard curve constructed producedOD's precisely 0.3-2.8 (Figure 4.1) meaning ODs that can interpolate the sample results are the ones that fall in the linear part of the standard curve and within this range.

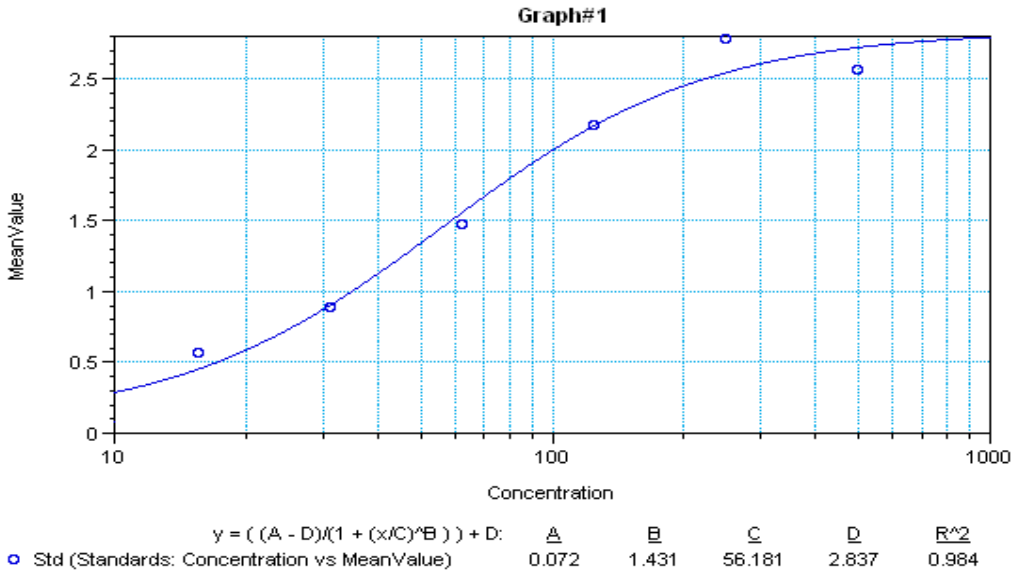

Figure 4.1: **Stardard curve for manufacturer recommended dilution of 1:8x10^4**

4.1.2 Initial sample dilution of 1:8x10^4 for TIgG as recommended by the manufacturer for both serum and plasma

From the initial dilution recommended by the manufacturer the samples were highly concentrated giving ODs of 2.9-3.6, that was outside the linear part of the curve **(**Figure4.1). With these ODs the sample concentration cannot be interpolated from the standard curve. The OD'S of 3.0 are above the linear part of the curve, meaning they are high as can be seen from figure 4.2**.**

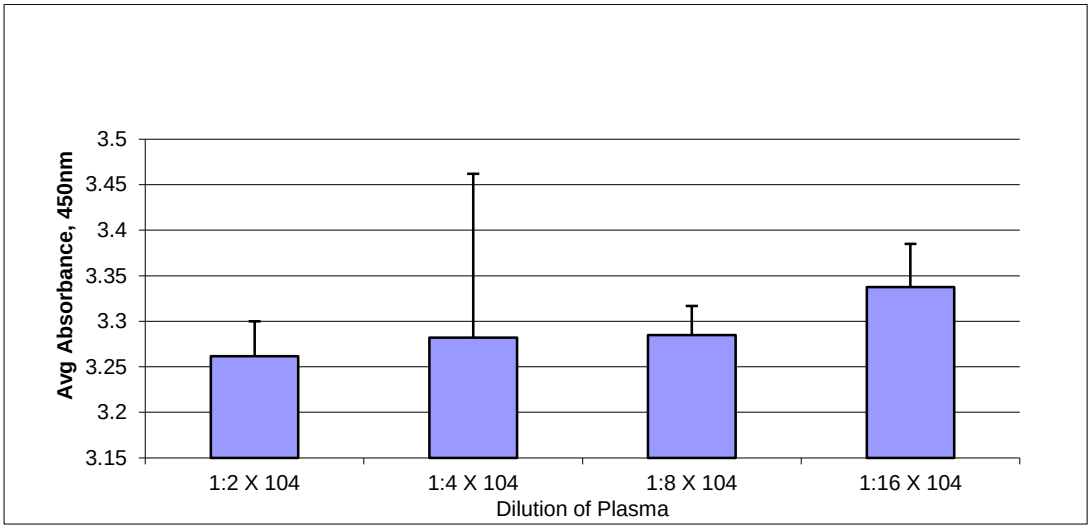

Figure 4.2: Initial recommended serial dilution of 1:2x10^4-1:16x10^5

4.1.3 Serial dilution of 1:1x10^5-1:8x10^5

The sample ODs at a lower dilution of 1:1x10^5-1:8x10^{5}were 0.3-2.5 and fell within the linear part of the standard curve (Figure4.3) hence can be interpolated to find the concentration from the standard curve constructed (Figure4.1).The actual dilution used in this study was1:4x10^{5}because at this dilution the sample ODs were all within the linear part of the standard curve.

