DEVELOPMENT OF ELISA AND LATERAL FLOW TEST KITS FOR HUMAN IMMUNODEFICIENCY VIRUS GUIDED BY THE PREVAILING CONSENSUS SEQUENCE OF HIV *ENV* GENE IN KENYA

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Development of ELISA and lateral flow test kits for Human Immunodeficiency Virus guided by the prevailing consensus sequence of HIV *Env* gene in Kenya

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DECLARATION

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

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This thesis has been submitted for examination with our approval as University supervisors

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DEDICATION

I dedicate this thesis to my wife, Margaret, my children Joy, Jesse and Mary-Christine, my mother, Teresia Waruthi, and my late grandmother, Isabella Warigia, for their inspiration and exceptional moral support.

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ABBREVIATIONS / ACRONYMNS

Ab	Antibodies
Ag	Antigen
AIS	AIDS indicator Survey
ANC	Antenatal clinic
ART	Antiretroviral therapy
ARV	Antiretroviral
bDNA	Branched chain Deoxyribonucleic acid
BED assay	A Recency assay that incorporates peptides from HIV-1 subtypes B, E and D into an IgG capture EIA
BSA	Bovine Serum Albumin
CAL	Calibrator
CBRD	Centre for Biotechnology Research and Development
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CDD	Conserved Domain Database
cDNA	Complementary Deoxyribonucleic acid
CI	Confidence Interval
CRFs	Circulating Recombinant Forms
CT	Cytoplasmic tail
CTLs	Cytotoxic-T-Lymphocytes

CVs	Correlation of variance
CXCR-4	C-X-C chemokine receptor type 4
DBS	Dried-blood spot
DDW	Double Diluted Water
DHS	Demographic and Health Survey
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ds-DNA	Double Stranded Deoxyribonucleic acid
D-SN	Diagnostic Sensitivity
D-SP	Diagnostic Specificity
ECDC	European Centre for Disease Prevention and Control
EIA	Enzyme Immuno Assay
ELISA	Enzyme-linked Immunosorbent Assay
***Env* gene**	*Envelop* gene
Fd	Fusion Domain
FRR	False-recent rate
Gag	Group-specific antigen
gp	Glycoprotein
GTR	Generalized time-reversible
HIV	Human Immunodeficiency Virus
HPC	High positive control

HR	Heptad repeat
HRP	Horse Radish Peroxidase
IDR	Immunodominant Region
IF	Indirect Immunofluorescence
Ig	Immunoglobulin
IPR	Intellectual Property Right
ITROMID	Institute of Tropical Medicine and Infectious Diseases
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KDHS	Kenya Demographic and Health Survey
KEMRI	Kenya Medical Research Institute
KHB	Shanghai Kehua Bio-engineering Co
LAg Avidity Assay	Limiting-Antigen Avidity Assay
LFA	Lateral Flow Assay
LFT	Lateral Flow Tests
LIAs	Line Immuno-assays
LLC	Limited Liability Company
LPC	Low Positive Control
MAFFT	Multiple Alignment using Fast Fourier Transform
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
MSF	***Médecins Sans Frontières***

NASBA	Nucleic acid sequence based amplification
NASCOP	National AIDS and STI Control Programme
NAT	Nucleic acid testing
NIAID	National Institute of Allergy and Infectious disease
NBTC	National Blood Transfusion Centers
NCBI	National Center for Biotechnology Information
NCST	National Council of Science and Technology
Nef	Negative regulatory factor
NP	Nano-particle
NPS	Nationally representative Population-based Surveys
NPV	Negative Predictive Value
NSI virus	Non-syncytium inducing Virus
OD	Optical Density
ODn	Normalized Optical Density
OIC-HIV	Orgenics Immunocomb Combfirm HIV confirmation test
PATH	Partner for Appropriate Technology in Health
PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline + Tween
PCR	Polymerase Chain Reaction
PEP	Post exposure prophylaxis
Pg	Pico gram

pH	Logarithmic measure of ***hydrogen ion concentration***
Phyre^2	Protein Homology/analogy Recognition Engine V 2.0
PoC diagnostics	Point-of-care diagnostics
Pol	Polymerase
PPV	Positive Predictive Value
PrEP	Pre-exposure prophylaxis
RBTC	Regional Blood Transfusion Centers
RDT	Rapid Diagnostic Test
Rev	Regulator of Virion Expression
RIBA	Recombinant Immunoblot Assay
r-IDR	Recombinant Immunodominant Region
RLS	Resource-limited settings
RNA	Ribonucleic Acid
ROC	Receiver operating characteristic
rpm	Revolutions per minute
RT	Reverse Transcriptase enzyme
SI Virus	Syncytium-inducing Virus
ssRNA-RT Virus	Single Stranded Ribonucleic Acid Reverse Transcribing Virus
STARHS	Serologic testing algorithm for recent HIV seroconversion
STI	Sexually transmitted infections
Tat	Trans-Activator of Transcription

T_h lymphocyte	T-helper lymphocyte
™	Trade mark
TM	Transmembrane
TMA	Transcription Mediated Amplification
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
UNAIDS	United Nations Programme on HIV/AIDS
VCT	***Voluntary Counseling and Testing***
Vif	Viral infectivity factor
vmRNA	viral messenger RNA
Vpr	Viral Protein R
Vpu	Viral Protein U
Vpx	Viral protein X
WB	Western Blot
WHO	World Health Organization
WTWG-HIAM	WHO Technical Working Group on HIV Incidence Assays Meeting

ABSTRACT

The *Env* gene of Human Immunodeficiency Virus (HIV) is vulnerable to mutations that could contribute to variability of diagnostic sensitivity of antibody-based HIV diagnostic tests. Previous studies established that some HIV Enzyme-linked Immunosorbent Assay (ELISA) kits displayed different diagnostic sensitivities when they were used to test blood samples from various geographical locations. Currently, the most widely used testing platforms for HIV in Kenya are the ELISA and Lateral Flow tests (LFTs) which are coated with peptides that have been developed using global consensus sequence of HIV *Env* gp41 Immunodominant Region (IDR) (and sometimes gp120) that may theoretically not be able to detect some local HIV strains.

This study was designed to develop and evaluate LFT and ELISA test kits for HIV 1/2 based on the prevailing consensus sequence of HIV *Env* gene in Kenya.

The study was a Laboratory-Based Experimental Design that involved collection of 200 HIV positive and a similar number of HIV negative blood samples from the four Regional Blood Transfusion Centers (RBTCs) in Kenya over a period of eighteen months. From the HIV positive samples, ribonucleic acid (RNA) was extracted, reverse transcribed and then amplified. The resultant cDNA was sent to Macrogen Europe (Amsterdam, Netherlands) for sequencing. The Consensus sequence of HIV *Env* gp41 IDR gene established in this study and the global consensus of HIV *Env* gp41 IDR were sent to LifeTein LLC (Hillsborough, NJ) for production of corresponding bulk peptides. These peptides were used with other commercially available reagents, through a series of optimization experiments, to produce ELISA and LFTs which were evaluated using the 400 characterized HIV panels prepared in this study. The study was approved by KEMRI Ethical Review Committee. Blood samples collected from the NBTCs concealed the identities of the donors. The data was collected and entered in the Excel software. Sequences were analyzed using various bioinformatics' tools that included Shannon-Two Entropy, Los Alamos PhyML 3.0 phylogenetic tree software, "RIP" software for ddetermination of HIV sub-types and recombinants and Phyre^2 software for peptide structure prediction, among others.

This study established the prevailing consensus sequence of *Env* gp41 IDR gene in Kenya and also developed two HIV testing kits (LFT and ELISA) using Consensus HIV *Env* gp41 IDR peptide from this gene. There was 100% sequence similarity in the

Cysteine Loop and CTL epitope of HIV *Env* gp41 IDR peptides between Kenya and global consensus peptide sequences. In general, 67.4% of the amino acid positions in Consensus HIV *Env* gp41 IDR peptide (Kenya) were highly conserved with the Entropy value of below 0.25 (baseline Entropy). Of the 91 samples that were sequenced the sub-type distribution of HIV was A1 (76.9%), C (6.6%), D (14.3%) and CRF A2.CY.94CY017_41 (2.2%). Samples that were tested in this study consisted of 31.9% "recent" HIV infections. In respect to Vironostika™ Uni-Form II Ag/Ab ELISA the developed LFT had a diagnostic sensitivity (D-SN) of 95.2% (95% CI: 90.3-98.0%) which was the same as that of Aware™ HIV-1/2 BSP LFT and close to the two kits that are currently in the National HIV testing Algorithm in Kenya: First Response™1-2.0 (95.9%, 95% CI: 91.2-97.4%) and KHB Colloidal Gold (95.9%, 95% CI: 91.2-98.5%). The developed LFT had a higher D-SN than the "Tie Breaker" kit in the Algorithm, Uni-Gold™ HIV Test (93.8%, 95% CI: 88.5-97.1). The ELISA test kit developed in this study had a D-SN of 97.2% (95% CI: 93.1-99.2%). The study also established that the overall D-SN of using LFTs in HIV testing in Kenya was 96.0% (95% CI: 92.3-98.3%) in respect to Vironostika™ Uni-Form II Ag/Ab ELISA and that among the LFTs, Determine™ HIV-1/2 showed the highest values of both D-SN and analytical sensitivity, 96.6% (95% CI: 92.2-98.9%) and 63pg/ml respectively, making it the LFT that would have been the most preferred choice of a Screening test in the National HIV testing Algorithm in Kenya were not for its being 10-16% more expensive than the current kits in this Algorithm. This study also established that although there were some variations of the HIV *Env* gp41 IDR peptide in Kenya in comparison with global consensus of the same, there were no differences in the performance of the kits developed using Consensus HIV *Env* gp41 r-IDR (Kenya) and Consensus HIV *Env* gp41 r-IDR (Global) peptides. The study also established that the analytical sensitivity of Determine™ HIV-1/2 Ag/Ab Combo and Vironostika™ Uni-Form II Ag/Ab ELISA in detection of p24 antigen were the same at 63pg/ml.

This study recommends the following: return of Determine™ HIV-1/2 to the National HIV Testing Algorithm due to its superior performance, regular monitor of the effectiveness of the HIV testing kits in Kenya for the possible emergence of HIV variants that could escape detection by these kits and seeking of more funds to support the commercialization of the prototype HIV kits developed in this study.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Globally more than 35 million people are living with HIV/AIDS with the global incidence being estimated at 3 million (UNAIDS, 2013). In Kenya, the Ministry of Health estimates the HIV prevalence of 6.0%, incidence of 0.44% and 1.6 million of people living with AIDS, of whom 191,840 are children (NASCOP, 2014). According to Kimani *et al* (2011) the HIV prevalence among voluntary donors in Kenya is estimated at 2.6% as compared with 7·4% of family replacement donors (family-sought donors).

The strains of HIV-1 are classified into four groups: the "major" group M; the "outlier" group O and two new groups, N and P (Plantier, 2009). Group M is further sub-divided into nine genetically distinct clades: A, B, C, D, E, F, G, H, J and K. Different HIV subtypes are also known to produce new hybrid virus by recombination of genomic RNA to produce Circulating Recombinant Forms (CRFs) (Bobkov *et al.*, 2004). The nucleotide variation within the *Env* gene is 5-15% intra-clade and 15-30% inter-clades (Leitner, 1996). This possibly explains the observation by Apetrei *et al.* (1996) of reduced screening test sensitivity during HIV-1 non-subtype B seroconversions. In Kenya 70.9% of HIV infections are caused by Clade A while 11.5% of infections are caused by Clade D as compared to Clade B in Europe which constitutes the majority of HIV infections (77.9%) with Clade A constituting only 3.8% (Los Alamos database, 2015).

The HIV is characterized by high rates of mutations of about 10^{-4} mutations per nucleotide that has been found to produce an estimated rate of mutation within the *Env* gene of 1% per year (Ho *et al.,* 1995). If these changes occur within Immunodominant region (IDR) of the *Env* gp41 peptide recognition by neutralizing antibodies can be reduced (Hutchinson; 2009). Studies carried out by Lihana *et al* (2009) showed a changing trend of HIV 1 subtypes in Nairobi where subtype A1/A1 was the most predominant at 65%. Other studies have established that some HIV Enzyme-linked Immunosorbent Assay (ELISA) kits showed different performance characteristics when tested using blood samples from various geographical locations (Thorstensson *et al.,* 1998). HIV-specific immune responses are usually measured using peptide sets based on consensus sequences, which frequently miss responses to regions where test set and infecting virus differ (Bordería *et al.,* 2010). Other studies have shown that HIV 1 group "O" antigens elicit antibodies that show either low reactivity or no reactivity at all with different ELISA recombinant assays. To overcome this challenge, HIV 1 group "O" antigens are nowadays included in design of HIV ELISA kits (van Binsbergen *et al.,* 1996).

1.2 Problem Statement

Effective immuno-assays for detection of HIV infection should be able to detect HIV infection irrespective of geographical location and related HIV sub-type distribution. Currently, the most widely used testing platforms for HIV in Kenya are the ELISA and LFTs which use peptides that have been developed using global consensus sequences of HIV *Env* gp41 IDR peptide. Various studies have demonstrated that HIV kits that have been developed using global consensus sequences of HIV *Env* gp41 IDR peptide

may not be able to detect some HIV strains (Thorstensson *et al.,* 1998; Wei *et al.,* 2010 and Kilembe *et al.,* 2012). However, some studies have demonstrated contrary performances showing that various HIV testing kits are not affected by the prevailing subtypes of HIV infections in a specific region (Masciotra *et al.,* 2000; Vallari *et al.,* 2010). This study is aimed at establishing whether the HIV ELISA and LFT developed using consensus sequences of HIV *Env* gp41 IDR peptide from Kenya HIV infection could perform differently in terms of Sensitivity and Specificity from the HIV ELISAs and LFTs that are currently in use in Kenya.

1.3 Justification of the Study

The need to develop ELISA and LFT for testing for HIV guided by the prevailing consensus sequence of HIV *Env* gene in Kenya arose from the fact that HIV shows high mutation rate (10^{-4} mutations per nucleotide per cycle) and substantial heterogeneity in types, groups, clades, Circulating Recombinant Forms (CRFs) and single mutants (Plantier, 2009) that could cause different HIV testing sensitivity patterns of HIV screening tests. Since HIV testing ELISAs and LFTs use peptides that have been developed using global consensus sequences of HIV *Env* gp41 IDR gene a potential existed for failure of these kits to detect some HIV strains in Kenya with adverse possibility of missing some HIV positive cases. The fear for such possibility was enhanced by previous studies that had demonstrated that the performances of various HIV testing kits were affected by the prevailing subtypes of HIV infections in a specific region (Thorstensson *et al.,* 1998; Wei *et al.,* 2010 and Kilembe *et al.,* 2012). Wei *et al.* (2010) found that HIV immunoassays developed using individual *Env* gp41 IDR peptides derived from respective subtypes failed to detect some infections by

other subtypes. When Thorstensson *et al.* (1998) compared the performance of 14 commercially available HIV-1/2 antibody assays from patient sera from Tanzania, Sweden and Guinea-Bissau it was found that 85.7% (n = 12) identified correctly all HIV-1 and HIV-2 antibody positive sera and that one Tanzanian HIV-1 antibody positive sample was not detected by two of the ELISAs employing synthetic peptides. Thorstensson *et al.* (1998) also showed a high specificity (99.2 to 100%) of these assays when they were used for analysis of Swedish blood donor sera while most of the assays showed a significantly lower specificity (91.9-99.6%) when used for testing African specimens. In Zambia and Rwanda a study by Kilembe *et al.* (2012) found that the new rapid antigen and antibody test to detect antigen-positive HIV infection could only detect less than 2% p24 antigen positive samples with analytical sensitivity of 248.3pg/mL (in comparison with 12.5pg/ml to 25pg/mL that was found with European samples). It is a well-known fact the extensive diversity of HIV *Env* glycoprotein poses a challenge to vaccine design as conventional vaccines have to protect against one or a limited number of strains. There is an existing notion of designing of region-specific vaccines, which will be required to protect populations infected by a specific viral strains circulating in discrete geographic areas (Salemi, 2011).

However, some studies had demonstrated that the performances of various HIV immunoassays were not affected by the prevailing subtypes of HIV infections in a specific region (Masciotra *et al.,* 2000; Vallari *et al.,* 2010). Masciotra *et al.,* (2000) had established that consensus HIV *Env* gp41 from group M peptides was able to detect all 130 group M sera (10 subtype A, 21 subtype B, 13 subtype B9, 20 subtype C, 21 subtype D, 14 subtype E, 25 subtype F, and 6 subtype G) resulting in a test

sensitivity of 100%. Vallari *et al.* (2010) noted that despite the high genetic divergence between HIV-1 groups M and N, all group N infections were detected using five commercial HIV immunoassays.

This study was therefore necessary to ascertain whether the HIV 1 and 2 ELISA and LFTs developed using prevailing consensus sequence of HIV *Env* gp41 IDR peptide in Kenya could be different in terms of Sensitivity and Specificity from that of other HIV immunoassays in the Kenyan market that were using global consensus sequence of HIV *Env* gp41 IDR peptide.

1.4 Hypothesis

Null Hypothesis

The Sensitivity and Specificity of HIV-1 and 2 ELISA and LFT developed using prevailing consensus sequence of HIV *Env* gp41 IDR peptide in Kenya are **NOT** different from that of other HIV immunoassays in the Kenyan market that use consensus Global sequence of HIV *Env* gp41 IDR peptide.

Alternative Hypothesis

The Sensitivity and Specificity of HIV-1 and 2 ELISA and LFT developed using prevailing consensus sequence of HIV *Env* gp41 IDR peptide in Kenya are different from that of other HIV immunoassays in the Kenyan market that use consensus Global sequence of HIV *Env* gp41 IDR peptide.

1.5 Objectives

1.5.1 General Objective

To develop ELISA and LFT for HIV guided by the prevailing consensus sequence of HIV *Env* gene in Kenya

1.5.2 Specific Objectives

1. To determine the prevailing consensus sequence of HIV *Env* gp41-IDR gene in Kenya
2. To produce Consensus HIV *Env* gp41-IDR peptide from the prevailing consensus sequence of HIV *Env* gp41-IDR gene in Kenya

3. To determine the proportion of the recent HIV infections among blood donors in Kenya using the developed HIV immunoassays

4. To develop HIV 1 & 2 ELISA and LFT prototypes using the generated Consensus HIV *Env* gp41-IDR peptides.

5. To evaluate the performance of the developed HIV 1 & 2 ELISA and LFT kits.

1.6 Ethical Considerations

The study was approved by KEMRI Ethical Review Committee (SCC Protocol no. 2170 dated 27th April 2012, *Appendix 9*). Blood samples collected from the NBTCs concealed the identities of the donors.

1.7 Limitations and assumptions of the study

The evaluations and designs of the developed HIV immunoassays considered testing of the plasma matrix only due to the limitations of funds to involve other matrices like saliva and whole blood. The source of samples in this study was donor's blood from RBTCs that had been rejected for being either overweight, underweight or infected. Since the donors' blood is treated with anti-coagulant, the plasma was obtained after centrifugation of the blood units. Otherwise, collection of other matrices of samples such whole blood unit and saliva would have made it mandatory to recruit the study subjects which would have made the study to be comparatively more expensive and time consuming. Generally, the results of tests that are carried out using plasma are usually replicated in other matrices albeit with minor modifications.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of HIV epidemic in Kenya

Globally more than 35 million people are living with HIV/AIDS with the global incidence being estimated at 3 million (UNAIDS, 2013). In Kenya, the Ministry of Health estimates the HIV prevalence of 6.0% with 1.6 million of people living with AIDS, of whom 191,840 are children (*Table 2-1)*. The HIV prevalence among young people aged 15-24 years is 2.2% (male, 1.7%; female, 2.7%) (NASCOP, 2014).

Table 2.1 Kenya National HIV estimates for 2013 (NASCOP, 2014)

Indicator	Value	Range
Number living with HIV	1.6 million	1.5 – 1.7 million
Prevalence 15+	6%	5.6 – 6.6%
New adult infections	89,000	69,000 – 110,000
New child infections	12,900	9,300 – 17,000
Annual AIDS deaths	58,000	49,000 – 72,000
Need for ART: adults	760,000	730,000 – 800,000
Need for ART: children	140,000	120,000 – 160,000
Mothers needing PMTCT	79,000	69,000 – 90,000

In 2013 HIV incidence in Kenya was 0.44% among the adults and it has been projected to stabilize at high rate of about 90,000 cases per year *(Figure 2.1)* (NASCOP, 2014).

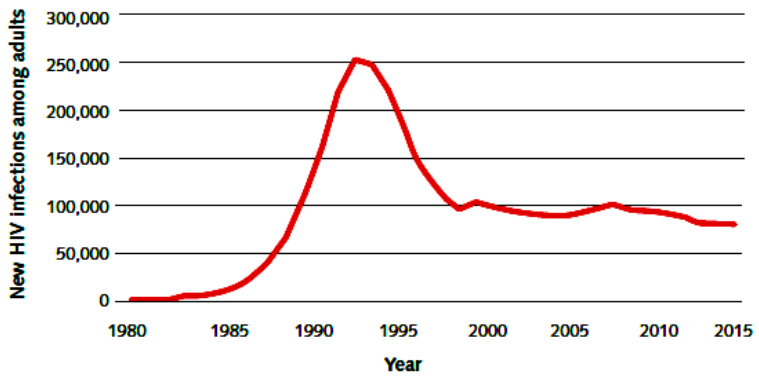


Figure 2.1 Estimated new HIV infections among adults (NASCOP, 2014)

According to Kimani *et al* (2011) the HIV prevalence among voluntary donors in Kenya is estimated at 2.6% as compared with 7·4% of family replacement donors.

2.2 HIV Infection and Prevention

HIV-1 belongs to viral *Group VI* (ssRNA-RT), family of *Retroviridae* and genera of *lentivirus*. There are two types of HIV: HIV-1 and HIV-2 (Korber, B.; Hahn, B. & Foley, B., 1997). Infection with HIV is followed by an acute flu-like illness that may remain unnoticed and it may not be associated with HIV infection. This is followed by an asymptomatic carrier state, which progresses to clinical AIDS in about 50% of infected individuals within 10 years after seroconversion. HIV p24 antigens can be detected during both acute phase and the symptomatic phase of infection while antibodies to HIV-1 and/or HIV-2 can be detected throughout the infection period, starting at, or shortly after the acute phase and lasting till the end stage of AIDS (Osmond, 1998).

Interventions for primary prevention of HIV/AIDS include the following; circumcision which is effective at preventing up to 60% of female-to-male transmissions (Bailey *et al.*, 2007), vaccine with an efficacy of about 30% (Rerks-Ngarm *et al.*, 2009),

microbicide with an efficacy of about 39% (Abdool-Karim *et al.*, 2010), physical barriers with effectiveness of up to 95% when used consistently (Pinkerton & Abramson, 1997) and pre-exposure prophylactics (PrEP) with efficacy of up to 44% in Men having Sex with Men (Grant *et al.*, 2010). Treatment with antiretrovirals (ART) can act as preventive measure too. In the test-and-treat approach, universal testing is carried out and cases which are positive for HIV put on ART to minimize their infectiousness to others (Simon & Abdool Karim, 2006). Post exposure prophylaxis (PEP) has demonstrated the rate of effectiveness higher than 80% (Albirt *et al.*, 2013).

2.3. Structure of the Human Immunodeficiency Virus

HIV is an icosahedral-shaped virus with a diameter of 80-120nm. It has two copies of single-stranded RNA enclosed by a conical capsid consisting of the viral protein p24, typical of lentiviruses *(Figure 2.2)*. This is in turn surrounded by a plasma membrane of host-cell origin. The single-strand RNA is tightly bound to the nucleocapsid proteins (p7) and reverse transcriptase and integrase enzyme.

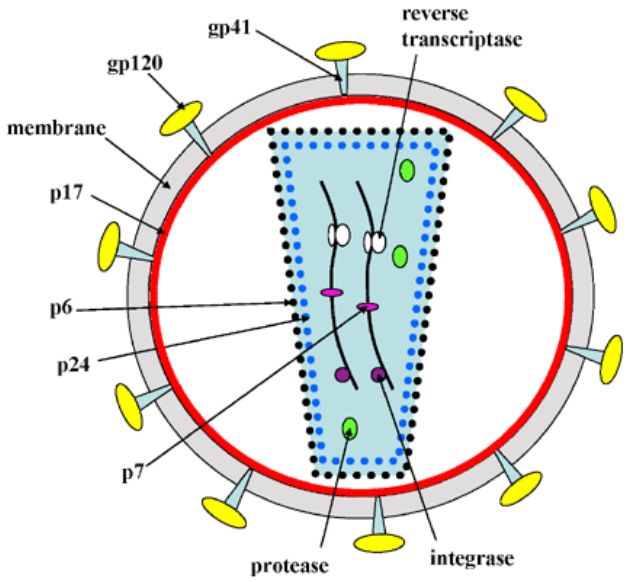


Figure 2.2 Simplified structure of HIV (Cleghorn, F.R.; Reitz, M.S.; Popovic, M. & Gallo, R.C.., 2005)

The nucleocapsid proteins associate with the genomic RNA (one molecule per hexamer) and protect the RNA from digestion by nucleases. A matrix composed of an association of the viral protein p17 surrounds the capsid, ensuring the integrity of the virion particle. Also enclosed within the virion particle are Vif, Vpr, Nef, p7 and viral protease. The envelope is formed when the capsid buds from the host cell, taking some of the host-cell membrane with it. The envelope includes the glycoproteins gp120 and gp41. The gp 120 binds to host cells that bear CD4 core-receptors of lymphocytes and monocytes (Cleghorn, F.R.; Reitz, M.S.; Popovic, M. & Gallo, R.C., 2005; Kartekayan, S.; Bharmal, R.N.; Tiwari, R. P. & Bisen, P.S., 1998).