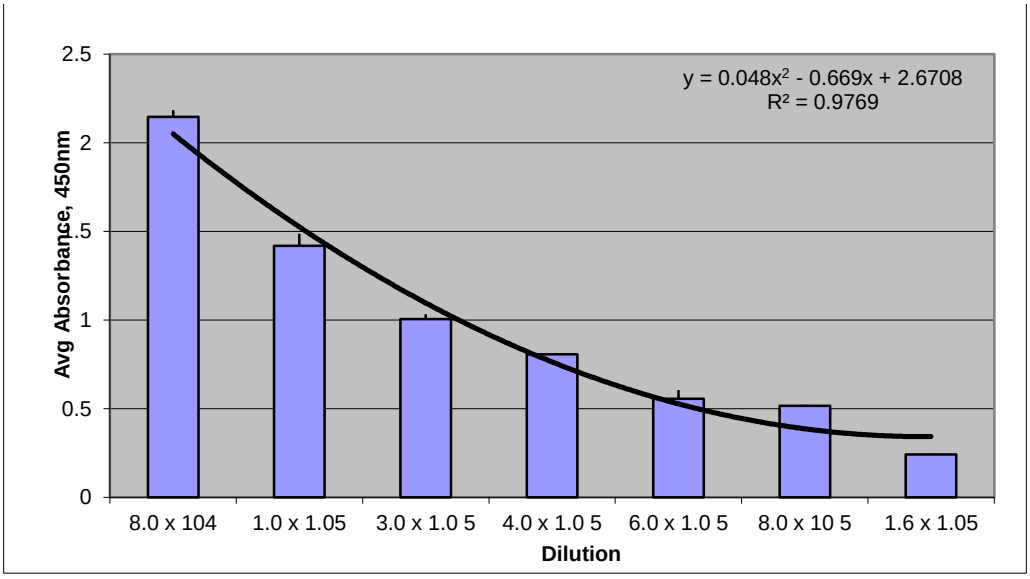

Figure4.3: **HIV Positive plasma dilution of $1:8x10^5$-$1:16x10^5$**

4.1.4Dilution of HIV negative Serum and plasma samples

The ODs for HIV negative serum at dilution of $1:2x10^4$-$1:8x10^4$ were 2.9-3.6 and the plasma at the same dilution was 3.0-3.6.Meaning the dilutions were high despite the sample type hence the ODs did not vary based on sample type, but rather individual sample (Figure4.4, Figure 4.5)