According to Frey *et al.* (2008) various segments of gp120 and gp41 are designated as, conserved regions 1–5 (C1–C5); variable regions 1–5 (V1–V5); fusion peptide (F); heptad repeat 1 (HR1); immunodominant loop with a conserved disulfide bond (C–C loop); heptad repeat 2 (HR2); transmembrane anchor (TM) and cytoplasmic tail (CT). Expression constructs are *(Figure 2.3)*: gp140, uncleaved ectodomain of gp160 with a C-terminal His tag; gp140-Fd, uncleaved ectodomain of gp160 with a trimerization tag and a C-terminal His tag; gp41-inter, gp41 in the prehairpin intermediate conformation trapped by an N-terminal HR2 peptide and a C-terminal foldon tag; gp41-post, gp41 in the six-helix conformation with partial MPER. The sequence from N- to C-terminal of the ECTO: the fusion peptide (FP), together with the N-terminal heptad repeat (NHR, or HR1), and the CHR (or HR2) are the three important functional regions closely related to fusion activity (Gallaher, W. R.; Ball, J. M. & Garry, R. F., 1989; Oldstone *et al.*1991).

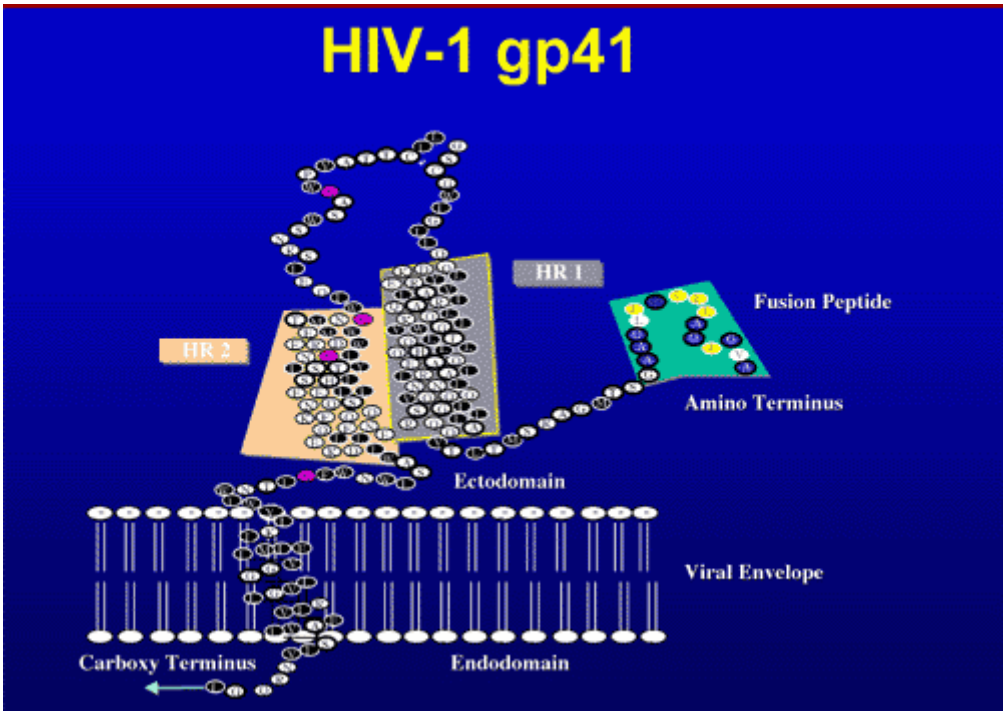


Figure 2.3. A fusion-intermediate state of HIV-1 gp41 targeted by broadly neutralizing antibodies (Gallaher, W. R.; Ball, J. M. & Garry, R. F., 1989)

2.4 HIV Genome

The HIV RNA, which is approximately 9749 nucleotides long, is mapped as shown in *Figure 2.4* below:

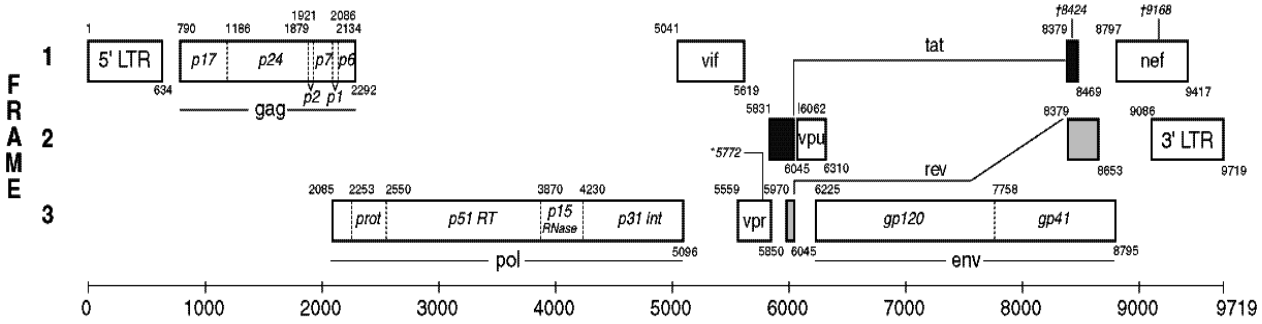


Figure 2.4 HIV genome (*http://www.hiv.lanl.gov/content/index, 2014)*

The positions of various genes within the HIV genome are shown and they include: gag polyprotein, pol, *Env* and transactivators' genes

The HIV genome has various genes. The group-specific antigen gene (gag) codes for the gag polyprotein, which is processed during maturation to matrix protein (p17), capsid protein (p24), spacer peptide 1(p2), nucleocapsid protein (p7), spacer peptide 2 (p1) and p6. Pol gene codes for viral enzymes reverse transcriptase, integrase, and HIV protease. The *Env* gene codes for gp160, the precursor to gp120 and gp41, proteins embedded in the viral envelope, which enable the virus to attach to and fuse with target cells. Transactivators include Trans-Activator of Transcription (tat), Regulator of Virion Expression (rev), Viral Protein R (vpr), viral infectivity factor (vif), Negative regulatory factor (nef) and Viral Protein U (vpu) (Kartekayan, S.; Bharmal, R.N.; Tiwari, R. P. & Bisen, P.S. , 1998) *(Figure 2.4)*.

2.5 HIV Replication

As illustrated by Evering and Markowitz (2008), National Institute of Allergy and Infectious diseases (2012) and Weiss *et al.* (2004) the replication of HIV occurs as described in *Figure 2.5.*

Successful HIV-1 replication requires the use of 3 enzymes: reverse transcriptase, integrase, and protease. The viral gp120 binds to CD4 molecule on T_h lymphocyte, macrophages, and/or microglial cells. Heparan sulfate proteoglycan stabilizes the interaction of the gp120 with CD4 antigen. This triggers a conformational change on cell envelope allowing binding of the C-C chemokine receptor type 5 (CCR5 or CD195) or C-X-C chemokine receptor type 4 (CXCR-4, or fusin or CD184). This further stabilizes the interaction and eventually the virus fusion protein (part of gp41) is

uncovered. The *Env* gp41 undergoes a conformational change and inserts itself into the membrane of the host cell to initiate the fusion of the two bilayers. This attachment and fusion process allows the HIV viral core to enter the host-cell.

Reverse transcriptase enzyme transcribes viral RNA into double-stranded DNA (dsDNA), which is then integrated, via the action of the integrase enzyme into the host-cell genome as described by Weiss *et al.*, (2004). Briefly, the viral reverse transcriptase synthesizes linear double-stranded cDNA, which is the template for the viral enzyme integrase. Integrase assembles in a stable complex with viral DNA - the pre-integration complex -and is chaperoned into the nucleus. Integration of HIV cDNA into the host genome is a two-step process catalyzed by the HIV-1 integrase enzyme. Initially, 2 nucleotides are excised from the 3´ ends of the nascent HIV-1 DNA. This is followed by the irreversible, covalent insertion of HIV-1 viral genomic DNA into the host chromosome. While the HIV-1 virus is known to preferentially target sites within transcribed host genes for integration - so called “hot spots”- the factors underlying these preferences are not entirely clear. The viral integrated dsDNA or 'provirus' then acts as a template for viral genomic and messenger RNA transcription by the host cell's nucleic acid replicating machinery.

Integration of the linear provirus dsDNA into the genome of the host-cell establishes an infection that lasts for the lifespan of the cell, and all its progeny, which usually means life-long infection for the host. During cellular replication, the provirus is transcribed by the host-cell RNA polymerase II enzyme, and the viral messenger RNA (vmRNA) and genomic RNA, are carried with the cellular mRNAs, to be translated into proteins. This vmRNA codes for a gag-pol precursor polypeptide that is ultimately cleaved by the viral-encoded protease enzyme to produce the gag and pol viral proteins. In addition, the

vmRNA is also spliced to produce other vmRNAs coding for the viral proteins transactivator of transcription *(tat)*, regulator of viral protein expression *(rev)*, negative regulatory factor *(nef)*, protein found in HIV-1 but not HIV-2 viral protein U *(vpu)* and protein found in HIV-2 and not HIV-1 viral protein X(vpx). Eventually, the env precursor polypeptide is cleaved by cellular (not viral) proteases, producing the envelope glycoproteins gp41 and gp120. These viral proteins, together with the replicated diploid viral genomic RNA, are assembled and enveloped by budding through the host-cell membrane, producing complete HIV virions.

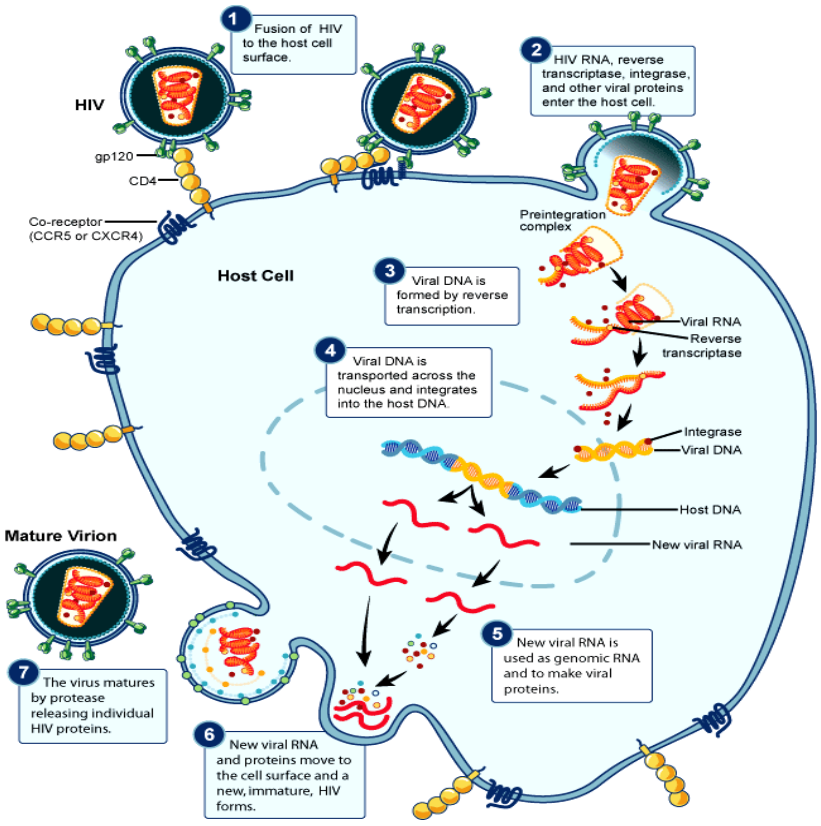


Figure 2.5. Diagrammatic illustration of Replication of HIV *(NIAID, 2012)*

It is worth to note that the CCR5 co-receptor are carried on macrophages, hence HIV strains requiring the CCR5 co-receptor for entry are referred to as 'macrophage-tropic' or R5 or non-syncytium inducing (NSI) although they also infect lymphocytes. Primary

HIV-1 infections tend to involve this R5 NSI macrophage-tropic phenotype. Sometimes individuals may have a homozygous deletion mutation in the CCR5 gene (CCR5Δ32) resulting in the absence of the CCR5 molecule on their macrophages. Therefore, these individuals cannot be infected by this R5 phenotype. The 'lymphotrophic' or X4 or syncytium-inducing, (SI) HIV strains use CXCR4 as the co-receptor. X4 viruses tend to appear later in about 50% of HIV-1 subtype B-infected individuals, but seldom with other subtypes, as they progress to AIDS (Weiss *et al.*, 2004).

2.6 HIV Types, Groups and Subtypes

There are two types of HIV, namely HIV-1 and HIV-2 (Korber, B.; Hahn, B. & Foley, B., 1997). Both viruses have similar morphology and lymphotropism, and the modes of transmission appear to be identical (Schim van der Loeff & Aaby, 1999). The HIV-1 and HIV-2 genomes exhibit about 60% homology in conserved genes such as gag and pol, and 39 - 45% homology in the *Env* genes (Guyader, M.; Emerman, M. & Sonigo, P., 1987). In terms of classification, HIV-2 strains have been classified into at least five subtypes (A through E). HIV 1 is more virulent and has shorter incubation period (Korber *et al.,* 1997). The strains of HIV-1 can be classified into four groups: the "major" group M (more than 90% of all HIV) the "Outlier" group “O” and two new groups, N and P (Korber *et al.,* 1997; Plantier, 2009). Group “O” distribution appears to be limited to West-Central Africa and group N strain, which was discovered in 1998 in Cameroon, is quite uncommon (Plantier, 2009). Group M is further divided into at least nine genetically distinct subtypes (or clades): A (predominant in West, Central Africa and Russia), B (predominant in Europe, the Americas, Japan and Australia), C (predominant in Southern and East Africa, India and Nepal, responsible for 50% of all global infections), D (predominant in East and Central Africa), F (predominant in Central Africa, South America and Eastern Europe), G (West and East Africa and

Central Europe), H (Central Africa), J (Central America) and K (Democratic Republic of Congo and Cameroon). Sometimes different HIV subtypes can be produced by recombination of genomic RNA to produce new hybrid virus. When an inter-subtype recombinant is transmitted between multiple individuals, i.e., has the potential to be of epidemiological significance, it is termed a Circulating Recombinant Form (CRF). As with the subtypes, these form distinct clusters in phylogenetic trees and some (CRF01 and 02 in particular) contribute disproportionately to the pandemic, as do certain subtypes (particularly C). Most recombinants though, do not mostly survive for long (Bobkov *et al.,* 2004; Quinones-Mateu & Arts, 1999). The nucleotide variations within the *Env* gene are 5% to 15% intra-clade and 15% to 30% inter-clades (Leitner, 1996). It is sometimes necessary to sequence *Env* gene as well as *gag* or *pol* genes in order to determine CRF (Quinones-Mateu & Arts, 1999).

2.7 HIV-1 Diversity and Its Implications in Diagnosis

The HIV is characterized by high rates of genetic variability *in vivo* due to rapid viral turnover in a patient with an active disease (about 10^{10} viral particles per day) and lack of proof-reading mechanisms during reverse transcription that produces high mutation rates of about 10^{-4} mutations per nucleotide (Ho *et al.,* 1995). Mutation rates and selection rates vary for different components of the HIV genome with *gag* and *pol* genes being less variable than the *Env* gene. There are five hypervariable regions, V1 to V5 within the *Env* gene. The V3 region, about 30 amino acids within the envelope protein gp120, is highly mutable, and changes of one amino acid in this region can restrict recognition by neutralizing antibodies. It has been estimated that the rate of mutation within the *Env* gene per year is 1%. This mutation rate allows the virus to evade host immune responses (Hutchinson, 2009).

Different HIV strains sometimes recombine to produce large genetic alterations that could, at times, produce genetic shifts (Temin, 1993). HIV subtype recombinations are known to yield heterogeneous viral populations, which could represent a large population jumps than what is known to occur in the nucleotide substitutions (Holland, 1994).

The *Env* gene encode for *Env* gp160 that is enzymatically cleaved into two peptides *Env* gp120 and *Env* gp41. The *Env* gp41 contains the immunodominant region (IDR) in the amino-terminal portion and more than 99% of HIV-1infected individuals produce antibodies directed to this region. The IDR of gp41 contains the following sub-regions: the CTL epitope (aa 591 to 602; AVERYLKDQQLL); the Cysteine Loop (aa 607 to 613; CSGKLIC) and ectodomain region (aa 671 to 676; ELDKWA) (13, 14). However, *Env* gp41 peptide fragment without the ectodomain region has been found to still detect more than 99% of HIV infected cases (Masciotra *et al.*, 2000). Mutations in the CTL epitope and Cysteine Loop have been shown to reduce or remove the capacity of Immunoassays to detect HIV infections. Dorn *et al.* (2000) reported a K597→R substitution that within the CTL epitope had been shown to abolish CTL recognition. An L611→H substitution within the cysteine loop has also been known to affect antibody reactivity (Horal *et al.*, 1991).

2.8 Immune Response after HIV Infection

Approximately 50% of infected individuals in primary HIV infection are symptomatic with fever and lymphadenopathy. After seroconversion there follows an asymptomatic period of 2-15 years. During this period, viral replication continues at a high rate of up to 10^{10} infectious virions/day, leading to approximately 10^{8}-10^{9} lymphocytes/day being

infected, which are replaced almost as quickly. HIV-infected lymphocytes are depleted through direct cytopathic effect of HIV (including the formation of syncytia by SI X4 HIV lymphotropic strains), immune destruction of HIV-infected cells by cytotoxic CD8 T lymphocytes that recognize HIV antigens presented on major histocompatibility complex (MHC) molecules and apoptosis due to lymphocyte activation in the presence of specific cytokines (Osmond, 1998; Tang & Chan, 2007).

In the absence of antiretroviral therapy, the natural course of the disease is generally as shown in *Figure 2.6*. The CD4 lymphocyte count drops rapidly for a short period, before recovering to almost normal levels. This is followed by the asymptomatic phase when the viruses evolve into a more heterogeneous population. Over this period, which may last between 2-15 years, there is a steady decline in the CD4 count. As the patient becomes more symptomatic, and the lymphotropic X4 viruses begin to predominate, the CD4 count drops even more quickly as the patient approaches end-stage disease. The degree of immune activation increases in a reciprocal relationship to the drop in CD4 count. The viral load and p24 antigen (Ag) levels initially peak during primary HIV infection, then decline to a 'set-point' during the early asymptomatic phase, the actual level depending on the degree of the immune response. There is then a gradual rise in viral load and p24 Ag, after about 2 years from initial infection, which becomes accelerated during the symptomatic phase. The levels of both the CD4 count and viral load in the early asymptomatic phase are highly predictive of disease progression (Osmond 1998).

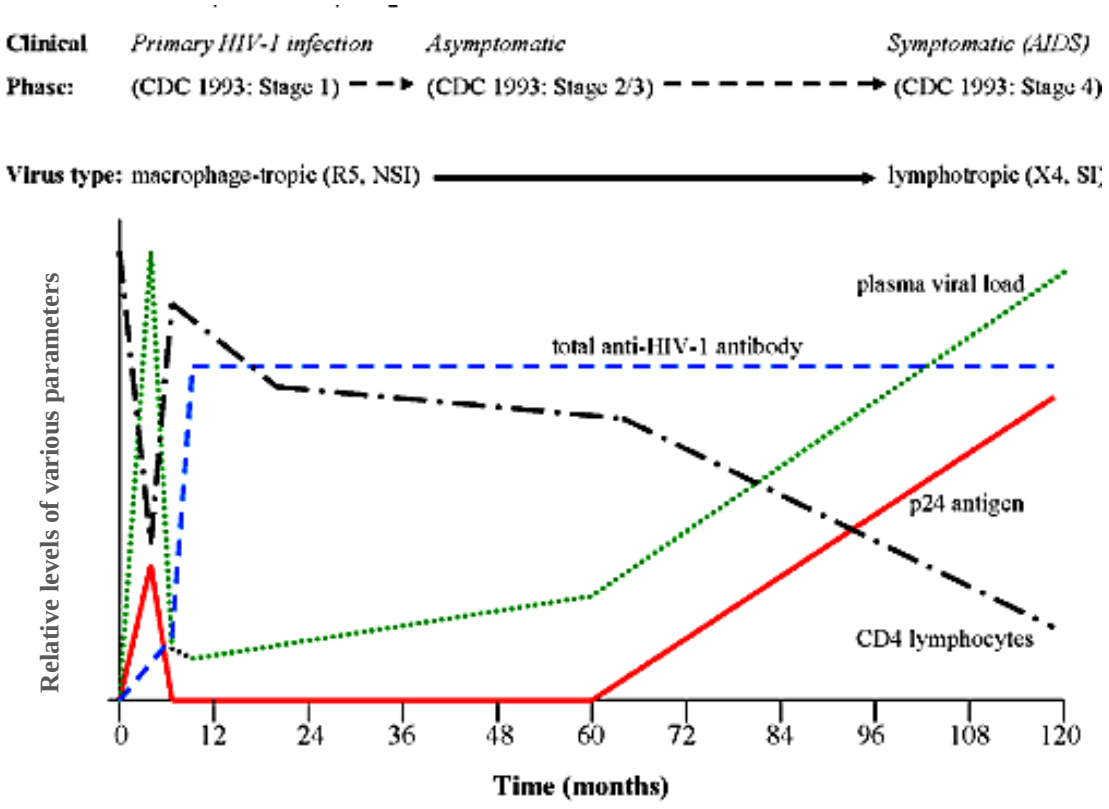


Figure 2.6. The course of immune response after HIV infection (Tang & Chan, 2007).

HIV antibodies (Ab) rise to maximum levels within 3-6 months after initial infection, remaining detectable for the duration of the disease. However, anti-p24 antibodies levels decline and may be indicative of disease progression. Markers of immune system activation, such as tumour necrosis factor (TNF) can be as useful as viral load at the time of infection, in predicting disease prognosis. With the use of antiretroviral therapy, and the suppression of viral loads to undetectable level, the antigenic stimulus has been removed in such patients, resulting in a decline in the level of anti-HIV antibodies. In some patients, these phases of natural HIV infection are considerably accelerated and merge, so that the HIV load remains high or increases with the p24 Ag, the CD4 count drops quickly and the patients progress to end-stage disease in less than 5 years (so-called rapid progressors). Alternatively, there are patients who remain clinically well in

the asymptomatic phase, with normal CD4 counts and low or undetectable viral loads, without the need for antiretroviral therapy (Osmond, 1998; Tang & Chan, 2007).

2.9 Laboratory Testing of HIV Infection

The main objectives of testing for HIV infections are: screening of donated blood to reduce spread of infection; diagnosis of HIV in individuals; surveillance of HIV trend over time in a population anonymous testing for statistical purpose; to prevent mother-to-child infection and to a lesser extent to detect and prevent HIV infection (CDC, 2001)

Antibodies or Cytotoxic-T-lymphocytes (CTLs) recognize various parts of *Env* gp120 and *Env* gp41. HIV antigens are generally detected either in acute phase or symptomatic phase of AIDS. Serological tests for detecting antibodies to HIV are classified as screening tests (initial) or confirmatory (supplemental) tests (WHO, 2006). The most widely used screening tests are ELISAs as they are the most appropriate for screening large numbers of specimens on a daily basis (Voluntary Counseling and Testing Operational Guidelines of India, 2004).

2.7.1 HIV ELISA tests

As illustrated in *Figure 2.7* there are different serological markers that can be used to detect the presence of HIV infection (Branson, 2010). The performance of the HIV testing kits has improved over time. The 1st generation assays were based on the lysate antigens derived from viruses that were grown in human T-lymphocyte lines and they could detect only IgG HIV antibodies after 6-12 weeks post-exposure. The presence of traces of host cell components in which the virions had been propagated could occasionally lead to cross-contamination and thus to very high rates of false-positive results. The 2nd generation kits, which were based on recombinant proteins and/or synthetic peptides, could similarly detect only IgG HIV antibodies after 6-12 weeks post

exposure. The 3rd generation (sandwich ELISAs) used labeled antigens as conjugate and they could detect all HIV isotypes antibodies (IgG, IgM, IgA and others) after 3-4 weeks post exposure. Detection of IgM antibodies is important as they are present only during the early stages of infection hence this shortened the antibody detection "window" period. The currently used 4th generation ELISA kits detect HIV antibodies after 2-3 weeks close to 1 week detection limit of nucleic acid test (NAT) based test kits.

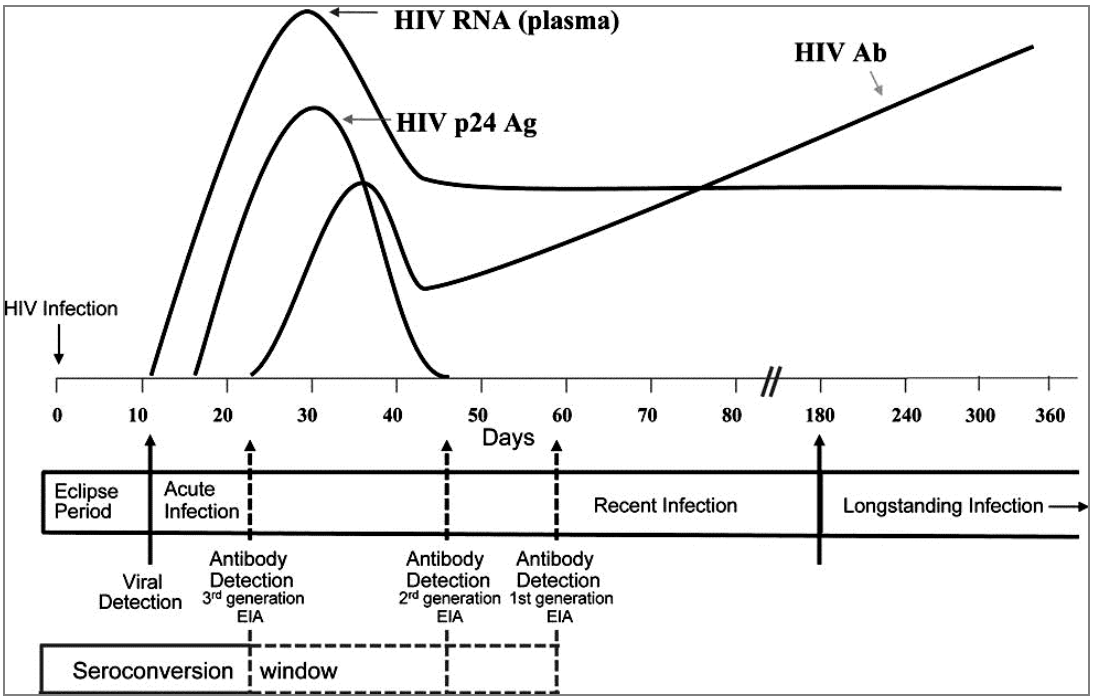


Figure 2.7. Sequence of appearance of laboratory markers in HIV infection (Branson, 2010)

HIV RNA markers are the first to appear in the blood followed by HIV p24 Ag and then HIV antibodies. Both HIV RNA and Ab persist in blood for life while p24 is only present during the active multiplication of the virus.

They use recombinant or synthetic antigens and monoclonal/polyclonal anti-p24 antibodies to detect both HIV viral capsid protein, p24 antigen and all HIV isotypes antibodies hence they are known as Ab/Ag combo assays (Brust *et al.*, 2000; Constantine & Zink, 2005; PishtazTeb Diagnostics, 2010).

The 3rd generation HIV 1 & 2 ELISA kits were produced as follows: ELISA micro wells were coated with calculated amounts of HIV antigens: *Env* gp41, (and sometimes *Env* gp120) and *Env* gp36. If specific antibodies (IgG, IgM, and IgA) for HIV were presented in the serum they could bind to HIV antigens through their individual Fab regions. After incubation at 37°C, the wells could be washed to remove unbound antibodies. To create a second sandwich component, HIV-1 and HIV-2 recombinant antigens conjugated to Horse Radish Peroxidase (HRP) could be added into the wells followed by incubation and a wash step. A solution of chromogen-substrate could be added and incubated for 15 minutes, resulting in the development of a blue color. The color development could be stopped with the addition of stop solution, and the color changed to yellow and measured spectrophotometrically at 450 nm (Best & Dax, 1997; PishtazTeb Diagnostics, 2010).

The 4th generation HIV 1 & 2 ELISA kits are produced as follows: polystyrene microwell strips are coated with calculated amounts of HIV *Env* gp41, *Env* gp41 "O" group, *Env* gp120 (sometimes), *Env* gp36 and anti-HIV p24 antibodies. Biotinylated anti-p24 antibodies are mixed and incubated with a sample in the microwells. Two types of binding occur; the first one is specific binding of HIV antibodies with coated antigens while the second one "sandwich ELISA" is the binding of p24 antigen / biotinylated anti-p24 antibodies complex binding to anti-p24 antibodies bound on microwells. The microwells are then washed to remove the unbound proteins. A mixture of two sets of detection systems are used; Horse Radish Peroxidase-Avidin to bind with biotin component (Ab-Ag-Ab-HRP "sandwich" complex) and a set of Horse Radish Peroxidase-HIV 1 & 2 recombinant antigens (to form Ag-Ab-Ag-HRP "sandwich" complex) (Wantai Company, 2007).

The first known case of development of ELISA test kit in Kenya was in 2002 by the Institute of Primate Research of Kenya. This kit could however detect HIV-1 only (Munene *et al.,* 2002) and there is no evidence that this kit was ever fully developed to commercialization level

2.7.2 HIV Western Blot Tests

For a long time the Western blot (WB) was used as HIV confirmatory test. However, its use has been proven to be very expensive and can, under some conditions, produce a relatively large number of indeterminate results (Uneke *et al.,* 2007). In USA, it was found that 13% to 48% of repeatedly HIV positive testing samples were indeterminate (or equivocal) with Western blot as compared with HIV ELISA tests (Uneke *et al.*, 2007). Confirmatory tests are designed to offer a greater specificity than the screening tests; they "confirm" positive results but not negative results. Therefore they rule out false positive not false negative. In the Western Blot test format, viral antigens (from actual viral lysate) are separated using electrophoretic techniques and blotted onto nitrocellulose membrane. When a sample is introduced, antibodies there in react with specific antigen on nitrocellulose membrane and they are visualized with an enzyme labeled anti-IgG and an enzyme substrate (comparable to ELISA) (Mahmood & Yang, 2012).

Line immuno-assays (LIAs) and Recombinant Immunoblot Assay (RIBA) are very similar to WB and they produce fewer indeterminate results as compared to WB, but are equally expensive (Prateek, 2005).

Studies have shown that combinations of ELISAs or Rapid assays can provide results as reliable as the WB at a much lower cost. WHO and UNAIDS therefore recommend that

countries need to consider testing strategies which use ELISAs and rapid assays rather than ELISA/WB for HIV antibody detection (WHO/UNAIDS, 1999).

2.7.3 Indirect Immunofluorescence (IF) Tests for HIV

Indirect Immunofluorescence (IF) assay involves fixing HIV infected lymphocytes with acetone which are incubated with the sample. This is followed by application of fluorescein conjugated anti-IgG. A specific pattern of cells staining is noted when the cells are observed under microscope. This method has the disadvantages of being less sensitive (as it detects IgG only), expensive, requires a lot of skills as well as being time consuming (Kvinesdal *et al.,* 1989)**.**

2.7.4 Nucleic Acid-Based Technology Tests for HIV

Currently nucleic acid technology (NAT) is the platform that gives the most sensitive results. The tests include the steps of sample preparation and/or nucleic acid extraction, nucleic acid amplification and quantification of amplicons. There are three methods that employ NAT technology: Reverse transcription PCR (RT-PCR), nucleic acid sequence-based amplification (NASBA)/Transcription Mediated Amplification (TMA) and branched chain DNA (bDNA) (Branson, 2010).

2.7.5 Lateral Flow (LF) Assays for HIV

The HIV antibody-detecting Lateral Flow Assays or Test (LFAs or LFTs) provide a good compromise between accuracy; cost, speed and overall effectiveness for Point-of-Care (PoC) use in Resource-Limited Settings (RLS) (WHO EMRO, 2004). There are two major platforms of immunochromatographic assays: Flow-through and Lateral-flow assays tests. In the Flow-through format, filter paper (porous membrane) is treated with proteins that target anti-HIV antibodies. A blood matrix sample such as whole blood,

plasma or serum is applied to the filter. Any anti-HIV antibodies present is immobilized on the filter. Following a wash, the addition of a gold nano-particle (NP) label that binds to the antibodies generates a visible color change to reveal the test result (Paek *et al.*, 2000; Peng *et al.*, 2008; Wong *et al.*, 2010).

In a LFT format (*Figure 2.8)*, HIV antigens are coated on a nitrocellulose membrane downstream as a thin band, while an antigen or an antibody is coated on the same membrane upstream as a similar thin band. A conjugate pad made up of anti-IgG or Protein A conjugated with colloidal gold or any other detection system is fixed before the test band. Once a HIV positive sample is applied on the HIV LFT, HIV antibodies are bound to the conjugate on the fc-region and migrate to the test band where some HIV antibodies carrying the gold conjugate are bound to the antigens to produce a distinct colour band. Some conjugates migrate to the control band and produce a distinct colour (RDT Info, 2008).

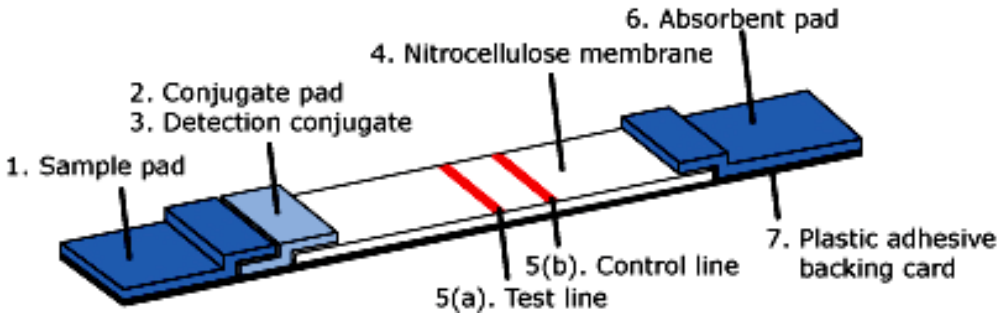


Figure 2.8 General design of the LF test kit (RDT Info, 2008)

Another format of LFT corresponds to the 3rd generation ELISA. In this sandwich format assay, HIV-1 and HIV-2 envelope recombinant antigens are coated on the membrane. The conjugate system contains HIV-1 and HIV-2 antigens conjugated to

colloidal gold. The HIV antibodies, in case they are present in a sample, bind to the HIV antigens conjugated to the gold colloids which then migrate and get bound to the antigens fixed on the test band on the nitrocellulose membrane to produce a visible red band (Arai *et al*., 1999).

2.10 Evaluation of HIV 1 and 2 ELISA and LFT kits

Diagnostic tools for the low resource settings should meet a stringent set of criteria to be of practical use. These tests should be accurate, inexpensive, rapid, simple to administer and robust. Moreover, it would be ideal if a test could function without electricity, refrigerated reagents or complex instrumentation as these may not be available in some places (Wong *et al.,* 2010).

Before HIV test kits are put to use, quality assurance programs are usually instituted to evaluate the tests, monitor their performance in given geographic regions and develop appropriate algorithms with clear instructions regarding on how to proceed with confirmatory testing in the event of an initial positive test result. In addition, the personnel employing these tests in the field must be properly trained not only in the technical aspects of the assay, since errors in operation or improper interpretation could result in an inaccurate diagnosis, but also to adequately counsel the patients following a diagnosis (Allain & Lee, 2005).

2.11 Causes of Errors in HIV Testing

Sometimes diagnostic tests for HIV give false results due to the nature of the samples and their storage. False-negative results can result from the following factors: performance and technical errors in pipetting, labeling of samples or wells and variability in test kits; biological, pathological or pharmaceutical errors such as pre-seroconversion (window) period, delayed antibodies production in infants, reduced

immunological response because of HIV related immune dysfunction, immunosuppressive therapy and concurrent infections with Epstein-barr virus or cytomegalovirus; congenital or drug-induced hypogammaglobulinemia and lastly factors related to specificity and sensitivity of the assay such as failure of the test to use sub-type-O specific antigen, limited antigenic determinants; hemodilution and non-denatured antigens (Allain and Lee, 2005).

2.12 Overview of reported failures of some HIV testing kits to detect common HIV markers

Wei *et al.* (2010) justified the development of multisubtype gp41 recombinant antigen (rIDR-M) with the fact that it could overcome the limitation of varying performance in different subtypes or populations that were shown by HIV kits developed using individual *Env* gp41 peptides derived from respective subtypes. During the evaluation of performance of the developed rIDR-M the study established that the rIDR-M protein was highly reactive with HIV antibodies in sera from different subtypes and equivalently detected antibodies to divergent subtypes B and AE from Thailand, in contrast to individual *Env* gp41 peptides derived from respective subtypes.

In a study carried out by Thorstensson *et al.* (1998) the performance of 14 commercially available HIV-1/2 antibody assays were compared with: HIV-negative blood donor sera from Sweden, unselected blood donor and patient sera from Tanzania; unselected sera from outpatient clinics in Guinea-Bissau; HIV-1 antibody positive sera from Sweden and Tanzania and HIV-2 antibody positive sera from Guinea-Bissau. The results of this study showed that 12 (85.7%) of the 14 assays identified correctly all HIV-1 and HIV-2 antibody positive sera. One Tanzanian HIV-1 antibody positive sample with complete banding pattern on Western blot was not detected by two of the ELISAs employing

synthetic peptides. The assays showed a high specificity ranging from 99.2 to 100% when used for analysis of Swedish blood donor sera, while most of the assays showed a significantly lower specificity, 91.9-99.6%, when used for testing African specimens.

A study by Apetrei *et al.* (1996) showed lack of screening test sensitivity during HIV-1 non-subtype B seronconversions raising the possibility of different HIV clades showing varying sensitivity to HIV test kits.

Despite some degree of immunological cross-reactivity between types and subtypes of HIV, reliable detection of the more divergent strains may only be achieved by incorporating specific sequences into the assay design. In one study in 1990's, detection of HIV-2 positive samples by licensed HIV-1 antibody kits ranged from 60% to 91%, depending on the test used (George, J.R.; Rayfield, M. & Philips, S., 1990). Detection of HIV-1 Group O samples by HIV-1 and HIV-1/HIV-2 assays varied from 0% to 100% in studies with USA licensed and European licensed test kits (Schable *et al.,* 1994). It was then concluded that HIV-1 Group "O" antigens elicit antibodies that show either low reactivity or no reactivity at all with various ELISA recombinant assays. To overcome this challenge, it has become imperative to include HIV-1 group "O" antigens in the design of HIV ELISA kits (Loussert-Ajaka *et al.,* 1994; van Binsbergen *et al.*, 1996).

However some studies have established that consensus *Env* gp41 from group M peptides (WGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTAVPWNASW) was able to detect all 130 group M sera (10 subtype A, 21 subtype B, 13 subtype B9, 20 subtype C, 21 subtype D, 14 subtype E, 25 subtype F, and 6 subtype G) resulting in a test sensitivity of 100% (Masciotra *et al.,* 2000). Despite the high genetic divergence between HIV-1 groups M and N, all group N infections in a study by Vallari *et al.* (2010) were detected using five commercial HIV immunoassays.

Failure of a new rapid antigen and antibody test to detect antigen-positive HIV infection has been reported by Kilembe *et al.* (2012) in samples taken from Zambia and Rwanda. They found that the new test could only detect less than 2% of p24 antigen positive samples. They also found that although the manufacture of the kit was claiming analytical sensitivity of 12.5pg/ml to 25pg/mL, they could only detect a concentration of 248.3pg/mL. The same study by Kilembe *et al.* (2012) had to modify the cut-off points of two p24 ELISA tests for them to be effectively used in the study using African samples or else the tests were giving high ratio of false positive results. A study by Miller *et al.* (2011) in Malawi found that 8 acute HIV infections (p24 antigen positive) could not be detected by the same kit. A study in France by Pavie *et al.* (2010) demonstrated failure of the same Combo test kit to detect two acute HIV infections, one with p24 concentration of 380 pg/mL. However, Pilcher *et al.* (2010) tested the same Combo test kit and it correctly identifies 7 out of 9 persons with acute infections. Kilembe *et al.* (2012) suggested that this variation in sensitivity could be related to the circulating strains of HIV.

In a study by Klarkowski *et al.* (2009) 2864 clients presenting to MSF VCT centers in Bukavu, Democratic Republic of the Congo, during January to May 2006 were tested using Determine HIV-1/2® and UniGold HIV® rapid tests in parallel by nurse counsellors. Plasma samples on 229 clients confirmed as double RDT positive by laboratory retesting were further tested using both WB and the Orgenics Immunocomb Combfirm® HIV confirmation test (OIC-HIV). Of these, 24 samples were negative or indeterminate by WB representing a false-positive rate of the WHO two-test algorithm of 10.5% (95% CI 6.6-15.2). Seventeen (17) of the 229 samples were weakly positive on rapid testing and all were negative or indeterminate by WB. The false-positive rate fell to 3.3% (95%CI 1.3–6.7) when only strong-positive rapid test results were

considered. Agreement between OIC-HIV and WB was 99.1% (95%CI 96.9–99.9%) with no false OIC-HIV positives when stringent criteria for positive OIC-HIV diagnoses were used.

The Global HIV Surveillance Program provides global specimen panels to demonstrate that assay performances are not affected by HIV strain diversity. The Ag detection side of one combination HIV antigen (Ag) and antibody (Ab) assay was evaluated using 47 viral strains originating from 21 different countries and representing the major HIV-1 group M subtypes and recombinant forms as well as group O (Chavez *et al.*, 2011). For evaluation of antibody detection, a panel of 693 specimens obtained from 13 different countries and representing HIV-1 groups M, N, and O and HIV-2 was used. In addition, using a diverse panel of HIV-1 virus isolates, it was determined that this assay could detect acute infections once the viral loads exceeded 58,000 RNA copies per mL regardless of HIV-1 strain. It should be noted that commercially available HIV Ag/Ab combination tests vary widely in their analytical sensitivity for HIV Ag, especially across diverse HIV-1 groups and subtypes (Ly *et al.*, 2012).

2.13 Estimation of HIV Incidence

Estimating HIV incidence is important for assessing the dynamics of HIV transmission and evaluating the impact of prevention policies. Several methods have been proposed for measuring HIV incidence in large areas, yet each presents specific challenges (Ghys, P.D; Kufa, E.; George, M.V., 2006; Mastro *et al.*, 2010). The original ‘‘Gold standard’’ method for measuring population level HIV incidence is a prospective cohort study that measures the occurrence of new infections in a well-defined HIV-negative population followed over time and tested at regular intervals for HIV infection. These studies,

however, are rare, difficult and expensive to implement, and prone to biases that could reduce generalizability of results (Kim *et al.*, 2011).

One of the earliest methods of estimating HIV incidence was carried out by Brookmeyer and Quinn (1995) from a cross-sectional survey. Briefly, a two-step algorithm that combined diagnostic tests for the p24 antigen and HIV-1 antibodies to determine the prevalence of p24 antigenaemia among antibody-negative individuals was used. The HIV incidence rate was calculated by using the classical epidemiologic relation between prevalence, incidence, and duration of the period between the onset of detectability of p24 and the first HIV antibodies. The disadvantage of this approach was that the time during which p24 antigen is detectable prior to sero-conversion was short. The mean duration of this period was 22.5 days in 1995 and it has become shorter since then due to the development of new diagnostic assays that allow detection of antibodies earlier (Busch *et al.*, 2005). The first consequence of this is that the estimation of this period comes with a considerable uncertainty which can have a large impact on the incidence estimate. The second consequence is that large samples and/or high HIV incidence are required to identify a sufficient number of individuals with detectable p24 antigen who have not seroconverted. Nevertheless, Brookmeyer and Quinn (1995) provided the conceptual framework for subsequent laboratory-based methods to estimate incidence from single cross-sectional surveys.

Testing of pooled blood for HIV RNA now seems to be the most appropriate approach because RNA can be detected earlier than p24 antigen allowing characterization of a longer time period. Pooling of specimens improves the predictive value of the amplification assays and substantially lowers the costs. However, in order to obtain

accurate incidence estimates, this method requires the inclusion of very large sample populations, such as those provided by blood donations (Busch *et al.*, 2005).

Most developing countries approximate adult HIV incidence using mathematical models that relate observed HIV prevalence to HIV incidence, which make assumptions on the average survival of HIV-infected individuals and the effect of antiretroviral (ARV) treatment on survival (Brown *et al.*, 2008). In countries with generalized epidemics, the primary sources of HIV prevalence data for these models are routine unlinked and anonymous HIV sero-surveys among pregnant women attending antenatal clinics (ANC) and nationally representative population-based surveys (NPS) with HIV testing, including demographic health surveys (DHS) and AIDS indicator surveys (AIS) (Diaz, T.; Garcia-Calleja, J.M.; Ghys, P.D. & Sabin, K., 2009). NPS have also been used to derive age-specific HIV incidence rates in the general population using HIV prevalence data from two sequential surveys in the country. This method has been broadly validated through comparison with cohort measures of incidence and has been applied to several settings where two such surveys exist. HIV incidence assays are a laboratory-based approach for detecting recently acquired HIV infection in cross-sectional samples of HIV-positive specimens and designed to estimate population-level HIV incidence (Kim *et al.*, 2011). These assays are based on the principle that antibody response to HIV infection matures over time and that immunological biomarkers of HIV disease progression can be used to distinguish recent from non-recent HIV infection. The ideal assay assumes that all HIV-infected persons eventually produce a non-recent test result. The mean time it takes to cross over the defined threshold value defines the assay's duration of recency. Incidence rates are estimated by combining the number testing as recent on the assay, the mean duration of recency for the assay, and the number at risk for recent HIV infection in an incidence formula. To correct for individuals in a

population who fail to progress out of the stage marked as 'recent' by the assay, the application of statistical adjustments in the incidence formula is required. A critical component to these adjustments is the assay's false-recent rate (FRR), defined as the probability that a chronically infected individual (that is, an HIV-infected individual infected 12 months) is misclassified as recent on the incidence assay. Some HIV-infected individuals that are undergoing treatment with ARVs are also misclassified as recent on the assay as a result of enhanced viral suppression and corresponding decrease in antibody response. Because of the significant impact that ARV use can have on incidence assay test results, all FRR and incidence surveys should have the ability to detect individuals that are currently taking ARVs to appropriately account for these individuals in the analysis (ECDC, 2013; Kim *et al.*, 2011).

Janssen *et al*., (1998) designed the first approach of estimating HIV incidence known as "Serologic testing algorithm for recent HIV seroconversion (STARHS)", which aimed at detecting a transient state reached after the antibody conversion. The approach has the advantage of testing only positive individuals and defining a period sufficiently short to fulfill the requirements of stationarity of the incidence over the study period, while sufficiently long to minimize the inaccuracy in its estimation. Suligoi *et al.* (2002) designed a procedure for detecting recent HIV infections by calculating the antibody avidity index. Around the same time Parekh *et al.*, (2002) devised an ELISA that detected increasing levels of anti-HIV IgG after seroconversion that could be used for detecting recent HIV-1 infection. This test used a branched peptide that included gp41 immunodominant sequences from HIV-1 subtypes B, E, and D that allowed similar detection of HIV-specific antibodies among various subtypes.

CHAPTER

THREE: MATERIALS AND METHODS

3.1 Research Design

The study design was the Laboratory-based Experimental Design. The collected samples were processed and experiments conducted in the laboratories at the KEMRI Production Department.

3.2 Sampling

Blood samples were collected from the Regional Blood Transfusion Centers (RBTCs) in Nairobi, Kisumu, Embu and Mombasa upon receipt of permission from the Director of the National Blood Transfusion Centers (NBTCs). The identity of the blood donors was not disclosed to me; only the sample numbers, dates of donation and their HIV sero-status were indicated on the pack.

A total of 400 samples (200 HIV positive and 200 HIV negative) were picked from the four RBTCs (uniformly spread among the four centers over a period of three months). This was the minimum sample size that is recommended by WHO and CDC for Evaluation of HIV LFTs and ELISA (WHO & CDC, 2002).

The sample size selected for sequencing was ninety one (91) based on the calculations done using Fisher's formula below:

$$N = Z^2 P (1- P) / D^2.$$

Where: N = Minimum sample size required

Z = 1.96 standard error; P = 6.3%, which is the prevalence of HIV in Kenya, (UNAIDS, 2013)

D = 0.05 is the inverse of 95% confidence limit (the allowable error).

Therefore: $N = 1.96^2 (0.063) (0.937) / (0.05)^2 = 91$

The collected blood units were stored at KEMRI Production Department at -80^0C.

3.3 Determination of the Prevailing Consensus Sequence of HIV *Env* gp41 IDR Gene in Kenya

In this study, the prevailing consensus gene of HIV *Env* gp41 IDR was determined from samples collected from RBTCs. Ninety one (91) HIV positive samples (out of 200) were picked for sequencing as per the procedure described by Masciotra *et al.* (2000). Briefly, viral RNA was extracted from plasma using the QIAamp viral RNA kit (Qiagen, Valencia, Calif.) according to the manufacturer's protocol. Two hundred microliters (200µl) of plasma was mixed with 800µl of lysis buffer. After a 10 minutes' incubation, 800µl of 100% ethanol was added to the lysate. The mixture was filtered through a silica gel spin column by centrifugation at the speed of 5000 RPM for 2 minutes. After being washed with buffer, the RNA was eluted from the column by adding 50 µl of RNase-free water. For negative controls, RNA from normal human plasma was also extracted. For reverse transcription (RT) and primary PCR, the primers were GP40F1 (forward; 5′TCTTAGGAGCAGCAGGAAGCACTATGGG) and GP41R1 (reverse; 5′AACGACAAAGGTGAGTATCCCTGCCTAA). For the nested PCR, the primers were GP46F2 (forward; 5′ACAATTATTGTCTGGTATAGTGCAACAGCA) and GP47R2 (reverse; 5′TTAAACCTATCAAGCCTCCTACTATCATTA). Three to 10µl of the RNA extract was used to synthesize cDNA with primer GP41R1 (20µM) and the GeneAmp RNA PCR kit following the manufacturer's protocol (Perkin-Elmer Cetus,

Norwalk, Conn.). The 20-µl cDNA reaction mixture was then added to a PCR mixture containing 50 µM GP40F1 and 30 µM GP41R1, 1× GeneAmp PCR buffer II, 1.25 mM $MgCl_2$, 1.25mM dNTPs and 2.5U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Foster City, Calif.) and was brought to a final volume of 100 µl with sterile distilled water. After initial denaturation at 94°C for 2 min, 35 cycles of PCR were performed in the GeneAmp 9600 thermocycler (Perkin-Elmer Cetus). Each cycle consists of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 5 min. For nested PCR, 5 µl of the primary PCR product was added to a 100-µl PCR mixture containing reagents similar to those in the primary PCR, except that the primers were replaced by 25 µM each GP46F2 and GP47R2. The PCR mixtures were subjected to 35 cycles under the same conditions as the primary PCR. After PCR, the nested PCR products were electrophoresed in 1.5% agarose gels along with a 1kb ladder (Gibco, Grand Island, N.Y.) and visualized under UV light by ethidium bromide staining.