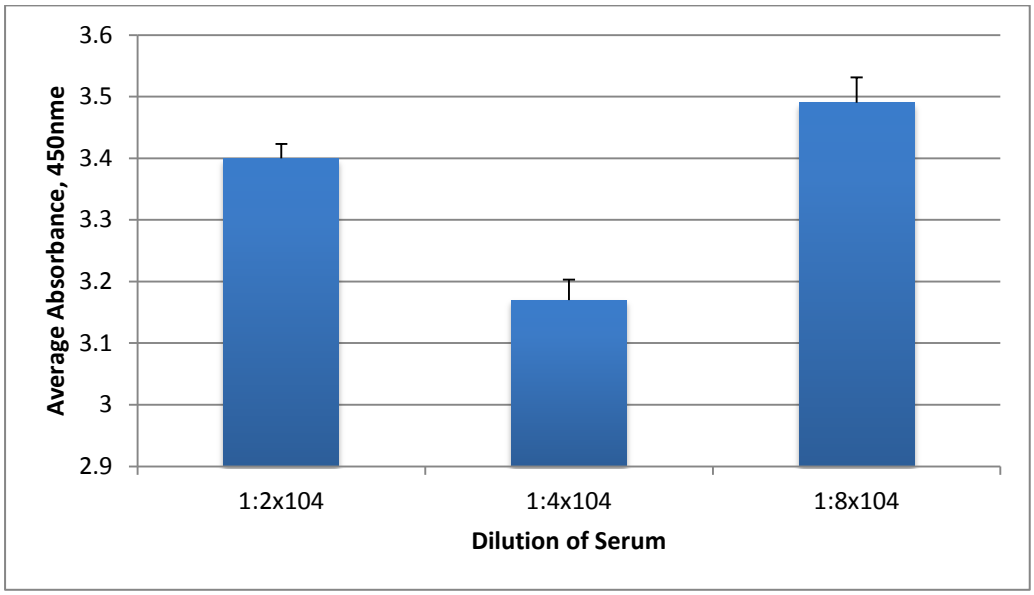

Figure 4.4**: Dilution of HIV negative serum at dilution of 1:4x10^4-1:8x10^4**

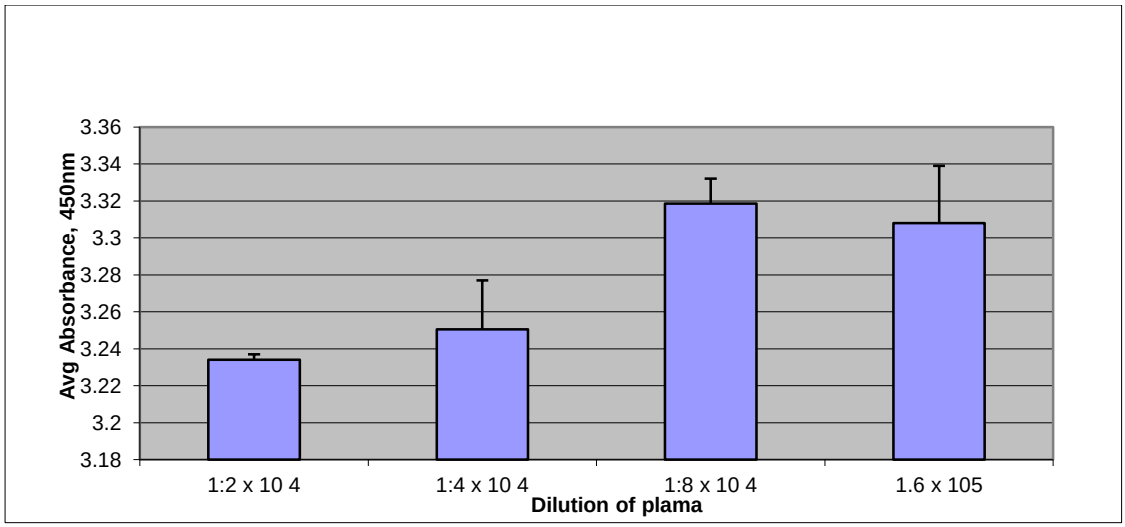

Figure 4.5: Dilution of HIV negative plasma at dilution of 1:2x10^4-1:16x10^5

4.1.5 Dilution of plasma from Nairobi and Mombasa cohort

Samples from Nairobi site cohort before ARVs at dilution of 1:4x10^4-1:4x10^5 gave ODs of 0.6-2.4 (Figure 4.6)and after ARVs were 0.2-2.3 so could be interpolated from the standard curve

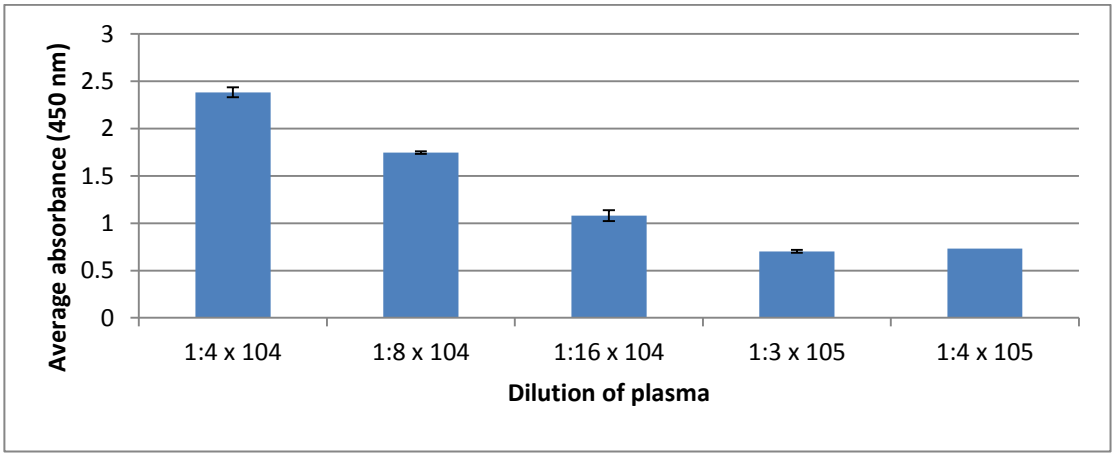

Figure 4.6: Dilution of plasma at 1:4x10^4-1:4x10^5 from Nairobi before ARVs

The plasma samples obtained from Mombasa After ARV's required a low dilution of 1:4x10^4-1:4x10^5producing OD's of 0.5-2.3 which could be interpolated of the standard curve(Figure4.7)

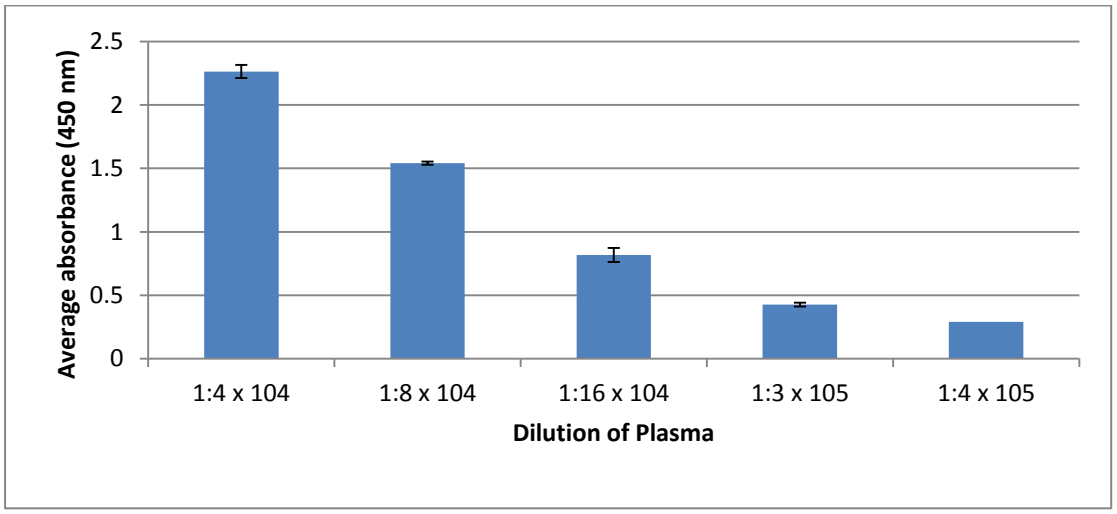

Figure 4.7: **Dilution of plasma at 1:4x10^4-1:4x10^5 from Mombasa after ARVs**

4.2. Determination of the precision of the optimal dilution factor for TIgG ELISA

4.2.1Reproducibilityofsamples ODs at dilutions of 1:1x10^5-1:16x10^5

Multiple dilutions of 1:1x10^5-1:16x10^{5}were conducted on each sample in duplicate and standard error calculated by SOFTmax PRO. Standard error bars were then drawn and were close with exception of a few as shown in figure 4.8,,the close error bars gave confidence to perform the tests in singlets.