Due to the financial constraints and lack of access to a sequencing machine the Complementary DNA was sent to Macrogen Europe (Amsterdam, Netherlands) for sequencing using a big dye terminator v. 3.1 (PE) and the same primers that were used for initial nested PCR (GP46F2 and GP47R2). The resultant sequences were aligned using "HIVAlign" software within Los Alamos HIV Data Base (Los Alamos Database, 2009) via Multiple Alignment using Fast Fourier Transform (MAFFT) multiple sequence alignment Program. The reference sequence used in these alignments was HXB2.

Prevailing HIV *Env* gp41 IDR consensus nucleotide sequence was generated by use of software "Advanced Consensus Maker" in the Los Alamos Database (Los Alamos

Database, 2009) using aligned sequences. Consensus HIV *Env* gp41 IDR nucleotide sequence was translated into Consensus HIV *Env* gp41 IDR peptide in the same database (Los Alamos).

3.4 Synthesis of *Env* gp41 IDR peptides

The Consensus sequence of HIV *Env* gp41 IDR nucleotide sequence (Kenya) established in this study and the Consensus HIV *Env* gp41 IDR nucleotide sequence (Global) were sent to LifeTein LLC (Hillsborough, NJ) for synthesis of HIV *Env* gp41 IDR peptides.

3.5 Peptide structure prediction of Consensus HIV *Env* gp41 IDR peptides (Kenya and Global)

Both consensus HIV *Env* gp41 IDR peptides (Kenya and Global) were fed separately into the Protein Homology/analogY Recognition Engine V 2.0, *(Phyre^2)* software (Kelley & Sternberg, 2009). The software gave an output of secondary and tertiary structures which were analyzed further using *"Interactive 3D view in JSmol"* and *"Secondary structure and disorder prediction"* software within the same program.

3.6 Analysis of diversity of HIV *Env* gp41-IDR peptide (Kenya) sequences by Shannon Entropy-Two

Shannon Entropy is a measure of variation in DNA and protein sequence alignments. Entropy-Two compares two sets of aligned sequences (named query and background sequences, HXB2 in this study), and determine if there is greater variability in one set relative to the other.

To determine Shannon Entropy-Two of 91 HIV Env gp41 IDR peptide sequences obtained in this study, the sequences were fed in the Los Alamos "Entropy" software

(Foley *et al.,* 2013) with HXB2 gp41 IDR peptide reference sequence as a background sequence. The entropy was calculated using the following options (default parameters as they were still appropriate for the study): statistical confidence using randomization with replacement, 100 randomizations and 5 random samples that could have higher entropy difference than the actual data.

3.7 Determination of HIV sub-types and recombinants

The 91 HIV *Env* gp41 IDR nucleotide sequences were fed into "RIP" software in Los Alamos Database (Foley *et al.,* 2013). The following conditions were selected to run the program: use of HIV subtypes A to K and CRF01_AE consensus alignment, 95% Confidence Threshold, stripping all gaps and plotting all window values and lastly scoring multistate characters as partial matches.

3.8 Reconstruction of the Phylogenetic tree

The phylogenetic tree was constructed using the software program "PhyML 3.0" (Guindon *et. al.,* 2010) within the Los Alamos Database. The 91 nucleotide sequences were aligned using "HIValign" software (Foley *et al.,* 2013) after which the aligned sequences were entered as inputs in the "PhyML 3.0" software and analyzed using the following default parameters: empirical equilibrium frequencies; Transition/Transversion ratio of 4; BioNJ starting tree, Generalized time-reversible M (GTR) model of nucleotides substitution and number of bootstrapped data sets of 1000.

3.9 Estimation of the Proportion of Recent HIV Infections among the Blood Donors in Kenya

Four hundred (400) samples (200 HIV positive and 200 HIV negative) were tested using Determine™ HIV-1/2 Ag/Ab Combo (Abbott Diagnostic Division, Hoofddorp, The Netherlands) and ELISA and Lateral flow assay (LFA) developed in this study to

determine p24-antigenemia. Determine™ HIV-1/2 Ag/Ab Combo LFA was picked in the study as its design allowed the detection of p24- antigen independent of system to detect HIV gp-41/gp36 antibodies.

In an effort to detect samples at acute HIV infection (AHI) stage 200 HIV negative samples by antibody tests were pooled into 10 equal pools after which HIV viral RNA was extracted from each pool using the QIAamp viral RNA kit (Qiagen, Valencia, Calif.) according to the manufacturer's protocol.

The US Centers for Disease Control (CDC) and Kenya Medical Research Institute (KEMRI) had signed a Technology Transfer agreement to develop a HIV Incidence ELISA.

In this arrangement CDC had constructed an oligonucleotide that codes for three variant sequences of Env gp41 peptides but joined to form a single multivalent peptide - the recombinant Immunodominant Region-M (r-IDR-M). The first sequence was for the subtypes A,B, C, F, G, H, J, and K and recombinants AG, AB, AC, BF, and BG.37. The second sequence was for subtype AE from Thailand, and the third sequence was for D and recombinant AD. The multivalent peptide had a terminal histadine tag to facilitate its purification using Ni-NTA. The r-IDR-M was expressed from *Escherichia Coli* BL21-CodonPlus DE3-RP (Stratagene, La Jolla, CA) donated by CDC Technology Transfer Office. The *E. coli* cells were lysed in 6M guanidine hydrochloride with occasional sonication over ice and purified by using Ni-NTA affinity column (QIAgen, Valencia, CA). The bound gp-41 rIDR-M was eluted at pH 3.5. The purified protein was dialyzed against 0.1 trifluoroacetic acid, lyophilized, dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml and stored at -70^{o}C until use.

The ELISA was conducted using the Two-Well Avidity-based assays (Wei *et al.*, 2010) using ELISA wells coated with 2.5 mg HIV Env gp41 peptides (rIDR-M; Consensus HIV *Env* gp41 Kenya and Consensus HIV *Env* gp41 Global). Before coating, the HIV Env gp41 peptides were reconstituted in 0.1M phosphate buffered saline (PBS) and incubated overnight. Plates were washed with PBS containing 0.1% Tween-20 and blocked with 5% non fat dry milk powder in PBS-Tween (milk buffer). A 100 ml aliquot of diluted sample in ratio of 1 to 400 with milk buffer was added to the two wells and incubated for 1 hr at 37^{o} C. The plate was then washed 4 times with 300 ml well of PBS-Tween. One well was incubated with pH 3.0 buffer (dissociation buffer) while the second well (control) was incubated with wash buffer at 37^{o} C for 15 min. The plate was then washed 4 times with 300ml of PBS-Tween. One hundred microliters (100µl) of goat-antihuman IgG peroxidase, diluted 1:5000 in milk buffer, was added and incubated at 37^{o} C for 30 min, followed by 4 washes with 300 ml of PBS-Tween. One hundred microliters (100 µl) of tetramethyl benzidine (TMB) was added and incubated at 25^{o} C for 15 min. The color development was stopped by the addition of 100 ml of 1N Sulphuric acid to each well and the optical density (OD) was read at 450 nm. The avidity index was calculated as a ratio of OD of the treated well divided by the OD of the control well, expressed as a percent.

3.10 Development of HIV 1/2 ELISA and LF Test Kits Guided by the Prevailing Consensus Sequence of HIV *Env* gp 41 IDR Gene in Kenya

3.10.1 Development of ELISA Test Kit for using synthetic peptide derived from the gp41 region representing the consensus sequence for HIV in Kenya.

The two synthetic *Env* gp41 IDR peptides (Kenya: GIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTNVPWNSSW and Global: GIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTAVPWNASW) were used to develop an ELISA as described by Wei *et al* (2010). Briefly, polyvinyl plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 2.5ug of synthetic peptide per ml (100ml / well) in 0.01 M carbonate buffer (pH 9.6) and incubated overnight at 4° C. The plates were washed six times with phosphate-buffered saline containing 0.05% Tween 20 (PBST); excess reactive sites were blocked by the addition of 5% bovine serum albumin in phosphate-buffered saline - 0.05% Tween 20. This step was followed by the addition of a 1:100 dilution of each test serum. The plates were incubated overnight at 4° C. After six more washes, Fc-specific, Horse Radish Peroxidase (HRP)-conjugated goat antibody to human immunoglobulin G (Sigma, St. Louis, Mo.) was added, and the plates were left at room temperature for 1hr. This step was followed by washing six times with PBST. This step was then followed by addition of 100µl of TMB) / hydrogen peroxide substrate (**MP** Biomedical, Santa Ana, Calif.) and 30 minutes incubation in the dark; stopping the reaction by adding 100 µl of 1M sulphuric acid (Sigma, St. Louis, Mo.); and measuring the O.D at 450 nm with Multiscan ELISA Reader (Thermo Fisher Scientific, Waltham, MA). The cutoff values were calculated by adding 0.1 to the mean optical densities plus 3 standard deviations of

normal control sera in the assay. The ELISA was optimized through a series of experiments as described by Crowther *et al.* (1995)

3.10.2 Development of LFT

The key step in the process of preparing LFT was the preparation of viable colloidal gold conjugates (p24-antibody, *Env* gp41 IDR peptides and *Env* gp36 IDR peptides). These colloidal gold were prepared by controlled reduction of a boiling solution of 0.02% chloroauric acid with 1% Sodium citrate according to method of Frens (1973). Briefly, 50 ml. of 0.02% chloroauric acid in double-distilled water was boiled. As soon as it started boiling, 1.2 ml of 1% sodium citrate solution was added with constant stirring. The colour changes from grey to blue to purple to violet to full red within 60 second. After the colour change, heat was turned off. The solution was stirred for 5-10 minutes and then allowed to cool at room temperature away from light. To 1 ml of colloidal gold solution, 5-10 micro liter of freshly prepared 1% K_2CO_3 solution was added to adjust the pH of the sol to 6.5. To this 15-20mg of p24-antibody or *Env* gp41 IDR peptides or *Env* gp36 IDR peptides were added while continually stirring the solution. It was incubated for 10 mins at room temperature and centrifuged for 5 mins. at 5,000 revolutions per minute (rpm). Nine hundred and fifty (950 µl) supernatant was discarded and the pellet resuspended (without washing) in the remaining 50 µl of supernatant. Later 5 µl of conjugate was added in each vial and lyophilized in individual vial, which were stored at 4º C. The LFT was optimized through a series of experiments as described by Crowther *et al.* (1995). Briefly, the baseline concentrations of reagents to be coated on the nitrocellulose membrane were obtained from the literature. These reagents included Avidin, *Env* gp41 IDR peptides and *Env* gp36 IDR peptides. Different types and concentrations of buffers were also tried with the three

coating reagents. The optimum concentrations of the reagents and buffers were identified from the quality of the signals obtained.

3.11 Evaluation of the Performance of the Developed HIV 1 & 2 ELISA and LFT

Four hundred blood samples, 200 HIV positives and 200 HIV negatives that had been collected from the Regional Blood Transfusion Centers (RBTCs) in Kenya (Nairobi, Nakuru, Kisumu, Embu and Mombasa) over a period of three months after removing donors' identifiers were used to make the Evaluation panels. This was done by using seven HIV diagnostic kits: (**HIV** (1 + 2) Antibody (**colloidal gold**) (KHB Shanghai Kehua Bio-engineering Co, Shanghai, China), First Response™ 1-2.0 (PMC Medical Pty. Ltd, Daman, India), Uni-Gold™ HIV test (Trinity Biotech, USA); Determine™ HIV-1/2 (Abbott Diagnostic Division, Hoofddorp, The Netherlands), Alere Determine™ HIV-1/2 Ag/Ab Combo (Orgenics Ltd, Yavne, Israel), Aware™ HIV-1/2 BSP (Calypte Biomedical Corporation, Oregon, USA) and Vironostika™ Uni-Form II Ag/Ab ELISA (bioMérieux, Marcy-l'Etoile, France). The first three kits were in the National HIV Testing Algorithm in Kenya while the fourth kit was in the immediate past National HIV Testing Algorithm in Kenya as a Screening Test. The fifth kit was the 4^{th} Generation HIV test that was designed to detect p24 antigen and acute HIV infection hence reducing the window period. The HIV status of the samples was determined by consensus of the six LFTs and Vironostika™ Uni-Form II Ag/Ab ELISA was used as a "Gold Standard". The two kits developed in this study were also tested. The Diagnostic Sensitivity (D-SN), Diagnostic Specificity (D-SP), Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were then calculated at 95% CI and compared.

The HIV positive panels were generated by pooling 30 HIV positive samples into three pools. The panels were double serial-diluted to 16^{th} dilution and each test kit ran against each set of double dilution. The detection limits for each kit were noted.

CHAPTER FOUR

RESULTS

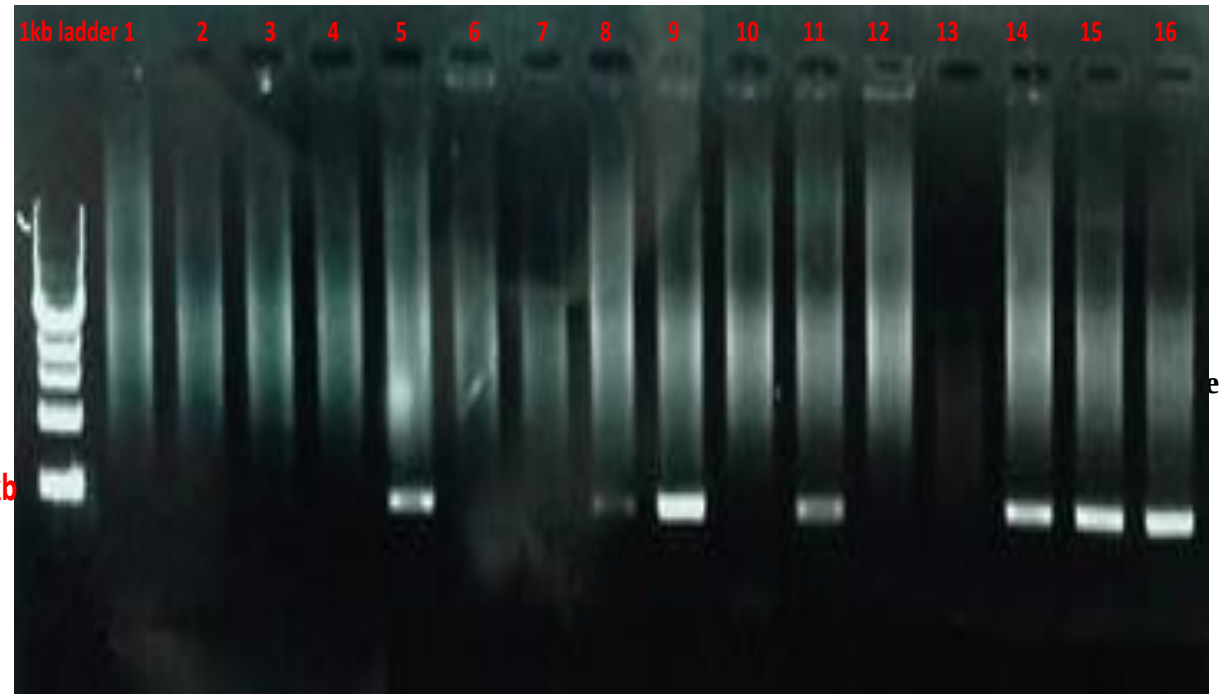
4.1 Presentation of results

This study established the prevailing consensus sequence of HIV *Env* gp41 IDR gene in Kenya. It showed that 67.4% of the amino acid positions in Consensus HIV *Env* gp41 IDR peptide (Kenya) were highly conserved as well as that of Cysteine Loop and CTL epitopes of the same peptide. Although the study noted some variations of the HIV *Env* gp41 IDR peptide in Kenya (in comparison with global consensus of the same) there were no differences in the performance of the kits that were developed using theses peptides. The detail of the findings are presented in the subsequent sub-sections of this chapter.

4.1.1 Prevailing consensus sequence of HIV *Env* gp41 IDR gene in Kenya

Extraction of HIV RNAHIV RNA was extracted, reverse transcribed and agarose gels ran as represented by *Plate 4.1*. In the first Lane, a 1 kb ladder (Gibco, Grand Island, N.Y.) was applied. In the lanes labeled 1 and 2 the known HIV RNA negative controls were applied while HIV ELISA positive (n = 200) and negative (n =200) samples were applied in other lanes. The HIV ELISA negative samples were applied in lanes 3, 4, 6, 7, 10, 12, & 13, which turned out as RNA negative. The HIV ELISA positive samples were applied in lanes 5, 9, 11, 14, 15, & 16, which turned out as RNA positive. The expected size of extracted RNA was $\leq$ 0.5 kb. These results also showed that the extractions of HIV RNA occurred without primer-dimers or other contaminants hence it was successful.

Plate 4.1 Representative electrophoretic gel photographs obtained during the RNA extractions from 91 samples.



HIV *Env* gp41 IDR Consensus nucleotide and peptide Sequences

The following Consensus HIV *Env* gp41 IDR nucleotide was generated from the 91 sequences in *Appendix 01* of this study:

tggggcattaaacagctgcaggcgcgcgtgctggcggtggaacgctatct gaaagatcagcagctgctgggcatttggggctgcagcggcaaactgattt gcaccaccaacgtgccgtggaacagcagctgg

This Consensus HIV *Env* gp41 IDR nucleotide was translated in the Los Alamos Database in the software "Translate Nucleotide' into Consensus HIV *Env* gp41 IDR peptide *(Figure 4.1)*

Figure 4.1 Consensus peptide sequence for HIV *Env* gp41 IDR (Kenya).

These results show that the process of HIV *Env* gp41 IDR RNA, sequencing and nucleotide translation were successfully carried out.

In order to facilitate the analysis of the results in the subsequent sections of the study, the Consensus HIV *Env* gp41 IDR peptide (Kenya) and the individual 91 sequences in the study were further analyzed.

The Consensus HIV *Env* gp41 IDR peptide sequence (Kenya) was aligned using "HIValign" software (Foley *et al.,* 2013) and compared with a Consensus HIV *Env* gp41 IDR peptide (Global) sequence (Bártolo & Taveira, 2012) and Consensus HIV *Env* gp41 IDR peptide (HXB2) sequence from Los Alamos Database (Foley *et al.,* 2013) (*Figure 4.2*)

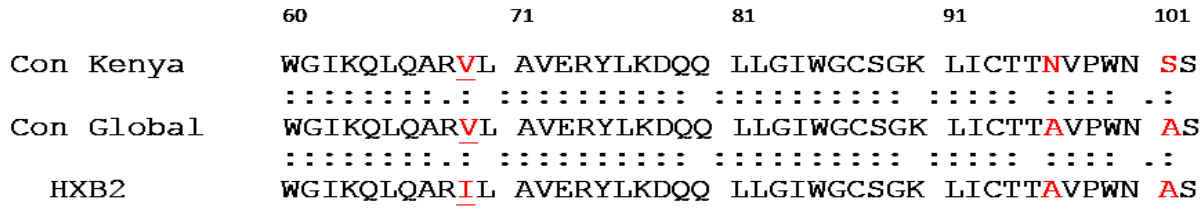
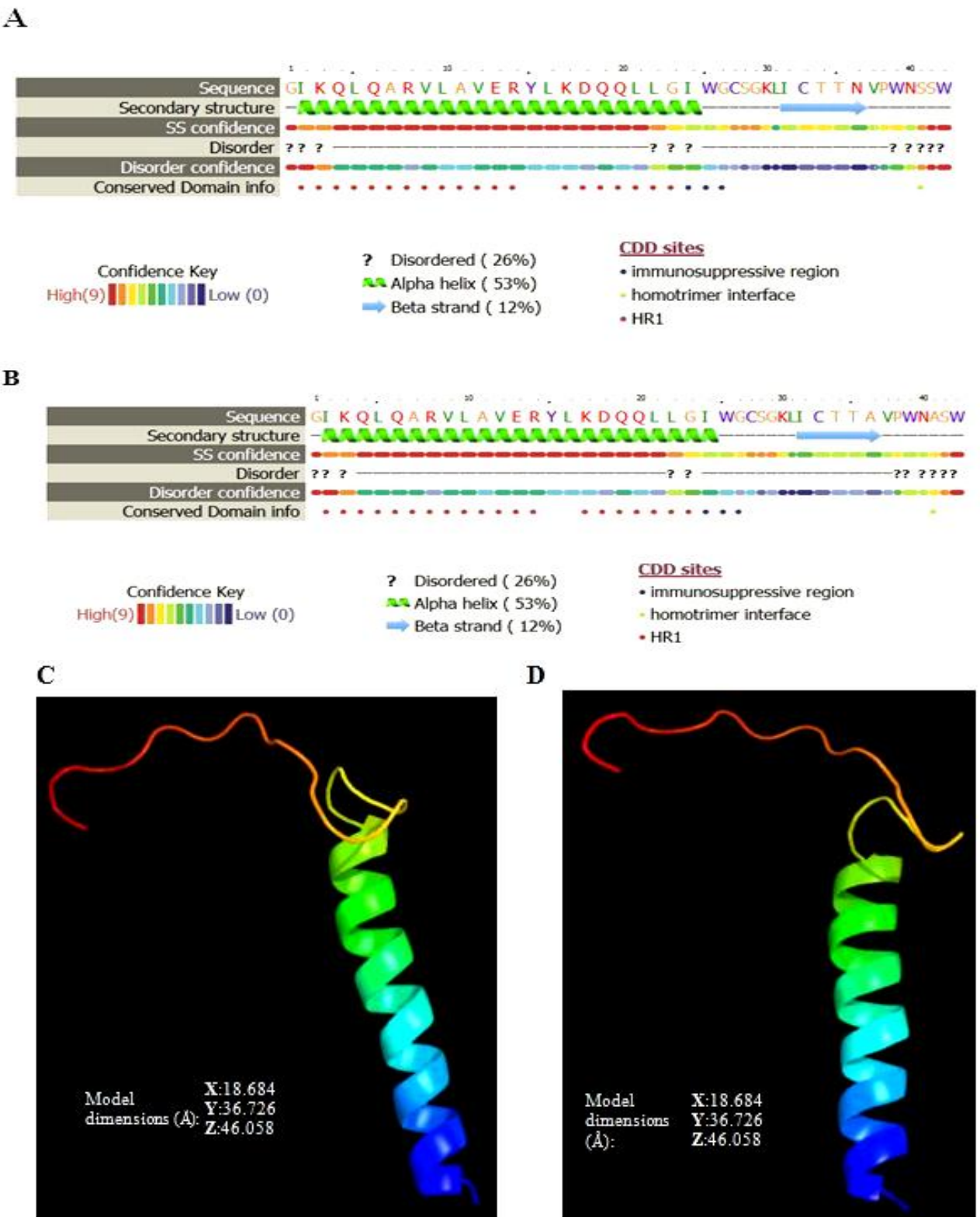


Figure 4.2 Alignment of Consensus HIV *Env* gp41-IDR peptides (Kenya, Global and HXB2).

Relative to Consensus HIV *Env* gp41 IDR peptide (HXB2), the Consensus HIV *Env* gp41 IDR peptide (Kenya) displayed three amino acid substitutions where I^{69} →V, A^{96}→N and A^{101}→S (93.0% similarity) and only two amino acid substitutions relative to Consensus HIV *Env* gp41 IDR peptide (Global) where A^{96}→N and A^{101}→S (95.3% similarity). In regards to the three consensus peptides there was 100% sequence similarity in the key sub-regions of IDR of gp41: the CTL epitope (aa 71 to 82; AVERYLKDQQLL) and the Cysteine Loop epitope (aa 87 to 93; CSGKLIC) *(Figure 4.2)*. This implies that the previously named substitutions within IDR of gp41 could generally have minimal effects on binding of antibodies to the peptides.

Peptide structure prediction of Consensus HIV *Env* gp41 IDR peptides (Kenya and Global)

The software **P**rotein **H**omology/analog**y** **R**ecognition **E**ngine V **2.0** *(Phyre^2)* modeled 100% of the amino acids of the Consensus HIV *Env* gp41 IDR peptides (Kenya and Global) with 99.9% confidence by the single highest scoring template. The secondary ;structure of Consensus HIV *Env* gp41 IDR peptide Kenya (A), Consensus HIV gp41 *Env* IDR peptide Global (B), and the 3D structure of Consensus HIV *Env* gp41 IDR



peptide Kenya (C) and Consensus HIV *Env* gp41 IDR peptide Global (D) are shown in *Figure 4.3*.

Figure 4.3 Secondary (A & B) and tertiary (C & D) structures of Kenya (A &C) and Global (B & D) Consensus HIV *Env* gp41 IDR peptides

The Secondary structures for the two peptides were the same irrespective of the 4.7% difference in amino acid constitution (where A^{96}→N and A^{101}→S in respect to Global Consensus) as shown by the arrows. Generally the tertiary (3D) structure for both Consensus Kenya and Global gp41 IDR peptides were the same apart from minor difference in folding at the junction of the Alpha helix and straight strand as shown by the arrows. This minor difference occurred even though the secondary structures were similar, which could possibly be caused by the 4.7% difference in the primary structure. It is possible that this minor difference in the tertiary structure could affect the activity of the two peptides.

4.2 Diversity of HIV *Env* gp41-IDR peptide (Kenya) of individual samples

4.2.1 Analysis of diversity HIV *Env* gp41-IDR peptide (Kenya) of individual samples

When the individual sequences in the study (*Appendix 3 and Appendix 6)* were analyzed for the substitutions in relation to the HIV Consensus HIV gp41-IDR peptide (Global) the following substitutions were obtained (*Table 4.1*):

Table 4.1 Diversity of HIV gp41-IDR of individual samples

HIV Sequence Identity	CTL Epitope	Cysteine Loop	Other Regions
KPDR_004(A1)	K^{77}→R	S^{88}→A; K^{90}→V; L^{91}→I	60-101(del 96; Ins(93a = P) A^{101}→S
KPDR_010(A1)	K^{77}→E; D^{78}→H	0	A^{96}→N; v^{69}→L

			A^{101}→S
KPDR_011(A1)	R^{68}→S; K^{77}→R	L^{91}→H	A^{96}→N; A^{101}→S
KPDR_014 (A1)	0	L^{91}→H	A^{96}→T
KPDR_017 (A1)	V^{72}→L	0	A^{96}→N; A^{101}→S
KPDR_019 (A1)	R^{68}→S; K^{77}→R	L^{91}→H	A^{96}→N; A^{101}→S
KPDR_047 (A1)	V^{72}→L	0	A^{96}→T; A^{101}→Y
KPDR_049 (A1)	K^{77}→Q	0	A^{96}→T
			A^{101}→S
KPDR_057 (D)	R^{68}→S; K^{77}→R	0	G^{83}→A; A^{101}→S
KPDR_063 (A1)	Q^{79}→R	0	A^{96}→N; A^{101}→S
KPDR_069 (A1)	V^{72}→L	K^{90}→R	A^{96}→N; T^{94}→P
		L^{91}→I	A^{101}→S
KPDR_078 (A2.CY.94CY017_41	K^{77}→Q	0	A^{96}→F; A^{101}→S
KPDR_088 (A1)	K^{77}→R	0	A^{96}→N; A^{101}→S
KPDR_095(A1)	0	0	A^{96}→T; A^{101}→S
KPDR_096 (A1)	K^{77}→V	0	A^{96}→N; A^{101}→S
KPDR_099_15_2_GP46F.ab1(A1)	A^{71}→G	L^{91}→I; I^{92}→L	I^{84}→F; A^{96}→F
	V^{72}→L	T^{94}→P	R^{99}→W; A^{101}→S
KPDR_101A1)	K^{77}→R	L^{91}→I; T^{94}→P	A^{96}→N; A^{101}→S
			A^{101}→S
KPDR_110 (A1)	0	0	I^{84}→L; A^{96}→N
KPDR_125 (A1)	V^{72}→L; K^{77}→V	0	A^{96}→N; A^{101}→S
KPDR_130 (A1)	0	0	A^{96}→N; A^{101}→S
KPDR_133 (A2.CY.94CY017_41)	K^{77}→Q	0	A^{96}→F; A^{101}→S
KPDR_140 (A1)	K^{77}→R	K^{90}→R	A^{96}→N; A^{101}→S
KPDR_141 (A1)	A^{71}→G; R^{68}→S	L^{91}→H	V^{77}→I; I^{84}→V
	K^{77}→R		A^{96}→T; A^{101}→S

KPDR_0146 (A1)	K^{77}→R	0	A^{96}→N; A^{101}→S
KPDR_151 (A1)	0	0	I^{84}→L; A^{96}→T
			T^{94}→S; A^{101}→S
KPDR_152 (A1)	0	0	T^{94}→P; A^{96}→N
			A^{101}→S
KPDR_155 (A1)	0	0	A^{96}→N; A^{101}→S
KPDR_1561(A2.CY.94CY017_41)	R^{68}→S	0	C^{87}→S;A^{101}→S
KPDR_162 (A1)	0	0	A^{96}→N; A^{101}→S
KPDR_164 (A1)	K^{77}→R	0	I^{84}→L; A^{96}→N
KPDR_176 (A1)	V^{72}→L; K^{77}→R	0	A^{96}→N
KPDR_177 (C)	V^{72}→I	0	Q^{66}→K; A^{67}→T
			T^{94}→P
KPDR_179 (A1)	V^{72}→L; K^{77}→R	L^{91}→I	A^{96}→N; A^{101}→T
KPDR_181(A1)	K^{77}→R	0	A^{96}→ N
KPDR_185 (A1)	0	L^{91}→H; K^{90}→R	I^{62}→V; V^{69}→I
			A^{96}→T; A^{101}→S
KPDR_188_11_07_GP46F.ab1(A1)	K^{77}→R	0	A^{96}→ N
KPDR_194 (A1)	0	0	Q^{66}→H; A^{96}→ N
			A^{101}→S
KPDR_198 (A1)	0	0	Q^{66}→R; A^{96}→ N
			A^{101}→S
KPDR_199(D)	R^{68}→S	0	V^{69}→I; I^{84}→L
			A^{101}→S
KPDR_200 (A1)	V^{72}→L; R^{68}→S	0	A^{96}→ N; A^{101}→S
	K^{77}→R		

KPDR_202 (A1)	0	0	A^{96}→T
KPDR_205 (A1)	0	0	L^{70}→ Q; I^{84}→L
			A^{96}→T; A^{101}→S
KPDR_212 (C)	0	0	A^{67}→T; T^{94}→P
			A^{101}→T
KPDR_216 (A1)	0	0	A^{96}→ N; A^{101}→S
KPDR_223 (A1)	0	K^{90}→R; L^{91}→H	I^{62}→V; V^{69}→I
			A^{96}→ N; A^{101}→S
KPDR_224 (A1)	K^{77}→Q	0	V^{69}→M; A^{96}→ T
			A^{101}→S
KPDR_230 (A1)	0	0	A^{96}→ N; I^{84}→L
KPDR_235_26_09_GP46F.ab1	K^{77}→R		A^{96}→ N; T^{94}→A
			A^{101}→S
KPDR_236 (A1)	Y^{75}→F	0	A^{96}→ T; A^{101}→S
KPDR_237 (A1)	0	0	V^{70}→L; I^{84}→L
			A^{96}→ T; A^{101}→T
KPDR_88 (A1)	K^{77}→G	0	A^{96}→ N; A^{101}→S
KPDR_96 (A1)	V^{72}→L; K^{77}→V	0	A^{96}→ N; A^{101}→S
KPDR_95 (A1)	0	0	A^{96}→ T; A^{101}→S
KPDR_110 (A1)	L^{81}→I	0	I^{84}→L; A^{96}→ T
			A^{101}→S
KPDR_146 (A1)	K^{77}→R	0	A^{96}→ N; A^{101}→S
KPDR_151(A1)	0	0	I^{84}→L; T^{94}→S
			A^{96}→ T; A^{101}→S
KPDR_177 (C)	V^{72}→I	0	A^{67}→T; T^{94}→S
			A^{101}→S
KPDR_199 (D)	R^{68}→S	0	V^{69}→I; I^{84}→L

			A^{101}→S
KPDR_202 (A1)	0	0	A^{96}→ T
KPDR_207 (D)	A^{71}→G; Q^{79}→H	C^{93}→S	T^{94}→P; A^{96}→ N
	L^{81}→P; L^{82}→P		
KPDR_216 (C)	R^{68}→K; K^{77}→R	0	G^{83}→R; A^{96}→ T
	L^{81}→I		A^{101}→S
KPDR_237b (A1)	L^{70}→Q	0	I^{84}→L; A^{96}→ T
			A^{101}→T
KPBM_258 (A1)	K^{77}→R	K^{90}→R; L^{91}→P	T^{94}→P
			60-101(del 96)
KPBM_262 (A1)	K^{77}→T; Q^{80}→R	0	A^{101}→S
KPBM_263 (A1)	R^{74}→G; K^{77}→R	0	A^{96}→ T; A^{101}→S
KPBM_264 (A1)	R^{74}→G; K^{77}→R	0	A^{96}→ T; A^{101}→S
KPBM_310 (A1)	A^{71}→G; K^{77}→R	0	60-101(del 63)
			A^{96}→ N
KPBM_314 (A1)	K^{77}→R	0	A^{96}→ T; A^{101}→S
KPBM_324_GP46F.ab1 (A1)	V^{72}→L	0	A^{96}→ N
			A^{101}→S
KPD_710 (A1)	K^{77}→R	0	T^{94}→P; A^{101}→S
KPD_721 (A1)	V^{72}→I; R^{74}→A	0	A^{96}→ S; A^{101}→S
KPD_724 (A1)	60-101(del 71)	0	Ins(68a = S, 68b = P)
			A^{96}→ N; A^{101}→S
KPD_738 (A1)	0	0	A^{96}→ N
KPD_764 (A1)	V^{72}→L; K^{77}→R		A^{96}→ N; A^{101}→S
KPD_830 (A1)	A^{71}→G		V^{69}→I
			I^{84}→L
			A^{96}→ N

			A^{101}→S
KPD_846_GP46F.ab1(A1)	60-101(del 75)	0	A^{96}→ N; Q^{66}→ P
			A^{96}→ N; R^{68}→E
			Ins(66a = G, 68a = S)
			60-101(del 70)
KPD_860 (A1)	K^{77}→R	O	I^{84}→L; A^{96}→ N
			A^{101}→S
KPD_982 (A1)	K^{77}→R	0	A^{67}→ T; A^{96}→ N
			A^{101}→S
KPD_1023 (D)	K^{77}→R	L^{91}→H	V^{69}→I; I^{84}→L
			A^{96}→ N; A^{101}→S
KPD_1034 (A1)	K^{77}→R	0	A^{96}→ N
KPD_1156 (A1)	0	0	A^{96}→ N; A^{101}→V
KPD_1168 (C)	V^{72}→L; K^{77}→R	L^{91}→R	T^{94}→P; P^{98}→R
			A^{101}→S
KPD_1178 (A1)	R^{74}→K	0	A^{96}→ N; T^{94}→P
			C^{87}→L; A^{101}→S
KPD_1216 (A1)	V^{72}→L	R^{74}→K	A^{96}→ N; T^{94}→P
			C^{87}→L; A^{101}→S
KPDR_151 (A1)	0	0	I^{84}→L; A^{96}→ T;
			T^{94}→S; A^{101}→S
KPDR_194 (A1)	V^{72}→L	0	A^{96}→ N; A^{101}→S
KPDR_199 (D)	R^{74}→S	0	V^{69}→I; I^{84}→L
			A^{101}→S
KPDR_202 (A1)	K^{77}→R	0	A^{96}→ T; A^{101}→S
KPDR_288 (A1)	V^{72}→L	0	A^{96}→ N; A^{101}→S
KPDR_289 (A1)	0	0	A^{96}→ T

KPDR_290 (A1)	0	0	$L^{70}\rightarrow Q$; $I^{84}\rightarrow L$; $A^{96}\rightarrow T$; $A^{101}\rightarrow T$

There were 331 (7.9%) substitutions in the Consensus HIV *Env* gp41 IDR peptides (Kenya) of the possible 4186 (46aa x 91 sequences). Out of these substitution 151 were due to the substitution in positions $A^{96}\rightarrow N$ (n = 79) and $A^{101}\rightarrow S$ (n = 75). Both Consensus HIV *Env* gp41 IDR peptides (Kenya) and Consensus HIV *Env* gp41 IDR peptides (Global) showed common substitutions rate of 4.2% (n = 151). Most (85%) substitutions occurred at positions G61 (n = 9); R68 (n = 11), V72 (n = 18), K77 (n = 41), I84 (n = 19), L91 (n = 14), T94 (n = 17), A96 (n = 79) and A101 (n = 75). Fifteen (15) amino acid positions (W60, K63, Q64, L65, V69, L76, W85, G86, G89, T95, V97, W99, N100, S102 and W103) were 100% conserved and they did not have any substitution. The most common substitutions were $A^{101}\rightarrow S$ (n = 68), $A^{96}\rightarrow N$ (n = 50), $K^{77}\rightarrow R$ (n = 31) and $I^{84}\rightarrow L$ (n = 17). It is worth noting that the only glycosylation site (out of 81 sites on the entire *Env* gene) in the sequence is N100 and it is 100% conserved. Thirty Nine (39) substitutions were single substitutions. The Study identified five cases of deletions {two cases of 60-101(del 96), 60-101(del 63) and 60-101(del 70), 60-101(del 71)} and three cases of insertions {Ins(93a = P), Ins(68a = S, 68b = P), Ins(66a = G, 68a = S)}. Substitutions involving Cysteine Loop were 25 and only one involved substitution of S ($S^{88}\rightarrow A$) *(Table 4.2 and Table 4.3)*.

Table 4.2 the frequency of amino acid substitutions at various positions in gp41-IDR peptide (Kenya)

Amino acid position	Frequency of substitutions	Amino acid Position	Frequency of substitutions
W60	0	G83	2
G61	9	I84	19
I62	2	W85	0

K63	0	G86	0
Q64	0	C87	3
L65	0	S88	1
Q66	4	G89	0
A67	4	K90	6
R68	11	L91	14
V69	0	I92	1
L70	3	C93	1
A71	5	T94	17
V72	18	T95	0
E73	0	A96	79
R74	6	V97	0
Y75	1	P98	1
L76	0	W99	0
K77	41	N100	0
D78	1	A101	75
Q79	2	S102	0
Q80	1	W103	0
L81	3		
L82	1	Total substitutions = 331 (151* after removing Con Global Sequence differences)	

It was noted that there were 74 different substitutions (in comparison with HXB2 sequences) with *Env* gp41 IDR from 91 sequenced samples. Out of these 74 substitutions 29 (39.2%) were unique with these Kenya sequences as "BLASTING" the substituted sequences in the NCBI Database did not yield any similar sequence *(Table 4.3)*.

Table 4.3 The frequency of various types of amino acid substitutions in HIV gp41-IDR peptide (Kenya) in comparison with HXB2 Reference Sequence

Amino acid and position	Frequency	Comment on substitution	Unique or common substitution(s)
A101→S	68	"A" specific for Subtype B, S for other subtypes	Not unique
K77→R	31	Documented with K.CD.97.EQTB11C	Not unique

A96→ N	50	"A" specific for Subtype B, N for other subtypes	Not unique
I84→L	17	Documented with CON_01_AE	Not unique
A96→ T	16	Documented with CON_A2	Not unique
V72→L	15	Documented with CON_A2	Not unique
T94→P	12	Documented – NCBI Acc.No. AHL45799.1	Not unique
A96→T	9	Documented with CON_A2	Not unique
R68→S	8	Not in NCBI data bank	Unique
L91→H	7	Documented with CON_D	Not unique
V69→I	7	Documented with CON_B	Not unique
A71→G	5	Not in NCBI data bank	Unique
L91→I	10	Documented with CON_01_AE	Not unique
K90→R	5	Documented – NCBI Acc. No AAT05976.1	Not unique
A101→T	5	Documented with CON_01_AE	Not unique
K77→Q	4	Documented with CON_A2	Not unique
T94→S	4	Not in NCBI data bank	Unique
K77→V	3	Not in NCBI data bank	Unique
V72→I	3	Documented with CON_C	Not unique
V69→I	7	Documented with CON_C	Not unique
A71→G	5	Not in NCBI data bank	Unique
K90→R	5	Documented – NCBI Acc. No. AEJ21211.1	Not unique
A101→T	5	Documented – NCBI Acc. No.AAM51932.1	Not unique
K77→Q	4	Documented – NCBI Acc. No. AAS72633.1	Not unique

T94→S	4	Not in NCBI data bank	Unique
K77→V	3	Not in NCBI data bank	Unique
V72→I	3	Documented – NCBI Acc. No. AAM51928.1	Not unique
A96→F	3	Documented – NCBI Acc. No. AAT05719.1	Not unique
A67→T	3	Documented – NCBI Acc. No. ABD33532.1	Not unique
I62→V	2	Not in NCBI data bank	Unique
L70→ Q	2	Not in NCBI data bank	Unique
C87→L	2	Not in NCBI data bank	Unique
R74→K	2	Documented – NCBI Acc. No. AAK92303.1	Not unique
R74→G	2	Documented – NCBI Acc. No. ACT87517.1	Not unique
L81→I	2	Documented – NCBI Acc. No. AAF71944.1	Not unique
K77→E	1	Documented – NCBI Acc. No. ACT87100.1	Not unique
D78→H	1	Not in NCBI data bank	Unique
Q^{79}→R	1	Documented – NCBI Acc. No. AHL45919.1	Not unique
S^{88}→A	1	Documented – NCBI Acc. No. AHL46250.1	Not unique
K^{90}→V	1	Not in NCBI data bank	Unique
A^{101}→Y	1	Not in NCBI data bank	Unique
G^{83}→A	1	Not in NCBI data bank	Unique
I^{84}→F	1	Not in NCBI data bank	Unique
R^{99}→W	1	Documented – NCBI Acc. No. ACT87100.1	Not unique
V^{77}→I	1	Documented – NCBI Acc. No. ACT87100.1	Not unique

I^{84}→V	1	Not in NCBI data bank	Unique
C^{87}→S	1	Not in NCBI data bank	Unique
Q^{66}→K	1	Not in NCBI data bank	Unique
Q^{66}→H	1	Not in NCBI data bank	Unique
Q^{66}→R	1	Documented – NCBI Acc. No. AHL46250.1	Not unique
R^{74}→S	1	Documented – NCBI Acc. No. AAD04415.1	Not unique
L^{91}→R	1	Not in NCBI data bank	Unique
P^{98}→R	1	Not in NCBI data bank	Unique
A^{101}→V	1	Not in NCBI data bank	Unique
A^{67}→ T	1	Documented – NCBI Acc. No. ABI23936.1	Not unique
R^{68}→E	1	Not in NCBI data bank	Unique
Q^{66}→ P	1	Documented – NCBI Acc. No. ADI60486.1	Not unique
A^{96}→ S	1	Documented – NCBI Acc. No. AAO65762.1	Not unique
R^{74}→A	1	Documented – NCBI Acc. No. AAL08731.1	Not unique
K^{77}→T	1	Documented – NCBI Acc. No. AFN07534.1	Not unique
Q^{80}→R	1	Documented – NCBI Acc. No. ACE74559.1	Not unique
L^{91}→P	1	Not in NCBI data bank	Unique
L^{70}→Q	1	Not in NCBI data bank	Unique
G^{83}→R	1	Documented – NCBI Acc. No. AEJ21241.1	Not unique
R^{68}→K	1	Documented – NCBI Acc. No.AAK92345.1	Not unique
Q^{79}→H	1	Not in NCBI data bank	Unique

C^{93}→S	1	Not in NCBI data bank	Unique
L^{81}→P	1	Not in NCBI data bank	Unique
L^{82}→P	1	Documented – NCBI Acc. No. AAT07342.1	Not unique
K^{77}→G	1	Documented – NCBI Acc. No. AAT74966.1	Not unique
V^{69}→L	1	Documented – NCBI Acc. No. AAT74965.1	Not unique
Y^{75}→F	1	Documented – NCBI Acc. No AHL46250.1	Not unique
T^{94}→A	1	Documented – NCBI Acc. No ADI60900.1	Not unique
V^{69}→M	1	Not in NCBI data bank	Unique

NB: The grey shaded substitutions are not documented in the NCBI database and they are unique in this study

4.2.2 Analysis of diversity by Shannon Entropy-Two of HIV *Env* gp41-IDR peptide (Kenya)

When Shannon Entropy-Two of alignment of ninety one HIV *Env* gp41 IDR peptide sequences obtained in this study was determined using Los Alamos "Entropy" software (Foley *et al.* 2013) with HXB2 gp41 IDR peptide as a background sequence, it was confirmed that position 77R was the most variable position with the Shannon Entropy value of 1.266 followed by N96 (1.217), 74R (0.813), 94T (0.755), 91L (0.742), 84 (I) and 101S (0.629) *(Table 4.4 and Figure 4.4)*. Sixty seven point four percent (67.4%) of the amino acid positions were conserved with the Entropy value of below 0.25 (baseline Entropy) with the most conserved positions being positions 60W (entropy value of 0.06) and 61G (entropy value of 0.0). This result agrees with those depicted in *Figure 4.2*.

Table 4.4 Values of Shannon-Two entropy of alignments of 91 HIV *Env* gp41 IDR peptide sequences

Position	Consensus in query set	Query entropy (Hq)
60.	W	**0.06**
61.	G	**0**
62.	I	**0.226**
63.	K	**0.121**
64.	Q	**0.121**
65.	L	**0.121**
66.	Q	**0.489**
67.	A	**0.3**
68.	R	**0.121**
69.	V	**0.52**
70.	L	**0.226**
71.	A	**0.241**
72.	V	**0.737**
73.	E	**0.121**
74.	R	**0.813**
75.	Y	**0.181**
76.	L	**0.181**
77.	R	**1.266**
78.	D	**0.226**
79.	Q	**0.181**
80.	Q	**0.166**
81.	L	**0.332**
82.	L	**0.121**
83.	G	**0.122**
84.	I	**0.686**
85.	W	**0.121**
86.	G	**0.121**
87.	C	**0.181**
88.	S	**0.166**
89.	G	**0.122**
90.	R	**0.441**
91.	L	**0.742**
92.	I	**0.181**
93.	C	**0.181**
94.	T	**0.755**
95.	T	**0.181**
96.	N	**1.217**
97.	V	**0.226**
98.	P	**0.286**
99.	W	**0.241**
100	N	**0.166**
101	S	**0.629**
102	S	**0.509**
103	W	**0.181**

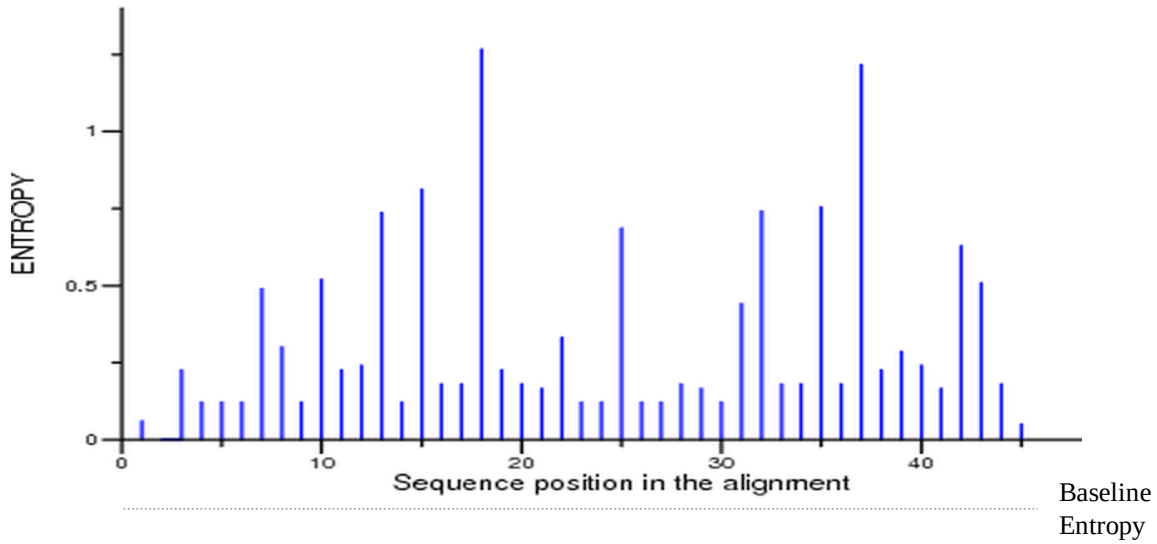


Figure 4.4 Shannon Entropy-Two of alignments of 91 gp41 IDR peptide 60 **ences. Posi** 70 **74R, 77R,** 80 **4T**and **N9(** 90 **the highest** 100 **py values while 67.4% of the amino acid positions were** highly **conserved with the Entropy value**s **of below 0.25.**

4.2.3 HIV Sub-types and Recombinants

Of the 91 samples that were sequenced the sub-type distribution was A1 (76.9%), C (6.6%), D (14.3%) and CRF A2.CY.94CY017_41 (2.2%).

4.2.4 The Phylogenetic Tree of selected nucleotide sequences of *Env* gp41 IDR (Kenya)

The Phylogenetic Tree of sequences of *Env* gp41 IDR showed six (6) major clusters (1-6). Cluster 1 contained most of the subtype D and some subtype A1. Cluster 2 contains mainly HIV subtype A1 and one subtype D. Cluster 3 contained mainly HIV subtype A1 and most of the subtype C. Cluster 4 contained subtype A1 only. Cluster 5 contained all subtype A1 and 1 subtype C mainly HIV subtype A1 and D and one subtype C *(Figure 4.5)*. Cluster 6 contained subtype A and D. The Bootstraps values (various) and confidence of branching (0.5) are indicate on the phylogenetic tree.