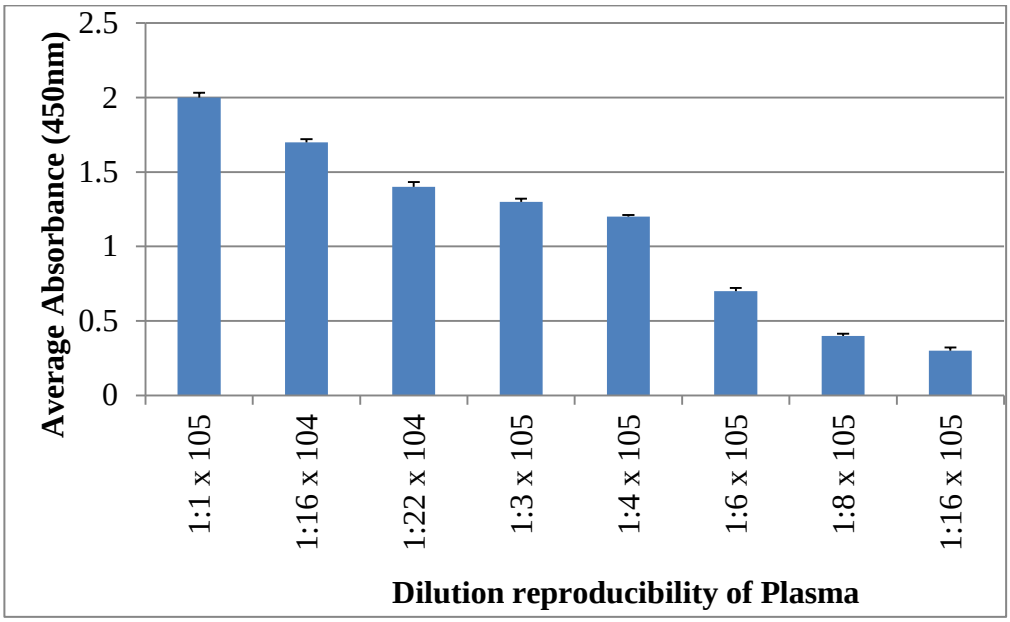

Figure 4.8: **Multiple dilution of a plasma at 1:1x10^5-1:16x10^5**

4.2.2 Precision to determine the reproducibility

Sample ODs at different dilutions was done using 8 samples with known concentrations of Total IgG. Test materials were the patient plasma specimens after determining the dilution factor. The samples were tested in duplicates so that the standard error could be determinedand the error was+-0.1 meaning it was close and duplicates gave similar results hence the samples were run in singlet's.

4.3 Qualitative antimeasles IgG results

From the total 163 tested plasma samples for anti-measles 146 were positive OD≥0.200, 10 were equivocal OD 0.100-0.200 while 7 were negativeOD≤0.100(Table 4.1).This means that there were more equivocal samples than the negative samples and the manufacturer recommend that they should be repeated.

Table4.1 **Qualitative analysis of anti-measles IgG**

Measles IgG	**N**	**%**
positive	146	**89**
Negative	7	**4**
Equivocal	10	**7**
Total	163	**100**

4.4 Total IgG and anti-measles IgG in HIV pregnant women before and after ART exposure

4.4.1 Total IgG titers before ART exposure in HIV infected pregnant women

The actual TIgG and antimeasles antibodies were determined in 164 HIV infected women before ART exposure. The mean in g/L before ART was 22.74 with a standard deviation of 14.29 and a standard error of 1.12

4.4.2 Changes of total IgG titers after ART exposure

The mean before was 22.74g/L with a standard deviation of 14.29 and a standard error of 1.12, while the mean after was 17.11g/L with a standard deviation of 11.93 and a standard error of 0.93. The findings show that there was a significant drop 4 weeks after ART exposure. The calculated overall mean difference between before and after was 5.62 with a standard error of 1.19. and the calculated t statistic was 4.703 (Appendix D)

4.4.3Antimeasles IgG titers before ART exposure in HIV infected pregnant women

The mean antibody titer in mIU/mL before ART exposure was 2549.9 with a standard deviation of 1988.6 and a standard error of 155.8(Appendix E)

4.4.4 Changes after ART exposure

The mean mIU/mL4 weeks after ART exposure was 2412.1 with a standard deviation of 1975.5 and a standard error of 154.31856. There was a significant drop between the mean mIU/mL after ART and the mean mIU/mL before ART. The calculated overall mean difference between before and after was -137.87 with a standard error of 443.3. The calculated t statistic was found to be -3.970 with an associated p-value of 0.000, and based on the p-value then there is a difference between the mean mIU/mL after ART and mean mIU/mL before ART with the results showing a significant drop in the mean mIU/mL after ART(Appendix E)