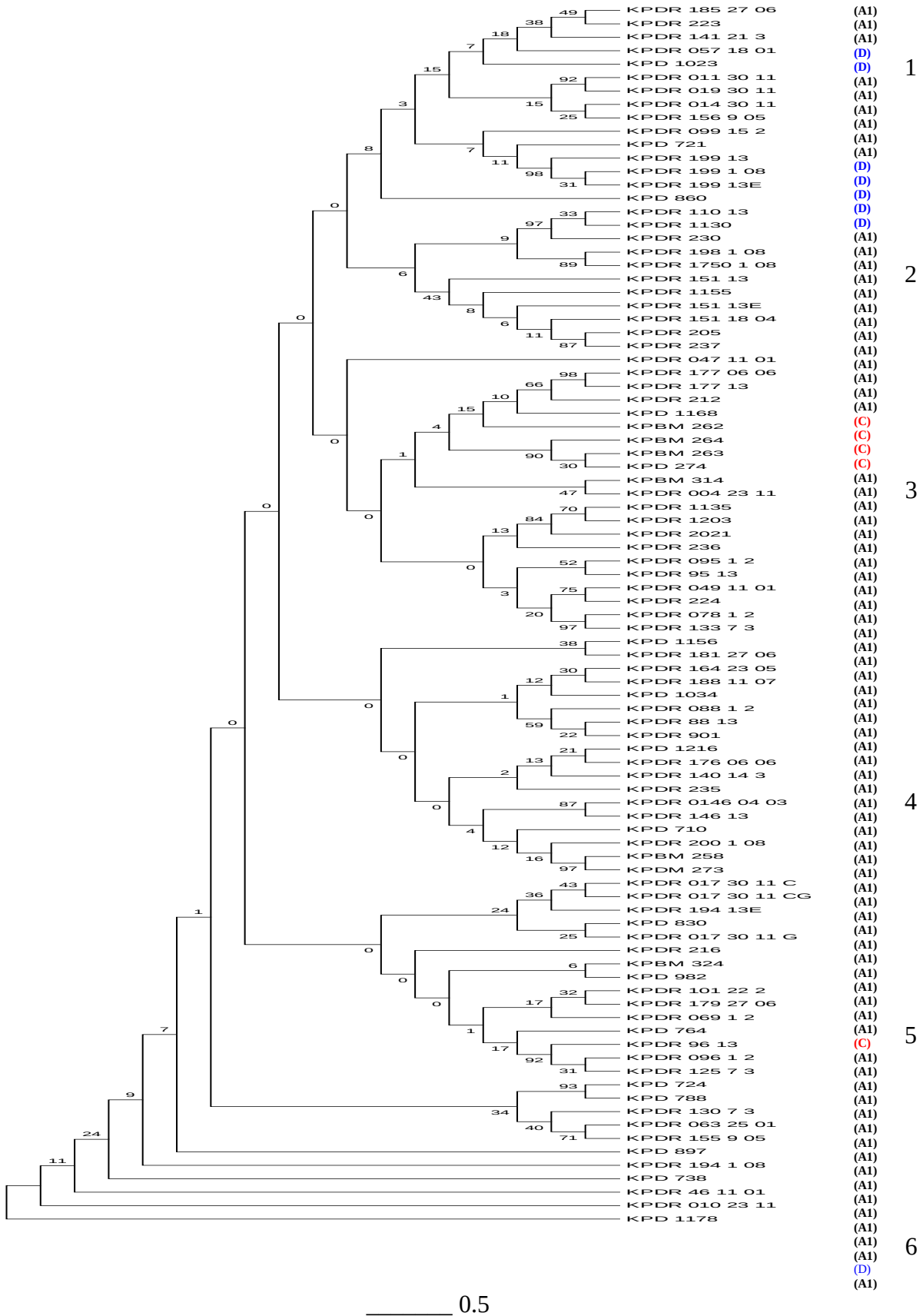


Figure 4.5 Phylogenetic tree of sequences of *Env* gp41 IDR (Kenya)

4.3 Determination of the proportion of the recent HIV infections among blood donors in Kenya

Determine™ HIV-1/2 Ag/Ab Combo LFA, did not detect any p24 antigen in all 400 samples (200 HIV positive and 200 HIV negative). Also, none of the 200 HIV ELISA negative samples that were screened for HIV viral RNA showed positive result.

4.3.1 HIV incidence

Confirmation of the performance of the r-IDR-M prepared in the study

The recombinant Immunodominant Region-M (r-IDR-M) was expressed from *Escherichia coli* BL21-CodonPlus DE3-RP (Stratagene, La Jolla, CA) and protein purified as stipulated in section 3.9. As a confirmatory step, the performance of the r-IDR-M generated in this study was compared with the standard r-IDR-M that had been provided by the Centers for Diseases Control and Prevention of USA (CDC). The ELISA was conducted as stipulated in section 3.9. The Signal: Noise Ratios (Optical density of the sample divide by Optical Density of Negative Control) were calculated for both ELISA tests system. Generally, the r-IDR-M expressed in this study showed a higher Signal: Noise ratio than the Standard r-IDR-M (*Figure 4.6)* implying that the in-house produced r-IDR-M displayed less non-specific signal, possibly due to its better quality of purification.

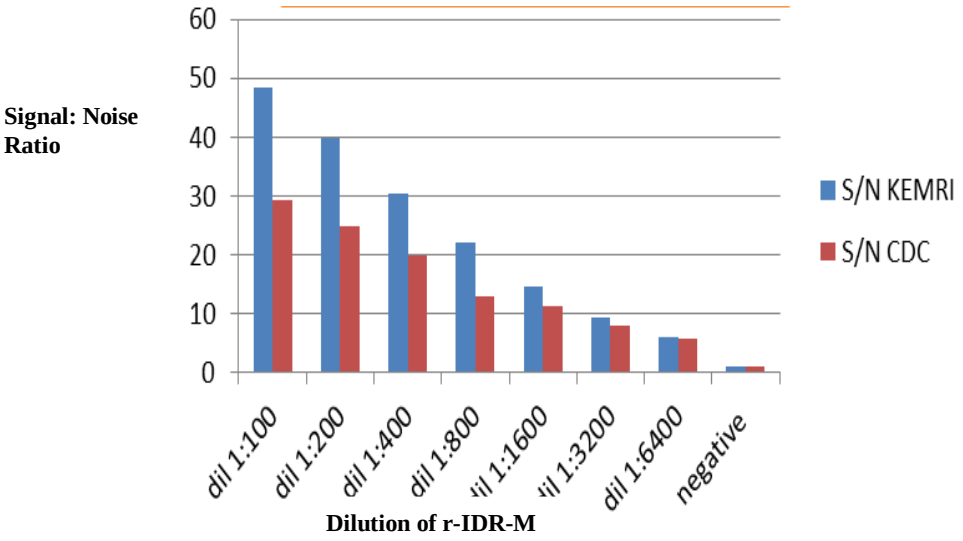


Figure 4.6 Comparative performance of in-house expressed r-IDR-M peptide (S/N KEMRI) and the Standard r-IDR-M (S/N CDC). Generally the in-house expressed peptide showed a higher Signal: Noise ratio than the Standard.

HIV incidence using wells coated with Consensus HIV *Env* gp41 IDR (Kenya), Consensus HIV *Env* gp41 IDR (Global) and r-IDR-M peptides

The Standard ELISA procedure (carried out using wells coated with rIDR-M peptide) gave the HIV Incidence of 31.9% while the tests that were carried out using wells coated with Consensus HIV *Env* gp41 IDR (Global) peptide and Consensus HIV *Env* gp41 IDR (Kenya) peptide gave the HIV Incidence of 76.9% and 41.8% respectively using the Avidity Index (AI) cut off point of 80% *(Figure 4.7 and Appendix 5)*. Tests that were carried out using wells coated with Consensus HIV *Env* gp41 IDR (Kenya) peptide were closer to the Standard than those from Consensus HIV *Env* gp41 IDR (Global) peptide.

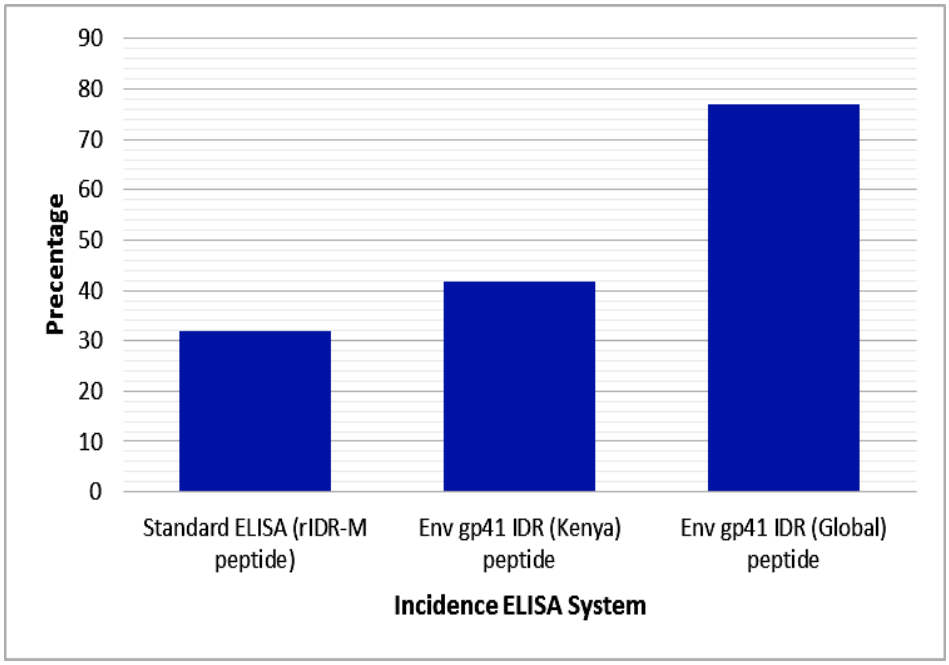


Figure 4.7 Comparison of the HIV incidence test results of ELISA system coated with three peptides: rIDR-M, Consensus HIV *Env* gp41 IDR (Kenya) and Consensus HIV *Env* gp41 IDR (Global) respectively.

4.4 Development of HIV 1 & 2 ELISA and LFT prototype kits using Consensus HIV *Env* gp41-IDR peptide generated in the project

4.4.1 Development of gp41 ELISA System

Initial experiment to check the performance of consensus HIV *Env* gp41 ELISA (Kenya)

The ELISA double strip was coated with Consensus HIV *Env* gp41 IDR (Kenya). A HIV positive sample (Lot no. 8139) was double serial diluted (in duplicate) to the 5th dilution. The standard ELISA was carried out using Goat anti-human IgG-HRP and TMB detection system. The results showed a characteristic serial dilution trend indicating that the Consensus HIV *Env* gp41 IDR (Kenya) system was responsive to the changes in the concentration of analyte and the experiment could be optimized to give better response *(Figure 4.8)*.

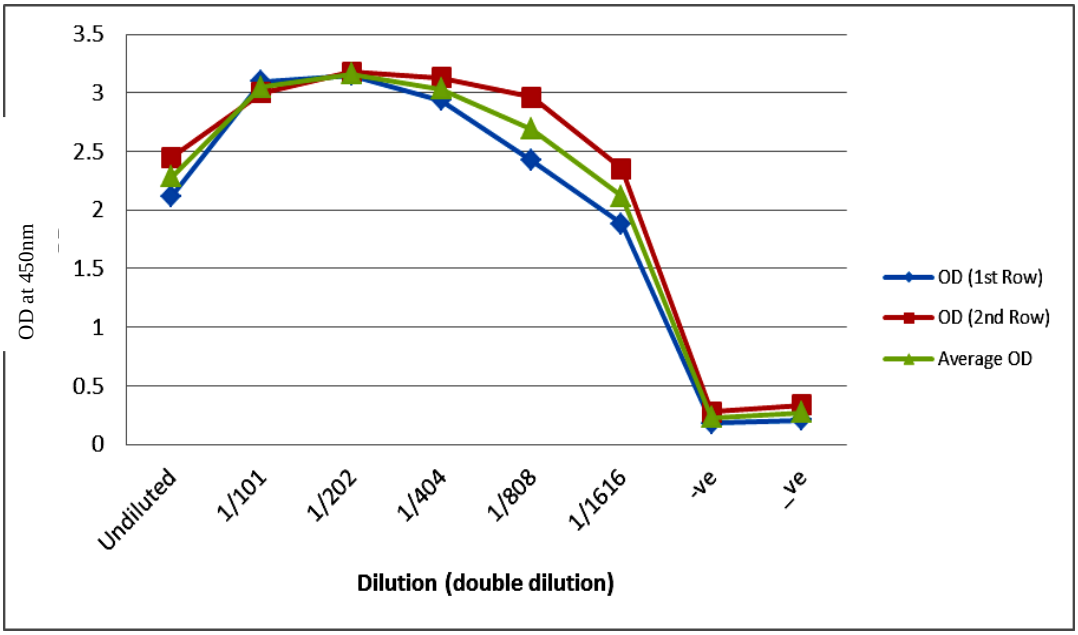


Figure 4.8 Performance of the initial ELISA plate coated with Consensus HIV *Env* gp41 IDR (Kenya).

Optimization of blocking buffers for Consensus HIV *Env* gp41 (Kenya) ELISA system

An experiment was carried out to establish the optimum concentration of Tween 20 as a diluent for gp41-HRP conjugate (Abcam, Cambridge, UK) which is the critical step for development of HIV ELISA. The same samples were run together with Vironostika™ Uni-Form II Ag/Ab ELISA (bioMérieux, Marcy-l'Etoile, France) as a reference Standard kit. Increased concentration of Tween 20 resulted in better performance of up to the concentration of 50% (results close to that of Vironostika System) when the performance started decreasing. The Tween 20 diluent buffers also gave low background signal with negative control *(Figure 4.9)*.

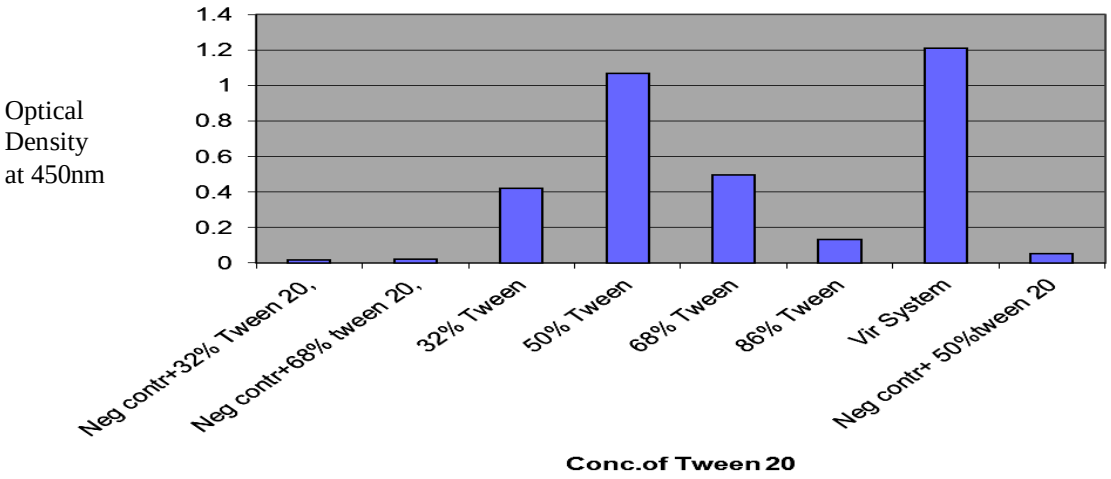


Figure 4.9 Performance of various concentrations of Tween 20 as diluents of gp41-HRP.

Effect of using various ratios of gp41-HRP and p120-HRP on gp41 ELISA

ELISA

The two conjugates, gp41-HRP (Abcam, Cambridge, UK) and gp120-HRP (Abcam, Cambridge, UK) were mixed in the ratios 75:25, 50:50 and 25:75 and used to run the ELISA with wells coated with Consensus HIV *Env* gp41 IDR (Kenya).

As the proportion of gp120-HRP was increased the optical activity decreased *(Figure 4.10)*. The results indicated that there was no need of including gp120-HRP in development process of HIV ELISA as gp41-HRP could work alone.

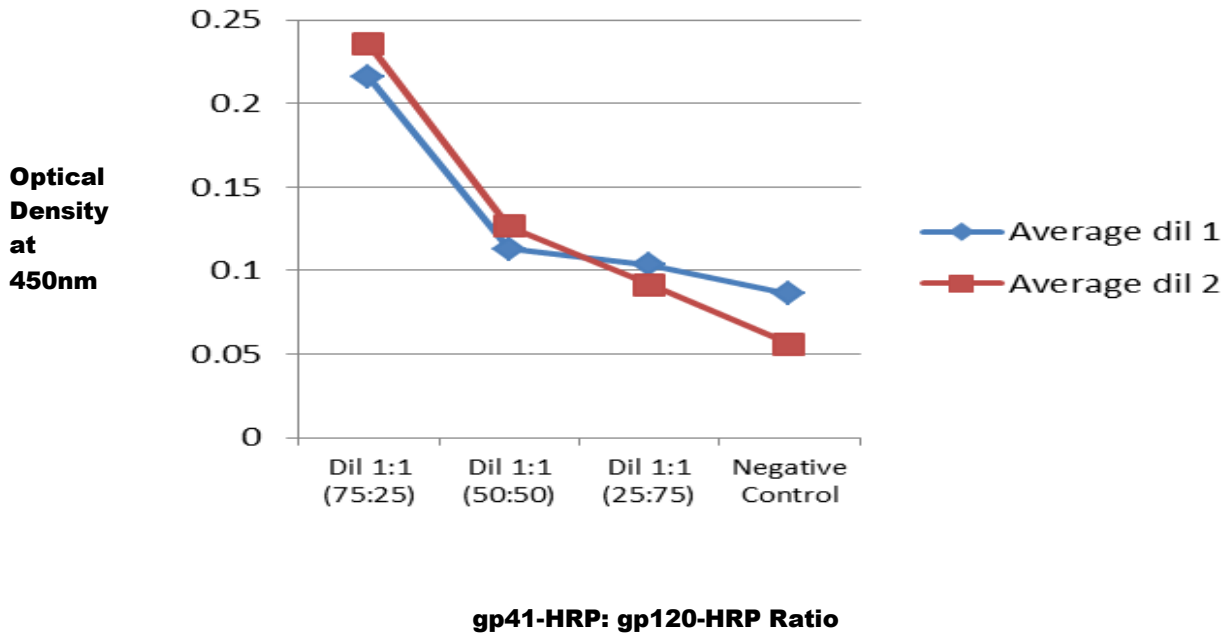


Figure 4.10 Effect of using various ratios of gp41-HRP and gp120-HRP on gp41 IDR coated ELISA

Comparison of the performance of consensus HIV *Env* gp41 IDR (Global) and consensus HIV *Env* gp41 IDR (Kenya)

Two sets of ELISA wells (16 wells each) were coated with consensus HIV *Env* gp41 IDR (Global) and consensus HIV *Env* gp41 IDR (Kenya) respectively and the ELISA tests carried out using the previously optimized conditions that included the use of gp41-HRP diluted in normal p24 dilution buffer (0.5% BSA, 0.05% Tween 20 in Tris buffer, pH 7.4). Although the wells that had been coated with consensus HIV *Env* gp41 IDR (Kenya) showed initial higher optical density values *(Figure 4.11)* both systems had the same Analytical Sensitivity (with limit of detection at the dilution of 1:400)

The two conjugates, gp41-HRP (Abcam, Cambridge, UK) and gp120-HRP (Abcam, Cambridge, UK) were mixed in the ratios 75:25, 50:50 and 25:75 and used to run the ELISA with wells coated with Consensus HIV *Env* gp41 IDR (Kenya).

As the proportion of gp120-HRP was increased the optical activity decreased *(Figure 4.10)*. The results indicated that there was no need of including gp120-HRP in development process of HIV ELISA as gp41-HRP could work alone.

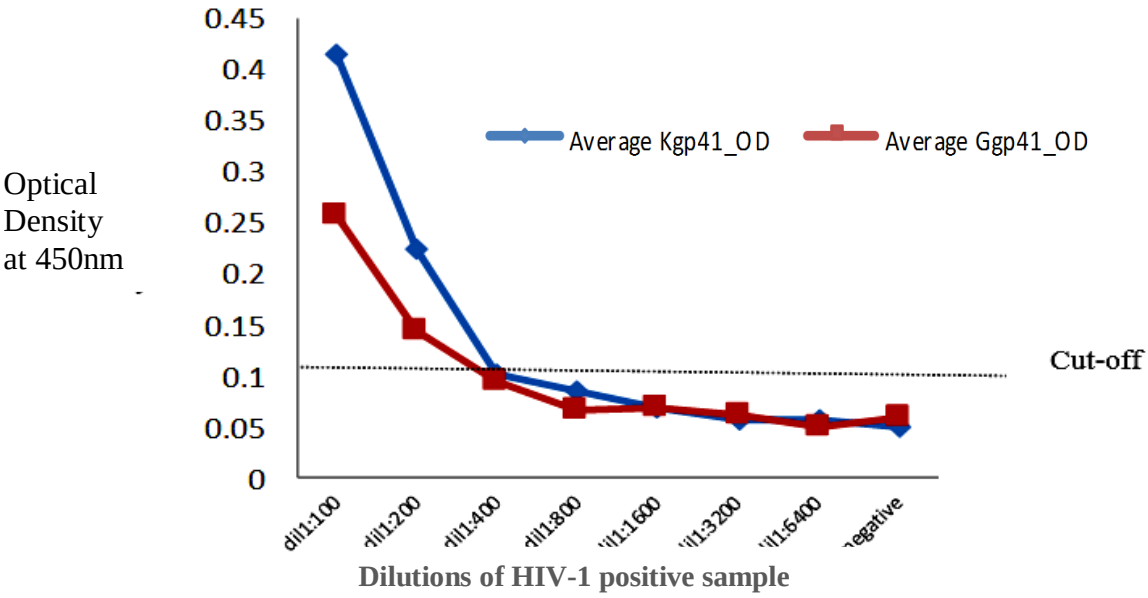


Figure 4.11 Comparison of performance of consensus HIV *Env* gp41 IDR (Global) and consensus HIV *Env* gp41 IDR (Kenya)

Development of p24 ELISA System

Comparative performance of conjugate diluent containing 50% Tween 20 and alternative p24 antibody dilution buffer

The difference between the performances of 50% Tween 20 (Sigma, St. Louis, Mo.) and p24 antibody dilution buffer as diluents for Goat anti-human IgG-HRP *(Figure 4.12)* was found not to be significant as the Two-Sample F-Test gave the F-Statistic value of 0.8931 and p-value of 0.8852 (F-Statistic value >P-value).

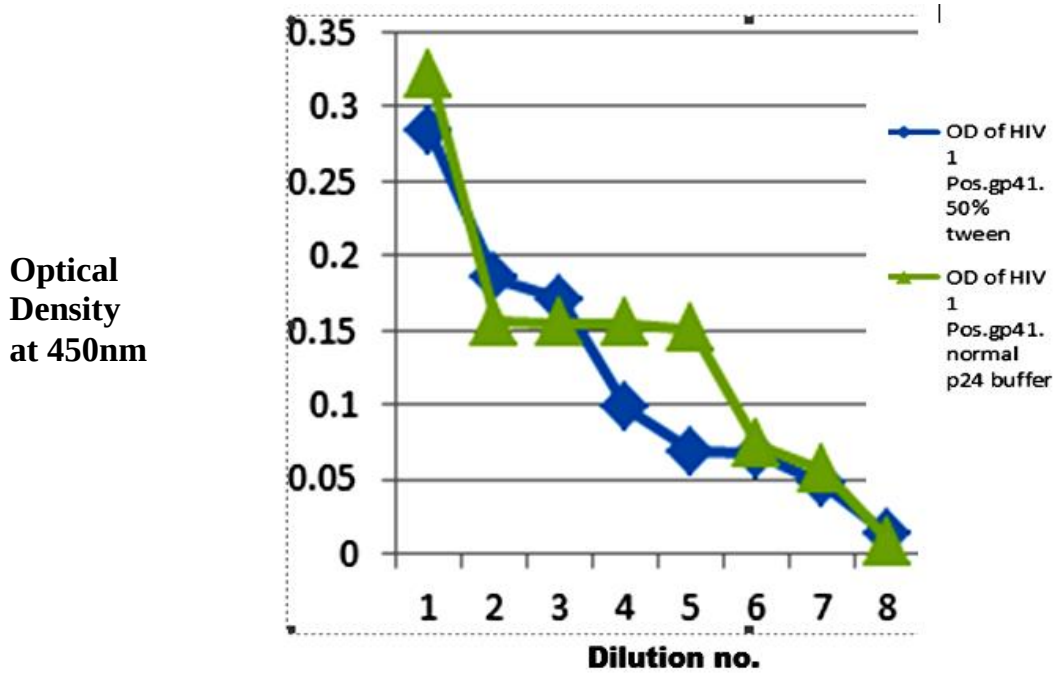


Figure 4.12 Comparative performance of two conjugate diluent: 50% Tween 20 and p24 antibody dilution buffer

Determination of the Standard curve trend for the p24 ELISA

A serial 2-fold diluted p24 antigen standard (Abcam, Cambridge, UK) was incubated in p24 antibody-coated wells starting with 1000pg/ml up to 7th dilution with 8thwell being the blank. The signal was detected using matching pair of anti-p24-HRP, Lot No 9072 (Abcam, Cambridge, UK) and TMB substrate system. The experiment showed a good repeatability of the two duplicate runs and a characteristic serial dilution pattern with a detection limit being 62.5pg/ml (*Figure 4.13)*

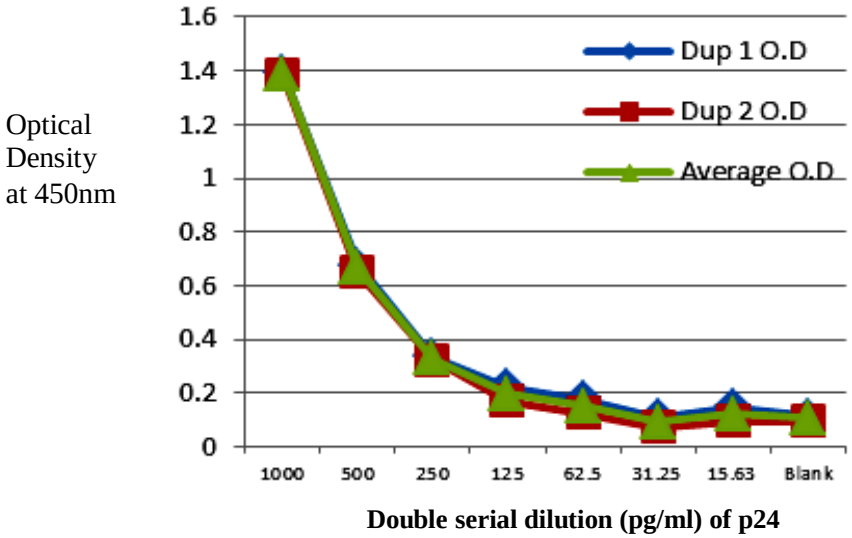


Figure 4.13 Serial dilution of p24 antigen ELISA to prepare a calibration curve

The calibration curve was prepared by calculation of the Log of the Optical densities and Log of the concentrations of p24 antigen pg/ml of the data illustrated in *Figure 4.14*. The calibration curve was used to read the concentrations of p24 antigen in samples using the optical densities.