CHAPTER FIVE

5.1 Discussion

5.1.1 Determination of the Optimum sample dilution for commercial TIgG ELISA

The TIgG reagent kits used in this study the optimum sample dilution needed to be determined to produceOD's0.1-2.5whichfalls on the linear part of the standard curve. The purpose of identifying the optimum dilution is to ensure high sensitivity and good linearity of the patient's antibody concentration. Example- if the curve plateaus at 2.0 OD and the sample OD is 2.1, this does not result in a proportional change in concentration as it would if the OD was in the linear part of the curve. This is because an ELISA standard curve expresses protein concentration as a function of spot intensity (Kemeny, 1991). Data from portions of the curve outside of the region approximating a straight line are not relevant because they would not be accurate, sensitive and hence would not enable quantification (Engvall, 1971). The standard curves constructed should be best fit meaning the correlation factor $r^2$is greater than 0.975 which is a measure of good linearity and sensitivity. Although no standard exists, a regression line's fit is commonly measured using a threshold value of the coefficient of determination (r^2) associated with the regression fit. The standard curves that were used for this study the r^2 was 0.9769. The manufacturer suggested the sample dilution to be done at $1:8x10^4$this dilution produced OD's that were between 3.18-3.5 which were high and the sample concentration could not be interpolated from the standard curves. The required maximum signal (OD) should not be more than 2.0(Immunology consultant laboratory) although not a strict cut off because some tests were leveling at 2.5.From this work a clear cut off could on be established, but rather an approximate OD range of 0.1-2.5. For these samples, the concentration obtained from the standard curve when analyzing the results was multiplied by the dilution factor. One standard curve was generated for each

assay run and so after the dilution coefficient determination standards we run with every assay. The actual dilution used was $1:4x10^5$ this was much lower than the manufacturer's recommendation of$1:8x10^4$.Studies have shown that very low dilution affect the results by lowering the concentration this were not determined in this study. A study carried out on malaria ELISA showed that results change depending on dilution factor selected (Miura *et al*.,2008)future studies need to be done to establish that in total IgG ELISA. Although multiple dilutions were done for each sample at the lower dilutions the results were consistent with Miura's study.

5.1.2Determination of optimum dilution of plasma and serum samples

Dilution of different sample types (plasma, serum) carried out showed that there was no significant difference in the OD's of the various sample types, because at the initial dilution of 1:80000 required by the manufacturer produced OD's that were high. Therefore the ODs did not vary based on sample type, but rather on individual sample. The expectation was that serum would require a lower dilution than plasma because of the proteins used up in the coagulation process(Jacobson, 1998).Dilution of HIV positive and HIV negative plasma was compared. HIV positive samples produced relatively higher OD's as compared to HIV negative samples though both were high at the required dilution by the manufacturer this is because HIV infection causes hypergammaglobinaemia (Goncalves *et al*., 2004).The high OD's could be because of the physiological and pathological variability of the study population under investigation or due to storage conditions of the samples. There have been reported cases of hypergammaglobinaemia in the tropics due to tropical diseases such as intestinal nematodes (Sussana *et al*., 2005). The reference interval of a test is simply defined as the range of values typically found in individuals who do not have the disease or condition that is being assayed by the test in the "normal" population (Horowitz *et al*.,2010). To be clinically useful, the reference interval must be appropriate for the population being served. Since these assays were imported from the USA the manufacturer maybe they

did not perform validation with adequate demographic information for the population to be studied, when the original dilution was determined. It had to be determined that the dilution is applicable to the population served by the research laboratory. Therefore if details for dilution were adequately provided, the laboratory needs to experimentally verify the dilution for its unique population (Westgard, 2008). There are many other causes of increased OD apart from high concentration of the analyte under study, for instance interference/cross-reactivity of other antibodies (Ravindranath *et al.*, 1994).

5.1.3 Determination of Normal ranges of TIgG antibodies from plasma

In this study the immunoglobulin levels were significantly high (mean titer of 22.74g/l) the reference ranges in a normal population are (7-16g/l).Usually the normal results range for a particular test 95% of healthy patients fall within the normal range. That means that 5% of healthy patients fall outside of the normal range. Thus positive test does not necessarily mean that there is an abnormality. Statistically 20 or 30 individual tests run as part of a panel, chances are 1 or 2 will be slightly outside the normal range.(Epner *et al.*,2013) Laboratory results may be outside the normal range for many reasons which may be due to race, dietetic preference, age, sex, menstrual cycle, and degree of physical activity, problems with collection and/or handling of the specimen, non-prescription drugs (aspirin, cold medications, and vitamins), prescription drugs, alcohol intake and a number of non-illness-related factors (Delean *et al*., 1998).In this study the high concentration could have been because the samples were from HIV infected Kenyan women and HIV infection causes hypergammaglobinaemia (Goncalves *et al*., 2004).

5.1.4Determination of optimum dilution of samples from Mombasa and Nairobi

Comparison of dilution of samples from Nairobi site did not show any difference with those from Mombasa site. The purpose for this comparison was to establish whether differences in sample storage conditions could influence the outcome of the dilution.

Studies have shown archived specimen storage conditions may interfere with the stability of antibodies (Karen *et al.,* 2012). In this study samples were from different sources, different freezers and different localities and they had been archived between 2005 and 2009.After comparing the dilution factor for Mombasa and Nairobi cohort the conclusion was that the stability of the antibodies was intact despite the fact that the freezer temperature chart was not regularly filled in Nairobi. Generally the samples had been stored at −80°C to ensure stability over time. In comparison to sensitive proteins such as enzymes, antibodies are highly stable proteins that retain activity in a wide range of biological conditions. As a result, storage is reasonably straightforward however repeated freeze/thaw cycles and excessive protein concentration dilution, particularly of purified antibody, can result in material losses and reductions in antibody stability (Miura *et al.,* 2008)

5.2 Determination of the precision of the optimum sample dilution

It is required to always run ELISA samples in duplicate or triplicate in most cases this is not feasible because of the cost of the reagents (Westgard, 2008).In this study this was done and because the error margin was small then the samples were run in singles to minimize on the cost of the reagents. It is insufficient to assess repeatability in a single run however in this study because precision was done several times for different samples types and the errors margin was small that is why the samples were run in singles (Horowitz *et al*., 2010).

5.3Assessment of distribution of specific antimeasles IgG in HIV infected women.

Measles antibodies were detected in 145 out of 163 tested which is 89%.From the results 6% of samples were equivocal which is a significant in relation the 4% negative individuals. According to the manufacturer's requirement equivocal sample should be tested twice and if the results still test equivocal then one should consider the individual

potentially susceptible to primary infection but in-house tests giving equivocal results should not be reported but should be repeated. Testing equivocal results twice may not be feasible especially with financial constraints especially in a research and so in many studies researchers choose to ignore the equivocal results. In this study the equivocal results were not used in the final data analysis and conclusion of the findings. Equivocal Measles IgM or IgG results indicate that the testing performed cannot definitively determine whether a significant level of IgM/IgG antibody to measles virus was detected.

Studies done on equivocal results show that if one has completed 2 full shot series of MMR and titer remains negative, non-immune or equivocal one is considered a Non-Responder to that immunization. According to recent seroprevalence studies, over 90% of UK adults have measurable measles antibody by commercial ELISA tests(Atkinson, 1995). Such antibody tests are likely to be specific and therefore have a high positive predictive value in adult populations. Neutralisation assays performed by Centre for Infections (CfI) on samples from individuals who tested positive or equivocal for measles antibody on commercial assays have shown that all have detectable measles neutralisation antibody (data from CfI Virus Reference Department (VRD).As routine antibody tests lack sensitivity, a high proportion of those found to be antibody equivocal or negative are likely to be truly immune so the issue that comes up is whether to report equivocal results negative or positive. Therefore for older women with a reliable history of measles, antibody testing is unnecessary and should be a Continual monitoring of test result trends will also reveal problems due to inhibition.

If a laboratory gathers sufficient data (100 to 500 specimens) and inhibition rates are found to be within acceptable limits considering the medical implications of a false-negative result, testing for inhibition may be relaxed or discontinued (Pan American Health Organisation, 1994).Inhibition rates were not done hence this was one limitation of the study so further studies need to be done to establish this. To prevent competition

and avoid adverse reductions in sensitivity, the internal control should be set at the lowest concentration that permits consistent detection of the control (Atkinson, 1995). Either before or after the sample is prepared. If added before sample preparation, the internal control can also serve as an extraction control voided (Pan American Health Organisation, 1994).

New methods for setting positive- negative thresholds suggests that discriminate analysis is better than the commonly used ones that set arbitrary limits for determining positive- negative thresholds in ELISA tests.(Kramer *et al.,* 1983), if it is a new method for which clinical relevance and clinical use must be established, or if the analyte poses a health threat (such as *Mycobacterium tuberculosis*) and a false-positive or false-negative result would be of significant risk to the patient or general public. Predictive values of test results can be used when one wants to interpolate results. (OIE Terrestrial manual 2013).In conclusion equivocal measles results should not be assumed during final analysis of data because they could potentially affect the diagnosis and/or may lead to make inaccurate conclusions decisions of the research.

5.4.1 Determination of actual TIgG levels in HIV infected pregnant women

From the results there was significantly high titer (mean 22.74g/l) of TIgGs antibodies above the reference ranges (7-16g/l) in a normal population. Hypergammaglobulinemia and defective humoral immunity are hallmarks of HIV-1 infection. These findings are in line with other studies done(De Milito *et al*., 2004).This is due to reduction of memory B lymphocytes and hyperactivation of naïve B cells which present the source of abnormal IgG production(De milito *et al*., 2004).The hyperactivation status of naïve B cell is confirmed by the down regulated expression of LAIR-1,a diffentiation antigen that is gradually lost during terminal differentiation of B lymphocytes in plasma cells(Angelo *et al*.,2003)Another reason for hypergammaglobinaemia could be, decreased B-cell proliferative responses to T-cell-independent B-cell mitogens, and