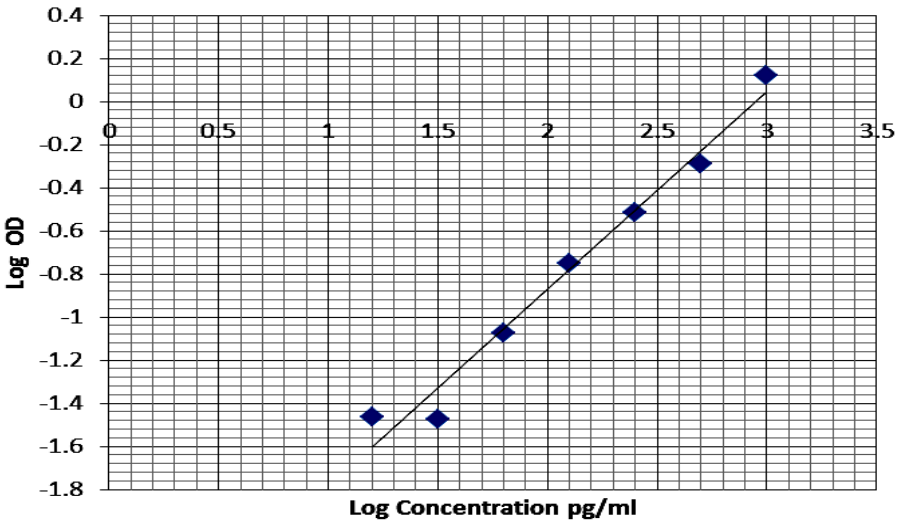
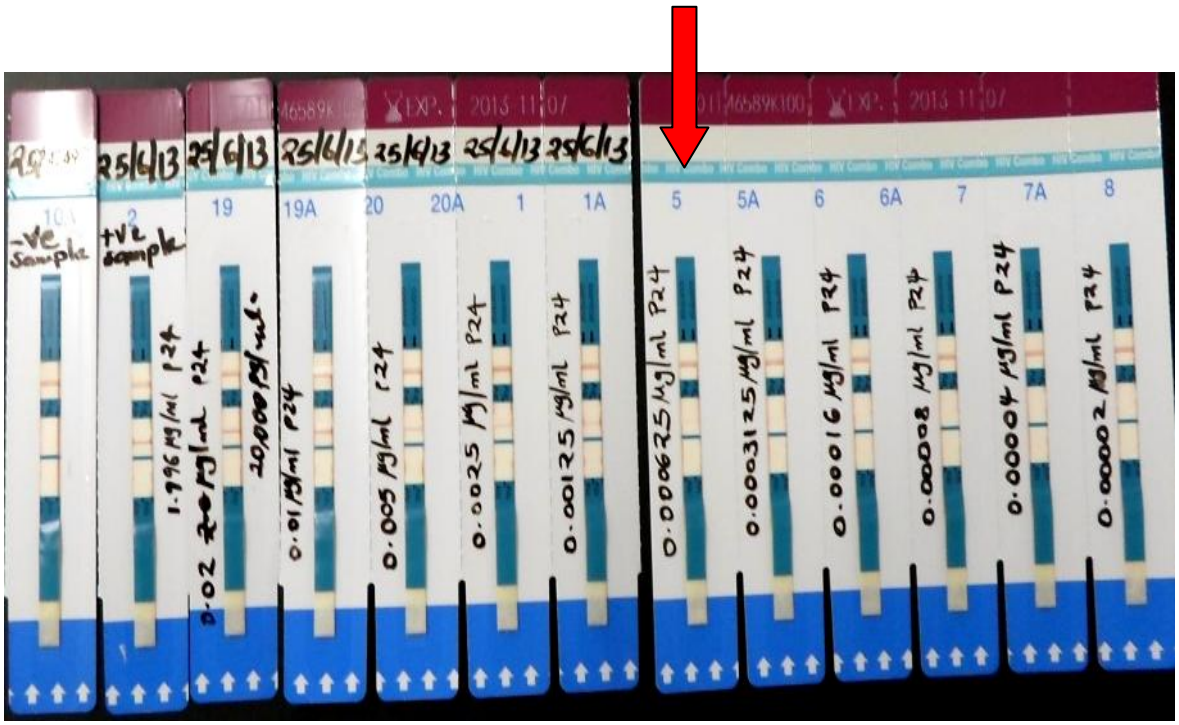


Figure 4.14 Calibration curve for p24 ELISA

Development of the Working Standard for p24 and determination of Sensitivity of Vironostika™ and Determine™ on P24 antigen

It was necessary to develop the Working Standards to be used for subsequent experiments in this study. It was also important to determine the comparative performance of Determine™ HIV-1/2 Ag/Ab Combo (Abbott Diagnostic Division, Hoofddorp, The Netherlands) and Vironostika™ Uni-Form II Ag/Ab ELISA (bioMérieux, Marcy-l'Etoile, France). To determine this, serial 2-fold diluted p24 antigen standard was prepared and tested using Determine™ HIV-1/2 Ag/Ab Combo and Vironostika™ Uni-Form II Ag/Ab ELISA on p24 antigen (Abcam, Cambridge, UK) analyte starting with 1000pg/ml up to 7th dilution. Both kits had a detection limit of 62.5pg/ml (*Plate 4.2 and Figure 4.15).*

Plate 4.3 The Limit of Detection (LoD) of the Determine™ HIV-1/2 Ag/Ab Combo on p24 antigen analyte. At the LoD the red positive band of the test is not visible any more.



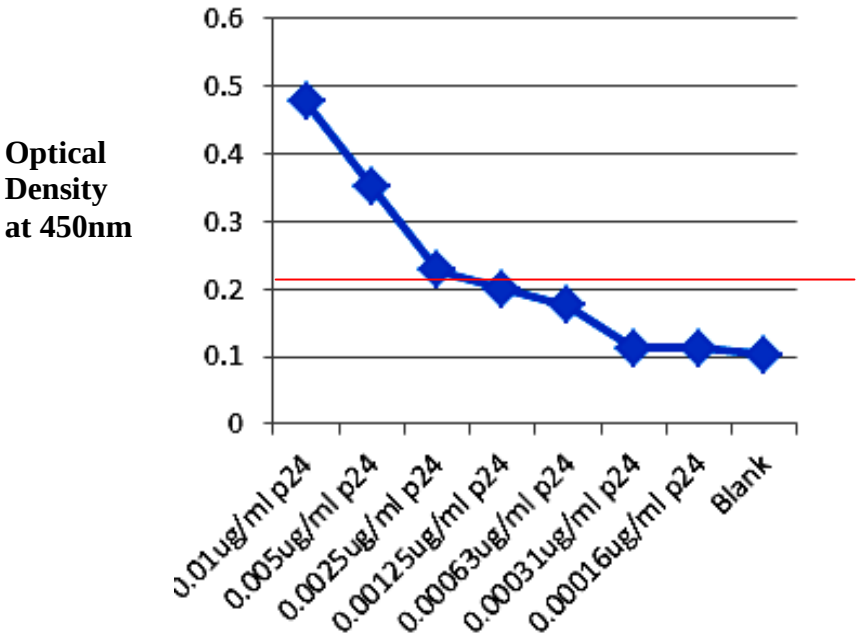


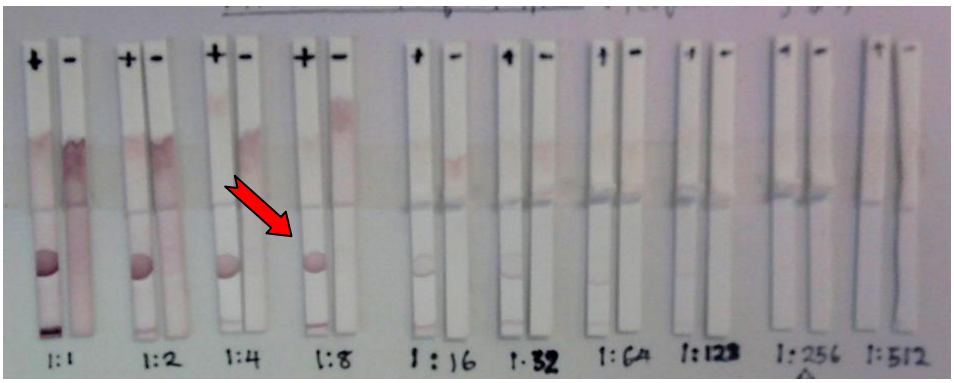
Figure 4.15 The limit of detection of the Vironostika™ Uni-Form II Ag/Ab ELISA (B) on p24 antigen analyte

4.4.2 Development of gp41 Lateral flow System

Optimization of Consensus HIV *Env* gp41 IDR (K) System in LFT

An experiment was carried out to optimize the gp41 IDR (K) system in rapid test kit. The experiments established that with gp41 IDR (K) spotting at a concentration of 1:5 and colloidal gold prepared from 5ug/ml gp41 (Arista Biologicals, Allentown, PA) and 11ml colloidal gold (Sigma, St. Louis, Mo.) the optimum dilution was 1:8 (Plate 4.3). The test picked HIV positive and negative sample without any nonspecific reaction.

Plate 4.4 Optimization of gp41 IDR (K) System in Lateral Flow Assay



Optimization of gp36 system in HIV Lateral Flow Assay

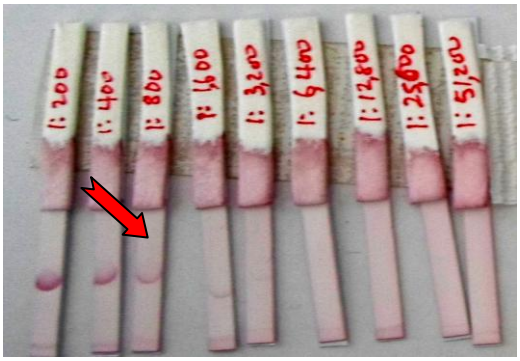
The aqueous dilution of 1:4 of 0.54 ug/ml of gp36 peptide (Arista Biologicals, Allentown, PA) was found to be the optimum dilution for coating of the nitrocellulose membrane in development of HIV lateral assay when gp36 colloidal gold conjugate (3.125 ul) diluted with 1% Tween 20 (25 ul) was used for signal detection. The dilution of 1:1 of 0.54 ug/ml gp36 had a high background signal while the dilution of 1:8 gave a faint signal *(Plate 4.4)*.

Plate 4.5 Optimization of gp36 system in Lateral Flow Assay

Optimization of concentration of anti-P24 Biotin in Lateral Flow Assay

The dilution of 1:800 biotinylated anti-p24 (ab 9072) was the maximum dilution that gave distinct signal *(Plate 4.5)* under the following conditions: use of nitrocellulose membrane (Merck, Darmstadt, Germany) coated with 25 ug/ml Avidin (Sigma, St. Louis, Mo.) in Double Diluted Water (DDW) (20ul), blocking using Casein 0.5% in 100mM Tris buffer at pH 7.6, use of anti-p24-biotinylated ab 9072 (Abcam, Cambridge, UK) at dilution of 1:100 and volume of 20 ul and detecting the signal using 5 ul of colloidal gold (Sigma, St. Louis, Mo.) conjugated with anti-p24 ab 9071 (Abcam, Cambridge, UK) at the dilution of 1:2. The dilution of 1:1600 biotinylated anti-p24 (ab 9072) was the limit of detection.

Plate 4.6 Optimization of concentration of anti-P24 Biotin in LFA



4.5 Evaluation of the performance of the developed HIV 1 & 2 ELISA and LFT

4.5.1 D-SN, D-SP, PPV and NPV using consensus testing from LFTs

As shown in *Table 4.5* the three (3) LFTs in the current HIV Testing Algorithm in Kenya (KHB Colloidal Gold**,** First Response™ 1-2.0 and Uni-Gold™ HIV) test showed a D-SN of 100% (95% CI: 97.4-100.0), 96.4% (95% CI: 91.8 - 98.8) and 100% (95%

CI: 97.4-100.0) respectively in relation to the Consensus status with LFTs. The other two kits that were also in use in Kenya, Determine™ HIV-1/2 and Aware™ HIV-of 1/2 BSP, had a D-SN of 100% (95% CI: 97.4-100.0%) and 99.3% (95% CI: 96.0 to 99.9%) respectively. The HIV 1/2 LFA developed in this study had D-SN of 99.3% (95% CI: 96.0 to 99.9%) that was comparable to Aware™ HIV. The KHB Colloidal Gold scored 100% (95% CI: 97.4-100.0) in D-SN, D-SP, PPV and NPV tests. Uni-Gold™ HIV test had the lowest D-SN but it scored 100% (95% CI: 97.4-100.0) in D-SP and PPV tests. The performance of the First Response™ 1-2.0 was the same as that of Determine™ HIV-1/2: D-SN of 100% (95% CI: 97.4-100.0) and PPV of 96.8% (95% CI: 83.2 - 99.5).

Table 4.5 Performance of various Rapid test kits in respect to Consensus status with LFAs

HIV Rapid Test Kit	D-SN (95% CI)	D-SP (95% CI)	PPV (95% CI)	NPV (95% CI)
Determine™ HIV-1/2	100 (97.4-100.0)	96.8 (83.2 - 99.5)	99.3 (96.1 - 99.9)	100 (88.3-100.0)
KHB Colloidal Gold	100 (97.4-100.0)	100 (88.7-100.0)	100 (97.4-100.0)	100 (88.7 - 100.0)
Uni-Gold™ HIV test	96.4 (91.8 - 98.8)	100.0 (88.7-100.0)	100.0 (97.3-100.0)	86.1 (70.5-95.3)
First Response™	100 (97.4-100.0)	96.8 (83.2 - 99.5)	99.3 (96.1- 99.9)	100 (88.3 - 100.0)
Aware™ HIV-1/2 BSP	99.3 (96.0 - 99.9)	100.0 (88.7-100.0)	100.0 (97.3 - 00.0)	96.9 (83.7-99.5)
In-House HIV 1/2 LFA	99.3 (96.0 - 99.9)	100.0 (88.7-100.0)	100.0 (97.3 - 00.0)	96.9 (83.7-99.5)

4.5.2 D-SN, D-SP, PPV and NPV using consensus testing from LFTs using ELISA

When compared with Vironostika™ Uni-Form II Ag/Ab ELISA (*Table 4.6)* as the Gold Standard the performance characteristics (D-SN, D-SP, PPV and NPV) of the LFTs were apparently reduced due higher Analytical Sensitivity of the ELISA. The three (3) LFTs in the current HIV Testing Algorithm in Kenya, KHB Colloidal Gold, First Response™ 1-2.0 and Uni-Gold™ HIV test, showed a D-SN of 95.9% (95% CI: 91.2-98.5%), 95.9% (95% CI: 91.2-97.4) and 93.8% (95% CI: 88.5-97.1) respectively. The D-SN of Determine™ HIV-1/2 that was the Screening kit in the immediate past HIV Testing Algorithm in Kenya HIV was 96.6% (95% CI: 92.2-98.9%), which was the highest among the tested LFTs. The Aware™ HIV-1/2 BSP had a D-SN of 95.2% (95%

CI: 90.3 - 98.0). All the LFTs tested in this study showed a D-SP of above 96.0% (95% CI: (79.6 – 99.3) in respect to Vironostika™ Uni-Form II Ag/Ab ELISA. All the LFTs tested displayed high PPV (above 98.6% (95% CI: 95.2-99.8) but low NPV, up to 73.5% (95% CI: 55.6-87.1) as shown by Uni-Gold™ HIV test. This implies that these LFTs had high probability of testing positive when the actual status of samples were positive but low probability of testing negative when the actual status of samples were negative.

Table 4.6 Performance of various Rapid test kits in respect to Vironostika™ Uni-Form II Ag/Ab ELISA

HIV Rapid Test Kit	D-SN	D-SP	PPV	NPV
	(95% CI)	(95% CI)	(95% CI)	(95% CI)
Determine™ HIV-1/2	96.6	100	98.6	83.3
	(92.2-98.9)	(86.2-100)	(95.2-99.8)	(65.3-94.30)
KHB Colloidal Gold	95.9	100	100	80.7
	(91.2-98.5)	(91.2-100.0)	(97.4-100.0)	(97.4 - 100)
Uni-Gold™ HIV test	93.8	100	100	73.5
	(88.5-97.1)	(86.2-100)	(97.3-100)	(55.6-87.1)
First Response™	95.9	96	99.3	80
1-2.0	(91.2-97.4)	(79.6 – 99.3)	(96.1 – 99.9)	(61.4-92.2)
Aware™ HIV-1/2 BSP	95.2	100	100	78.1
	(90.3 – 98.0)	(86.2 -100.0)	(97.4-100.0)	(60.0 – 90.7)
In-House HIV 1/2 LFA	95.2 (90.3 – 98.0)	100 (86.2 -100.0)	100 (97.4-100.0)	78.1 (60.0 – 90.7)
In-House HIV 1/2 ELISA (K)	97.24 (93.1-99.2)	100 (93.1 - 99.2)	100.0 (97.4-100.0)	86.2 (68.3 - 96.0)
In-House HIV 1/2 ELISA (G)	97.24 (93.1-99.2)	100 (93.1 - 99.2)	100.0 (97.4-100.0)	86.2 (68.3 - 96.0)

As illustrated in *Table 4.7* the number of observed agreements between the evaluation of the LFTs by use of Consensus test from five LFTs and Vironostika™ Uni-Form II Ag/Ab ELISA was 392 (98.0% of the observations) with Kappa = 0.967 (95% CI: 0.94-0.99) and P value of less than 0.0001 implying that the strength of agreement was 'very good'. The D-SN, D-SP, PPV and NPV of LFTs were 96.0% (95% CI: 92.3 – 98.3%),

100.0% (95% CI: 98.2100.0%), 100.0% (98.1- 100.0%) and 96.2% (95%CI: 92.6 – 98.3%) respectively.

Table 4.7 Diagnostic Sensitivity of Consensus LFTs in comparison with Vironostika™ Uni-Form II Ag/Ab ELISA

		Status as determined by Vironostika™ Uni-Form II Ag/Ab ELISA		
		Positive	*Negative*	
Status as determined by Consensus Status of 6 LFTs	*Positive*	192	0	192
	Negative	8	200	208
		200	200	

4.5.3 Analytical Sensitivity of the LFTs

In regards to testing of Analytical Sensitivity of the HIV LFTs, Determine™ HIV-1/2 showed a highest sensitivity in comparison with other HIV LFTs (*Figure 4.16)*. The performance of KHB ***Colloidal Gold*** and First Response™ 1-2.0 were comparable (13 and 11 for panel P-A-15; 9 and 11 for panel P-B-15 and 12 and 12 for P-C-15). Uni-Gold™ HIV showed the lowest Analytical Sensitivity (with detection limit at 9, 5 and 8 dilutions of the three HIV panels respectively).

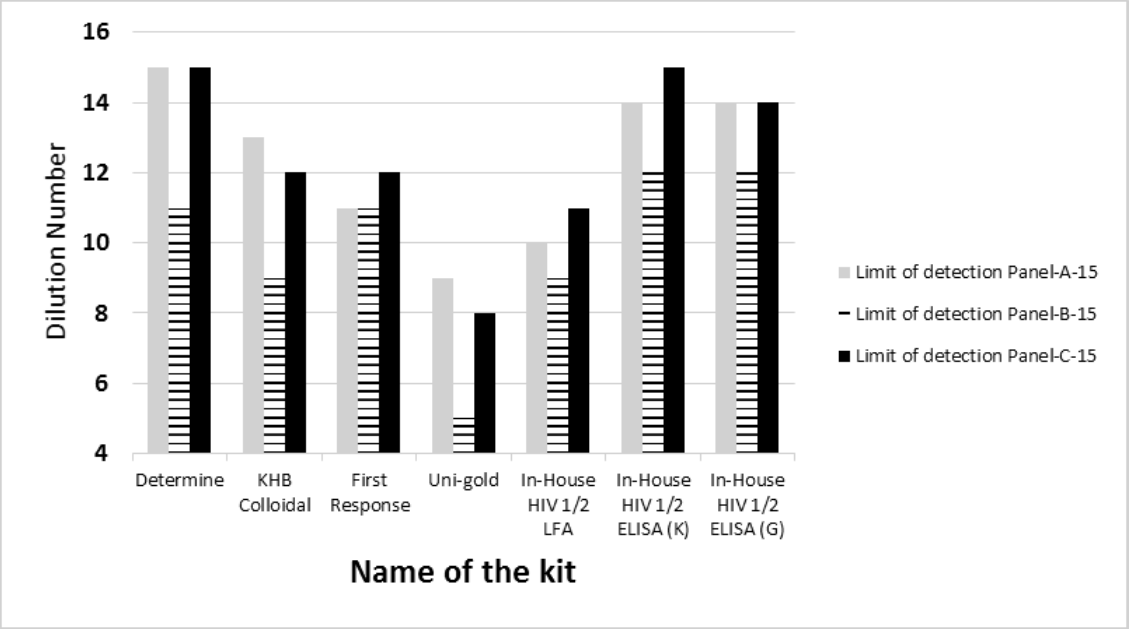


Figure 4.16. The relative Analytical Sensitivity of HIV LFTs in Kenya using panels prepared in-house

4.5.4 RNA Extraction from HIV negative samples with antibody tests

Out of 10 equal pools of the 250 HIV negative samples by immunological-based kits none yielded HIV viral RNA after extraction.

4.5.5 The need of a tie-breaker kit

With the testing of 400 samples in this study all the HIV positive samples with ***KHB Colloidal Gold*** were also positive with the First Response™ 1-2.0 and hence there was no need of re-testing with Uni-Gold™ HIV test if the tests were carried out using the current HIV Testing Algorithm in Kenya.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION5.1.1 The prevailing consensus sequence of HIV *Env* gp41 gene in Kenya

The study established the prevailing consensus sequence of Immunodominant Region (IDR) of HIV *Env* gene in Kenya to be "tggggcattaaacagctgcaggcgcgcgtgctggc

ggtggaacgctatctgaaagatcagcagctgctgggcatttggggctgcagcggcaaactgatttgcac

caccaacgtgccgtggaacagcagctgg". When this sequence was translated it gave peptide sequence: WGIKQLQARVLAVER YLKDQQLLGIWGCSGKLICTT**N**VP

WN**S**SW. This study established that relative to the consensus HIV *Env* gp41-IDR (global) from group M peptides (WGIKQLQARVLA VERYLKDQQLLGIWGCSGKLICTT

AVPWNASW) (Masciotra *et al.*, 2000) there were only two amino acid substitutions (A^{96}→N and A^{101}→S) and similarity of 95.3%. There was 100% sequence similarity in the key sub-regions of IDR of gp41: the CTL epitope (aa 71 to 82; AVERYLKDQQLL) and the Cysteine Loop (aa 87 to 93; CSGKLIC. In general, 67.4% of the amino acid positions were highly conserved with the Entropy value of below 0.25 (baseline Entropy) with the most conserved positions being positions 60W (entropy value of 0.06) and 61G (entropy value of 0.0). The nucleotide variation of 4.7% of consensus HIV *Env* gp41 IDR gene noted in this study is close to 5-15% intra-clade and 15-30% inter-clades that had been reported by Leitner (1996) given that 76.9% of *Env* gp41 IDR gene sequences analyzed were found to belong to clade A.

The amino acid residues, 82L, 85W, 86G, 99W and a basic residue, 90K are required for fusion function and for association of gp41 with gp120 (Cao *et al.,* 1993). In this study there was one substitution (L82→P), six substitutions K90 (Valine replacing lysine and arginine which is also basic replacing lysine in 5 cases) and no substitutions in positions W85, G86 and W99. The acidic residues, 73E (with no substitution) and 78D (with one substitution: D78→H) are also essential for fusion activity of gp41 with gp120. These acidic residues form intermonomer -73E with basic residue 68R and intramonomer -78D with basic residue 77K (Maerz *et al.,* 2001).

The study found that 29 amino acid substitutions on *Env* gp41 IDR that were unique with the HIV sequences in Kenya, which did not affect the detection of HIV antibodies.

5.1.2 Proportion of the recent HIV infections

Among the 400 panels tested, none tested positive for p24 antigen. Extraction of RNA from the 200 HIV negative panels did not yield any positive results. Brown *et al.* (1991) found that the prevalence of p24 antigenemia in sera from 200 asymptomatic HIV-1-infected individuals was 3.5% compared with 7% in individuals in the USA hence this study was expected to capture some p24 antigenemia. According to Constantine (2001) the common causes of an indeterminate neutralization test are: nonspecific reaction in an uninfected person, low p24 antigen levels in an infected person, or sample deterioration or antigen-antibody complex formation during storage. At the opportune time it may be necessary to repeat testing for p24 antigen using fresh samples rather than blood from blood donors' services.

Theoretically, some ELISA negative samples would have been expected to be positive

after nucleic acid test (NAT) as NAT is known to detect HIV earlier before sero-conversion (Branson, 2010). However, some studies have found no difference in sensitivity of the NAT and ELISA (Thangam *et al.*, 2014) which could be a similar case in this study. This could also mean that the HIV testing at NBTCs in Kenya was very effective.