qualitatively deficient helper T cells(Lane *et al.*,2005). After administration of ARVs there was a significant drop in the titers P– value - 0.068 to 17.11g/l meaning that there was immune reconstitution. The mechanism behind this needs to be investigated because it is contrary to a study done in Japan that suggested that HIV-1 infection affects peripheral CD27- (naive) B cells as well as CD27+ (memory) B cells and that CD27- B cells might be activated and rendered highly susceptible to apoptosis by HIV-1 infection and that some phenotypic alterations in CD27- B cells may continue after the reduction of HIV-1 loads by effective antiviral therapy (Chong *et al.,* 2004).Previous studies have suggested that the efficiency of transplacental transport of IgG is lower when the maternal levels are very high, whether for total immunoglobin G or for specific antibodies to measles or tetanus(Caceres *et al.,* 2000, Farquhar *et al.,* 2005). This means after administration of ARV`s there is likely would of not only improvement of the immune status of the mother but the same is likely to be transferred to the new born. This is because although there is active transportation of IgG across the placenta the number of cellular receptors of IgG limits transplacental antibody transfer (Kohler *et al*., 1996). Antigen specific antibodies like measles antibodies are preferentially transferred across the placenta than exists for TIgG and only a slight decrease intransplacental concentration efficiently across the range maternal antibody levels (Goncalves *et al*., 1998; Lennon *et al*., 1996). Raised maternal total serum IgG titres are associated with reduction of measles IgG transfer 87% as discovered by (De morae Pinto *et al.,* 1998) during a study done in Malawian population. The concern in this study is whether ARV`s cause a reduction that would cause an increase in the transfer of the antigens specific IgG`s (Michaux *et al.,* 1996) were the first to observe that the pattern of placental IgG may change when maternal total serum IgG titers reach15g/L. Cord total IgG titers tend to be lower than the corresponding maternal values. Further studies need to be done to compare the decrease of the maternal TIgG levels and the cord serum values.

5.4.2Determination of the specific anti-measles antibody levels in HIV infected pregnant women

Measles antibodies means were much higher(2412.1)before ARV`s administration which was higher than the protective value (≥ 320 mlu /ml) (Arpibol *et al.*,2006).Data suggested that average mean were higher and in agreement with other studies carried out on HIV infected women (De morae pinto *et al.,* 1998). Factors associated with high levels of measles antibodies could be low social economic status through increased levels of exposure to natural infection in overcrowded housing or increased infection in mothers through poor nutrition status although this could not be determined from the study. After administration of ARV`s there was a significant drop in the average titers (p- value 0.000) though the values we still higher in comparison to the protective value of ≥ 320 mlu /ml**.** A decrease of maternal measles antibody level may increase transplacental transfer; this is because Transplacental Transport Efficiency of MIgG decreases with increased maternal levels (Gon calves *etal.,* 1998).

Factors associated with low levels of measles antibodies in women include vaccine induced rather than the wild type (wt) measles virus), high parity and early gestation age of the foetus probably due to hemodilution several clinical conditions can interfere with the maternal fetal transport of specific IgG antibodies across the human placenta. These include prematurity maternal hypergammaglobinaemia maternal HIV infection and placental, malaria (de Morae *et al.,* 1998).These factors were also not determined in this study. In Africa the condition co-exists and the prevalence of infectious diseases preventable by vaccine are high among infant populations. Previous studies have reported that infants born with low level of maternal measles continue to have lower level over time compared to those who are born with higher levels (W.H.O, 1997, Christine *et al*., 1990). Furthermore a proportion of adults have shown negative results for measles antibodies. Mass measles vaccination campaigns targeting children less than 15 years old are being conducted throughout Africa with the support of consortium of

donors including the American Red Cross, CDC, UNAID. The reduction of measles through these campaigns will provide indirect protection to young infants in the short terms.

CHAPTER SIX
CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The actual dilution used in this study was $1:4x10^{5}$ which was low as compared to$1:8x10^{4}$ required by the manufacturer (Immunology Consultant Laboratory, Inc).

2. The precision of the sample dilution was constant for all samples in regardless of the type, geographical location, and source or HIV status of the patient.

3. Equivocal antimeasles IgG results were more than the negative results meaning they all needed to be repeated which is not visible in a research with constrained funding.

4. Maternal HIV infection is associated with reduced measles antibodies titers and hypergammaglobinaemia. This is of concern in the sub- Saharan African countries where up to 40% of women of child bearing age may be HIV infected and measles still causes much preventable mortality. The results of this study suggests that administration of ARV`s cause a significant drop of antibodies level.

The hypothesis was rejected because validated commercial TIgG ELISA kits need determination of the optimum sample dilution before use.

6.2 Limitations

There were financial constrains therefore the equivocal anti-measles ELISAs could not be repeated. The demographic information about the women could not be obtained and so the data from the women could not be interpreted further.

6.3 Recommendations

Based on this study, it is recommended that:

1. Every laboratory should determine the best dilution to use for quantitative TIgG assays
2. Further studies need to be done on how best to interpret equivocal results because they maybe more than the negative results and omitting them may lead to wrong conclusions about the study.
3. Further studies need to be done to assess whether the drop of the TIgG antibodies cause an increase in the antimeasles antibodies which are subsequently transferred to the infant to confer protection to the infant.
4. Further studies are required to determine the optimum age range for campaigns for long term measles reduction and the optimum age routine vaccination, especially in areas of high prevalence.

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APPENDICES

Appendix A: SSC Research Permit

KENYA MEDICAL RESEARCH INSTITUTE

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Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/9505 **15th June, 2011**

Alice Murugi

Thro'
Director, CVR
NAIROBI

DIRECTOR
CENTRE FOR VIRUS RESEARCH
P. O. Box 54628
NAIROBI

REF:SSC No.2047 (Revised) – Effects of exposure antiretroviral drugs on total IgG and measles antibodies in HIV infected pregnant women

Thank you for your letter dated 13th June, 2011 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval.

Sammy Njenga, PhD
SECRETARY, SSC

CENTRE FOR VIRUS RESEARCH
17 JUN 2011
P.O. Box 54628 - NAIROBI

Appendix B: ERC Research Permit

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **March 1, 2012**

TO: **LILLY ALICE MURUGI NJUE (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. FRED OKOTH,**
THE DIRECTOR, CVR,
NAIROBI

DIRECTOR
CENTRE FOR VIRUS RESEARCH
P. O. Box 54628
NAIROBI

Dear Madam,

RE: SSC PROTOCOL No. 2047 – 2ND REVISED (*RE-SUBMISSION*): EFFECTS OF EXPOSURE TO ANTIRETROVIRAL MEDICINES ON TOTAL AND ANTIMEASLES ANTIBODIES IN HIV INFECTED PREGNANT WOMEN

Reference is made to your letter dated February 21, 2012.

This is to inform you that the Committee determines that the issues raised at the 191st meeting of 12th July 2012 and on 6th February 2012 are adequately addressed. Consequently, the study is granted approval for implementation effective this **1st day of March 2012** for a period of one year.

Please note that authorization to conduct this study will automatically expire on **February 28, 2013**. If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **January 17, 2013**. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the ERC. You are also required to submit any proposed changes to this study to the SSC and ERC for review and approval prior to initiation and advise the ERC when the study is completed or discontinued.

Work on this project may begin.

Sincerely,

CHRISTINE WASUNNA,
Ag. SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

CENTRE FOR VIRUS RESEARCH
02 MAR 2012
P.O. Box 54628 - NAIROBI

Appendix C: QC variables for failing assays

New kit/ CTRL incubator									
					Reference Validation	Reference Validation		Reference Validation	
Sample	**OD Ag**	**OD CoAG**	**Δ Abs** *(OD AG-OD CoAg)*	**Avg ΔAbs**	**Margins**	**% change ref start and avg ref**	**Pass or fail**	**% change ref end and avg ref**	**Pass or fail**
Ref Start	0.73	0.015	0.715	0.7045	pass /pass	-1.5	pass/pass	1.5	pass/pass
Ref End	0.702	0.008	0.694	N/A	pass /pass	N/A	N/A	N/A	N/A
New kit/ UoN incubator									
					Reference Validation	Reference Validation		Reference Validation	
Sample	**OD Ag**	**OD CoAG**	**Δ Abs** *(OD AG-OD CoAg)*	**Avg ΔAbs**	**Margins**	**% change ref start and avg ref**	**Pass or fail**	**% change ref end and avg ref**	**Pass or fail**
Ref Start	0.784	0.004	0.78	0.744	pass /pass	-4.8	pass/pass	4.8	pass/pass
Ref End	0.716	0.008	0.708	N/A	pass /pass	N/A	N/A	N/A	N/A
					Reference Validation	Reference Validation		Reference Validation	
Sample	**OD Ag**	**OD CoAG**	**Δ Abs** *(OD AG-OD CoAg)*	**Avg ΔAbs**	**Margins**	**% change ref start and avg ref**	**Pass or fail**	**% change ref end and avg ref**	**Pass or fail**
Ref Start	0.005	0.021	-0.016	0.0955	pass/fail	116.8	fail/pass	-116.8	pass/fail
Ref End	0.214	0.007	0.207	N/A	pass/fail	N/A	N/A	N/A	N/A

Old kit/ CTRL incubator

					Reference Validation	Reference Validation		Reference Validation	
Sample	**OD Ag**	**OD CoAG**	**Δ Abs** *(OD AG-OD CoAg)*	**Avg ΔAbs**	**Margins**	**% change ref start and avg ref**	**Pass or fail**	**% change ref end and avg ref**	**Pass or fail**
Ref Start	0.005	0.021	-0.016	0.0955	pass/fail	116.8	fail/pass	-116.8	pass/fail
Ref End	0.214	0.007	0.207	N/A	pass/fail	N/A	N/A	N/A	N/A
Old kit/ UoN incubator									
					Reference Validation	Reference Validation		Reference Validation	
Sample	**OD Ag**	**OD CoAG**	**Δ Abs** *(OD AG-OD CoAg)*	**Avg ΔAbs**	**Margins**	**% change ref start and avg ref**	**Pass or fail**	**% change ref end and avg ref**	**Pass or fail**
Ref Start	0.238	0.009	0.229	0.224	pass/fail	-2.2	pass/pass	2.2	pass/pass
Ref End	0.229	0.01	0.219	N/A	pass/fail	N/A	N/A	N/A	N/A

Appendix D: Paired Sample Statistics for TIgG

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Adj.Res g/L beforeART	22.74595	164	14.292766	1.116077
	Adj.Res g/L after ART	17.11860	164	11.930848	0.931643

	Paired Differences						
	Mean antibody	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
			Lower	Upper			

	Paired Differences				t	df	Sig. (2-tailed)
	Mean antibody	Std. Error Mean	95% Confidence Interval of the Difference				
			Lower	Upper			
Adj.Res g/L before ART - Adj.Res g/L after ART	5.627354	1.196608	3.264501	7.990206	4.703	163	.000

Appendix E: Paired Sample Statistics for antimeasles IgG

	Mean antibody	**N**	**Std. Deviation**	**Std. Error Mean**

mIU_mLafter ART	2412.0774	163	1975.48410	1547.31856
mIU_mLbefore ART	2549.9480	163	1988.62713	1557.61298

				95% Confidence Interval of the Difference				
	Mean	Std. Deviation	Std. Error Mean	Lower	Upper	t	df	p-value
After ART – before ART	-137.87	443.3	34.72	-20.64	-69.30	-3.970	162	0.000