The HIV Incidence (or Recency) testing conducted in this study using the Two-Well Avidity-Based assays (Wei *et al.*, 2010) found that 31.9% of 91 samples tested using ELISA wells coated with consensus HIV *Env* gp41 rIDR-M peptide (as the standard) were recent. When the same samples were tested for recency using wells coated with Consensus HIV *Env* gp41 IDR (Global) and Consensus HIV *Env* gp41 IDR (Kenya) peptides the HIV Incidence was found to be 76.9% and 41.8% respectively. Previous studies in Kenya reported the rate of recently HIV infection of 11% among the HIV seropositive individuals, using the BED CEIA testing protocol, with males aged 25-35 years old showing the highest percentage of recent HIV infection at 47%. The study also found that the age group with the largest discrepancy in recent infection between males and females was the 15-24 year old age group (36% in females vs. 13% in males) (WTWG-HIAM Report, 2009). According to NASCOP (2014) the current HIV Incidence in Kenya is 0.44% of the general population (or 7.3% of the HIV positive cases). In Kenya, about 70% of blood donors are aged between 15-19 years old while 30% are about 20-64 years old. This may partly explain the HIV Incidence of 31.9% noted in this study. The False Recency Rate (FRR) is reported to be about 5% due to presence of Elite controllers (individuals who naturally maintain low or undetectable HIV RNA levels and have low antibody responses) (WHO/UNAIDS, 2013) hence after adjustment the HIV Incidence is 26.9%.

5.1.3 Performance of the developed HIV 1 and 2 ELISA and LFT

Some important data was established during the pre-requisite stage of preparation of the panels for evaluation of the developed HIV kits in this study. The study observed an agreement of results of 98.0% (Kappa = 0.967 (95% CI: 0.941-0.993) and P value of less than 0.0001 between the evaluation of the LFTs by use of Consensus test from five LFTs and Vironostika™ Uni-Form II Ag/Ab ELISA implying that the strength of agreement was 'very good. The overall D-SN of using LFTs in HIV testing in Kenya was found to be 96.0% (95% CI: 92.3-98.3%). The D-SN of individual kits was as follows: KHB ***Colloidal Gold*** (95.9%), First Response™ 1-2.0 (95.9%), Uni-Gold™ HIV test (93.5%); Determine™ HIV-1/2 (96.6%), Aware™ HIV-1/2 BSP (95.2%), LFTs developed in this study (95.2%) and ELISA developed in this study (97.2%. Among the LFTs, Determine™ HIV-1/2 showed the highest D-SN of 96.6% with Uni-Gold™ HIV test showing the lowest D-SN of 93.5%. These results generally show slightly lower values in comparison with a number of studies that had established high sensitivity of LFTs when HIV ELISA kits were used as Gold Standard. Mine *et al.* (2015) found a D-SN and D-SP of 98.2% and 100% respectively for **KHB Colloidal Gold** against Vironostika™ Uni-Form II Ag/Ab ELISA as Gold Standard and D-SN and D-SP of 98.2% and 98.1% respectively for Uni-Gold™ HIV test against the same ELISA kit. However, Fabiani *et al.* (2005) found a low sensitivity of the HIV Testing Algorithm using Rapid Tests of 90.6% (95% CI: 78.6 -96.5) when compared with an ELISA system.

This study established that there was no significant difference between the performance of the HIV ELISA Testing kit that was developed with Consensus HIV *Env* gp41-IDR peptide (Kenya) and that which was developed using Consensus HIV *Env* gp41-IDR peptide (Global) (p-value = 1, the result is *not* significant at $p < 0.05$). The study also

established that the HIV testing kits that are used in Kenya are able to detect all HIV infections irrespective of numerous amino acid substitutions in the Immunodominant Region of p41 and distribution of subtypes. This study is in agreement with a study by Masciotra *et al.* (2000) who established that consensus HIV *Env* gp41-IDR from group M peptides (WGIKQLQARVLAVERYLKDQQLLGIWGCSGKL ICTTAVPWNASW) was able to detect all 130 group M sera (10 subtype A, 21 subtype B, 13 subtype B9, 20 subtype C, 21 subtype D, 14 subtype E, 25 subtype F, and 6 subtype G). The findings also agree with the findings by Bártolo and Taveira (2012) that despite the high genetic divergence between HIV-1 groups M and N, all group N infections had been detected using five commercial HIV immunoassays.

The study established the following HIV sub-type distribution among the blood donors in Kenya: A1 (76.9%), C (6.6%), D (14.3%) and CRF A2.CY.94CY017_41 (2.2%). The HIV sub-type distribution in Kenya has been reported in the Los Alamos Database (2015) as follows: A (77.8%), C (6.0%), D (15%) and others (1.2%). The difference then between the results that were obtained in this study and the data in Los Alamos data base was not significant (P-Value = 1; $p < 0.05$).

5.2 Conclusions

The study;

- Established that the prevailing consensus sequence of Immunodominant Region (IDR) of HIV *Env* gene in Kenya is "tggggcattaaacagctgcaggcgcg cgtgctggcggtggaacgctatctgaaagatcagcagctgctgggcatttggggctgcagcggc aaactgatttgcaccaccaacgtgccgtggaacagcagctgg".

- Prepared the Consensus HIV *Env* gp41-IDR peptide with the following amino sequence: WGIKQLQARVLAVER YLKDQQLLGIWGCSGKLICTT**N**VP WN**S**SW

- Determined the proportion of the recent HIV infections among blood donors in Kenya as 31.9% (adjusted to 26.9% using False Recency Rate of 5%)

- Developed LFT and ELISA using consensus HIV *Env* gp41 IDR peptide from Kenya and demonstrated that their performance not different from other HIV testing kits in use in Kenya.

- Found that although the Consensus HIV *Env* gp41 IDR (Kenya) and Consensus HIV *Env* gp41 IDR (Global) peptides had almost similar primary, secondary and tertiary structures and they showed almost similar performance with normal ELISA and LFT applications.

- Found that among the LFTs in use in Kenya Determine™ HIV-1/2 had the highest D-SN and Analytical Sensitivity. Its D-SP was also high. Unfortunately

it was removed from the HIV Testing Algorithm in Kenya as it is 10-15% more expensive than the kits in the current HIV Testing Algorithm.

- Found that the Analytical Sensitivity of Determine™ HIV-1/2 Combo Ag/Ab LFT (Abbott Diagnostic Division, Hoofddorp, The Netherlands) in respect to testing for HIV p24 Antigen was the same as that of Vironostika™ Uni-Form II Ag/Ab ELISA (bioMérieux, Marcy-l'Etoile, France).

- Found that the HIV sub-type distribution among the blood donors in Kenya was to be A1 (76.9%), C (6.6%), D (14.3%) and CRF A2.CY.94CY017_41 (2.2%). This was not significantly different from the results that are recorded in Los Alamos data base for HIV (P-Value = 1; $p < 0.05$).

- Found 29 amino acid substitutions on HIV *Env* gp41 IDR that were unique with the HIV sequences in Kenya.

5.3 Recommendations and Future Prospects

- The study recommends the return of Determine™ HIV-1/2 to the National HIV Testing Algorithm due to its superior perform`ance.

- Since this study and others have established the existence of variations of the HIV gp41 IDR gene the Kenya National HIV Reference Laboratory needs to continually monitor the effectiveness of the HIV testing kits in use in case of emergence of variants that could escape detection by the existing HIV testing kits.

- There is a need of seeking more funds to support the commercialization of the prototype HIV kits developed in this study.

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APPENDICES

Appendix 1 Sequences of the HIV *HIV Env* gp41 IDR

>KPBM_258

TGGGGCATTAAACAGCTCCAGGCACGAGTCCTTGCTGTGGAGAGATATCTAAGGGATCAACAGCTCCTA
GGAATTTGGGGCTGCTCTGGAAGACTCATCTGCACCACTAATGTGCCCTGGAACTCTAGTTGG

>KPBM_262

TGGGGCATTAAACAGCTCCAGGCAAGAGTCCTGGCTGTGGAAAGATACCTAACGGATCAACGGCTCCT
AGGGATTTGGGGCTGCTCTGGGAAACTCATCTGCACCACTGCTGTGCCTTGGAACTCTAGTTGG

>KPBM_263

TGGGGCATTAAACAGCTCCAGGCCAGAGTCCTGGCTGTGGAAGGATACCTAAGGGATCAACAGCTCCT
AGGGATTTGGGGCTGCTCTGGAAAACTCATCTGCACCACTACTGTGCCCTGGAACTCTAGTTGG

>KPBM_314

TGGGGCATTAAACAGCTCCAGGCAAGAGTCCTGGCTGTGGAAAGATACCTAAGGGATCAACAGCTCCT
AGGAATTTGGGGCTGCTCTGGAAAACTCATCTGCACCACTACTGTGCCCTGGAACTCTAGTTGG

>KPBM_324

TGGGGCATTAAACAGCTCCCGGCAAGAGTCCTGGCTCTGGAAAGATACCTAAAGGATCAACAGCTCCT
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>KPD_710

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Appendix 2 Sequences alignment of the HIV Env. Gp41 IDR

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>KPD_830

WGIKQLQARILSVERYLKDQQLLGLWGCSGKLICTTNVPWNSSW

>KPD_860

WGIKQLQARVLAVERYLRDQQLLGLWGCSGKLICTTNVPWNSSW

>KPD_982

WGIKQLQTRVLAIERYLKDQQLLGIWGCSGKLICTTNVPWNSSW

>KPD_1023

WGIKQLQARILAVERYLRDQQLLGLWGCSGKHICTTNVPWNSSW

>KPD_1034

WGIKQLQARVLAVERYLRDQQLLGIWGCSGKLICTTNVPWNSSW

>KPD_1156

WGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTNVPWNVSW

>KPD_1168

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>KPD_1178

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>KPD_1216

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>KPDR_004_23_11

WGIKQLQARVLAVERYLRDQQLLGIWGCAGKVICPTTVPWNSSW

>KPDR_010_23_11

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>KPDR_011_30_11

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>KPDR_014_30_11

WGIKQLQARVLAVENYLKDQQLLGIWGCSGKHICTTTVPWNSSW

>KPDR_017_30_11_G

WGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTNVPWNSSW

>KPDR_017_30_11_C

WGIKQLQARVLALERYLKDQQLLGIWGCSGKLICTTNVPWNSSW

>KPDR_017_30_11_CG

WGIKQLQARVLALERYLKDQQLLGIWGCSGKLICTTNVPWNSSW

>KPDR_019_30_11

WGIKQLQARVLAVESYLRDQQLLGIWGCSGKHICTTNVPWNSSW

>KPDR_46_11_01

WGIKQLQARLLAVERYLEHQQLLGIWGCSGKLICTTNVPWNSSW

>KPDR_047_11_01

WGIKQLQARVLALERYLKDQQLLGIWGCSGKLICTTTVPWNYSW

>KPDR_049_11_01

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>KPDR_057_18_01

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>KPDR_063_25_01

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>KPDR_069_1_2

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>KPDR_078_1_2

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>KPDR_088_1_2

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>KPDR_88_13

WGIKQLQARVLAVERYLGDQQLLGIWGCSGKLICTTNVPWNSSW

>KPDR_095_1_2

WGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTTVPWNSSW

>KPDR_95_13

WGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTTVPWNSSW

>KPDR_096_1_2

WGIKQLQARVLALERYLVDQQLLGIWGCSGKLICTTNVPWNSSW

>KPDR_96_13

WGIKQLQARVLALERYLVDQQLLGIWGCSGKLICTTNVPWNSSW

>KPDR_099_15_2

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>KPDR_101_22_2

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>KPDR_110_13

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>KPDR_125_7_3

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>KPDR_130_7_3

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>KPDR_133_7_3

WGIKQLQARVLAVERYLQDQQLLGIWGCSGKLICTTFVPWNSSW

>KPDR_140_14_3

WGIKQLQARVLAVERYLRDQQLLGIWGCSGRLICTTNVPWNSSW

>KPDR_141_21_3

WGIKQLQARILAVESYLKDQQLLGVWGCSGRHICTTTVPWNSSW

>KPDR_0146_04_03

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>KPDR_146_13

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>KPDR_151_13

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>KPDR_151_13E

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>KPDR_151_18_04

WGIKQLQARVLAVERYLKDQQLLGLWGCSGKLICSTTVPWNSSW

>KPDR_155_9_05

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>KPDR_156_9_05

WGIKQLQARVLAVESYLKDQQLLGIWGCSGKHICTTSVPWNASW

>KPDR_164_23_05

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>KPDR_176_06_06

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>KPDR_177_06_06

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>KPDR_177_13

WGIKQLKTRVLAIERYLKDQQLLGIWGCSGKLICPTAVPWNTTW

>KPDR_179_27_06

WGIKQLQARVLALERYLRDQQLLGIWGCSGKIICATNVPWNTSW

>KPDR_181_27_06

WGIKQLQARVLAVERYLRDQQLLGIWGCSGKLICTTNVPWNASW

>KPDR_185_27_06

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>KPDR_194_1_08

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>KPDR_194_13E

WGIKQLQARVLALERYLKDQQLLGIWGCSGKLICTTNVPWNSSW

>KPDR_198_1_08

WGIKQLRARVLAVERYLKDQQLLGLWGCSGKLICTTNVPWNSSW

>KPDR_199_1_08

WGIKQLQARILAVESYLKDQQLLGLWGCSGKLICTTAVPWNSTW

>KPDR_199_13

WGIKQLQARILAVESYLKDQQLLGLWGCSGKLICTTAVPWNSTW

>KPDR_199_13E

WGIKQLQARILAVESYLKDQQLLGLWGCSGKLICTTAVPWNSTW

>KPDR_200_1_08

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>KPDR_1135

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>KPDR_2021

WGIKQLQARVLAVERYLRDQQLLGIWGCSGKLICTTTVPWNASW

>KPDR_1203

WGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTTVPWNASW

>KPDR_205

WGIKQLQARVQAVERYLKDQQLLGLWGCSGKLICTTTVPWNTSW

>KPDR_212

WGIKQLQTRVLAMERYLKDQQLLGIWGCSGKVICPTAVPWNSSW

>KPDR_216

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>KPDR_223

WGVKQLQARILAVERYLKDQQLLGIWGCSGRHICTTNVPWNSSW

>KPDR_224

SGALNSSRQECWLWKDTYRINSS*EFGVALENSSAPLLCPGTLVX

>KPDR_230

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>KPDR_235

WGIKQLQARVLAVERYLRDQQLLGIWGCSGKLICATNVPWNSSW

>KPDR_236

WGIKQLQARVLAVERFLKDQQLLGIWGCSGKLICTTTVPWNSSW

>KPDR_237

WGIKQLQARVQAVERYLKDQQLLGLWGCSGKLICTTTVPWNTSW

>KPDM_273

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>KPD_274

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>KPD_788

WGIKQLQARVLAVERYLKDQQILGIWGCSGKLICTTNVPWNSSW

>KPD_897

WGIKQLQARVLAVEKYLKDQQLLGIWGCSGKLICPTNVPWNSSW

>KPDR_901

WGIKQLQARVLAVERYLGDQQLLGIWGCSGKLICTTNVPWNSSW

>KPDR_1155

WGIKQLQARVLAVERYLKDQQLLGLWGCSGKLICSTTVPWNSSW

>KPDR_1130

WGIKQLQARVLAVERYLKDQQILGLWGCSGKLICTTNVPWNSSW

>KPDR_1750_1_08

WGIKQLRARVLAVERYLKDQQLLGLWGCSGKLICTTNVPWNSSW

Appendix 4 Data for performance of various HIV testing kit in Kenya

Sample No.	DETERMINE 4th GEN HIV-1	DETERMINE 1/2 (Old kit)	KHB COLLOIDAL	UNIGOLD RAPID	FIRST RESPONSE HIV 1-2	AWARE HIV KIT	True Status_Serial Rapids	Vironostika ELISA	Gp41 Consensus LFA (K)	Gp41 Consensus ELISA (K)	Gp41 Consensus ELISA (G)
2012/05/25/512	+	+	+	+	+	+	+	+	+	+	+
2012/05/25/513	+	+	+	+	+	+	+	+	+	+	+
2012/05/25/514	+	+	+	+	+	+	+	+	+	+	+
2012/05/25/515	+	+	+	+	+	+	+	+	+	+	+
2012/05/25/516	+	+	+	+	+	+	+	+	+	+	+
2012/05/25/517	+	+	+	+	+	+	+	+	+	+	+
2012/05/25/518	+	+	+	+	+	+	+	+	+	+	+
2012/05/25/519	+	+	+	+	+	+	+	+	+	+	+
2012/05/25/520	+	+	+	+	+	+	+	+	+	+	+
2012/05/25/521	+	+	+	+	+	+	+	+	+	+	+
2012/05/25/522	+	+	+	+	+	+	+	+	+	+	+
2012/05/30/525	+	+	+	+	+	+	+	+	+	+	+
2012/05/30/526	+	+	+	+	+	+	+	+	+	+	+
2012/05/30/527	+	+	+	+	+	+	+	+	+	+	+
2012/05/30/528	+	+	+	+	+	+	+	+	+	+	+
2012/05/30/529	+	+	+	+	+	+	+	+	+	+	+
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2012/05/30/534	+	+	+	+	+	+	+	+	+	+	+
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2012/05/30/536	+	+	+	+	+	+	+	+	+	+	+

2012/05/30/537	+	+	+	+	+	+	+	+	+	+	+
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2012/05/30/540	+	+	+	+	+	+	+	+	+	+	+
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2012/05/30/542	+	+	+	+	+	+	+	+	+	+	+
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2012/05/30/544	+	+	+	+	+	+	+	+	+	+	+
2012/05/30/545	+	+	+	+	+	+	+	+	+	+	+
2012/05/30/546	+	+	+	+	+	+	+	+	+	+	+
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2012/06/15/558	+	+	+	+	+	+	+	+	+	+	+
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2012/06/15/562	+	+	+	+	+	+	+	+	+	+	+
2012/06/15/563	+	+	+	+	+	+	+	+	+	+	+
2012/06/15/564	+	+	+	+	+	+	+	+	+	+	+
2012/06/15/565	+	+	+	+	+	+	+	+	+	+	+

2012/06/15/566	+	+	+	+	+	+	+	+	+	+	+
2012/06/15/567	+	+	+	+	+	+	+	+	+	+	+
2012/06/15/568	+	+	+	+	+	+	+	+	+	+	+
2012/06/15/569	+	+	+	+	+	+	+	+	+	+	+
2012/06/15/570	+	+	+	+	+	+	+	+	+	+	+
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2012/06/15/580	+	+	+	+	+	+	+	+	+	+	+
2012/06/15/581	+	+	+	+	+	+	+	+	+	+	+
2012/06/15/582	+	+	+	+	+	+	+	+	+	+	+
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2012/06/15/584	+	+	+	+	+	+	+	+	+	+	+
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2012/06/15/596	+	+	+	+	+	+	+	+	+	+	+
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2012/08/02/807	-	-	-	-	-	-	-	-	-	-	-
2012/08/02/808	-	-	-	-	-	-	-	+	-	-	-
2012/08/02/809	-	-	-	-	-	-	-	-	-	-	-

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2012/08/02/816	-	-	-	-	-	-	-	-	-	-	-
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2012/08/02/819	-	-	-	-	-	-	-	+	-	+	+
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2012/08/10/830	+	+	+	-	+	+	+	+	+	+	+
2012/11/23/982	+	+	+	+	+	+	+	+	+	+	+
2012/11/23/983	-	-	-	-	-	-	-	-	-	-	-
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2012/11/23/985	-	-	-	-	-	-	-	-	-	-	-
2012/11/23/986	+	+	+	-	+	-	+	+	-	+	+
2013/03/15/998	+	+	+	+	+	+	+	+	+	+	+
2013/03/15/1000	+	+	+	+	+	+	+	+	+	+	+
2013/09/27/1001	-	-	-	-	-	-	-	-	-	-	-
2013/09/27/1002	+	+	+	+	+	+	+	+	+	+	+
2013/09/27/1006	-	-	-	-	-	-	-	-	-	-	-
2013/11/08/1111	-	-	-	-	-	-	-	-	-	-	-
2013/11/08/1113	+	+	+	+	+	+	+	+	+	+	+
2013/11/15/1115	-	-	-	-	-	-	-	-	-	-	-
2013/11/15/1116	-	-	-	-	-	-	-	-	-	-	-
2013/11/15/1117	-	-	-	-	-	-	-	-	-	-	-
2013/11/15/1118	-	-	-	-	-	-	-	+	-	-	-
2013/11/15/1119	-	-	-	-	-	-	-	+	-	-	-
2013/11/15/1130	+	+	+	+	+	+	+	+	+	+	+
2013/11/15/1131	-	-	-	-	-	-	-	-	-	-	-

2013/11/15/1150	-	-	-	-	-	-	-	-	-	-	-
2013/11/15/1151	-	+	-	-	-	-	-	+	-	-	-

Appendix 5 Results of testing of HIV infection Incidence

(Using three ELISA systems where ELISA well are coated with Consensus *HIV Env* gp41 peptide (Kenya), Consensus *HIV Env* gp41 peptide (Global) and rIDR-M peptide respectively)

Sample ID	OD Control-KE	OD PH3 - KE	A.1-KE	OD Control-rIDR	OD PH3 rIDR	A.1 - rIDR	OD Control-G	OD PH3-G	A.1-G
KPDR_004)	0.63	0.73	116.06	0.58	0.67	115.46	0.59	0.49	82.29
KPDR_010(A1)	0.55	0.41	75.46	0.40	0.32	79.50	0.48	0.25	51.36
KPDR_011(A1)	0.46	0.47	100.65	0.49	0.52	105.88	0.42	0.24	57.42
KPDR_014 (A1)	0.47	0.41	88.44	0.28	0.45	161.82	0.40	0.27	69.37
KPDR_017 (A1)	0.33	0.28	84.15	0.44	0.46	104.82	0.31	0.14	47.06
KPDR_019 (A1)	0.54	0.41	74.77	0.54	0.42	78.44	0.48	0.31	64.26
KPDR_047 (A1)	0.60	0.61	102.01	0.50	0.49	97.41	0.51	0.37	73.62
KPDR_049 (A1)	0.64	0.65	101.72	0.59	0.69	115.91	0.61	0.49	80.43
KPDR_057 (D)	0.67	0.60	89.88	0.60	0.50	84.03	0.64	0.38	59.87
KPDR_063 (A1)	0.76	0.70	92.26	0.63	0.58	91.00	0.70	0.54	77.13
KPDR_069 (A1)	0.72	0.68	94.58	0.70	0.60	86.35	0.68	0.43	62.87
KPDR_078 (A2.CY.94CY017_41	0.71	0.72	101.27	0.66	0.78	116.87	0.69	0.41	59.74
KPDR_088 (A1)	0.50	0.47	94.38	0.63	0.66	104.73	0.45	0.27	61.07
KPDR_095(A1)	0.06	0.05	82.81	0.10	0.05	47.12	0.08	0.06	78.21
KPDR_096 (A1)	0.60	0.21	34.33	0.50	0.25	50.30	0.55	0.13	23.33
KPDR_099_15_2_GP46F.ab1(A1)	0.77	0.59	76.30	0.62	0.70	113.17	0.72	0.39	54.52
KPDR_101A1)	0.54	0.55	100.55	0.66	0.70	105.89	0.51	0.29	55.97
KPDR_110 (A1)	0.62	0.60	95.99	0.61	0.63	104.11	0.54	0.37	68.41
KPDR_125 (A1)	0.68	0.65	95.74	0.55	0.55	98.91	0.58	0.39	67.19

KPDR_130 (A1)	0.33	0.17	50.60	0.41	0.32	78.13	0.28	0.10	35.84
KPDR_133 (A2.CY.94CY017_41)	0.48	0.25	51.88	0.38	0.43	113.23	0.43	0.12	29.18
KPDR_140 (A1)	0.57	0.37	64.80	0.60	0.39	64.74	0.55	0.20	36.00
KPDR_141 (A1)	0.66	0.33	50.46	0.56	0.29	51.61	0.60	0.20	32.95
KPDR_0146 (A1)	0.44	0.34	76.48	0.42	0.48	113.84	0.37	0.20	54.52
KPDR_151 (A1)	0.56	0.27	47.52	0.47	0.43	91.33	0.51	0.19	36.91
KPDR_152 (A1)	0.53	0.53	99.06	0.51	0.50	98.04	0.48	0.30	63.05
KPDR_155 (A1)	0.60	0.62	103.50	0.48	0.59	121.28	0.58	0.41	71.06
KPDR_1561(A2.CY.94CY017_41)	0.47	0.21	44.16	0.26	0.34	128.63	0.52	0.16	31.53
KPDR_162 (A1)	0.60	0.36	60.50	0.54	0.41	75.61	0.55	0.25	44.48
KPDR_164 (A1)	0.48	0.22	45.23	0.30	0.23	76.97	0.41	0.19	46.14
KPDR_176 (A1)	0.60	0.56	92.38	0.59	0.47	79.90	0.46	0.38	81.68
KPDR_177 (C)	0.73	0.47	64.84	0.57	0.43	75.58	0.69	0.34	48.98
KPDR_179 (A1)	0.70	0.78	111.52	0.64	0.72	113.50	0.61	0.57	92.65
KPDR_181(A1)	0.68	0.59	86.36	0.54	0.32	58.88	0.61	0.39	64.11
KPDR_185 (A1)	0.19	0.19	102.69	0.15	0.17	118.62	0.16	0.15	97.45
KPDR_188_11_07_GP46F.ab1(A1)	0.38	0.33	86.72	0.45	0.52	116.78	0.34	0.20	57.60
KPDR_194 (A1)	0.24	0.17	70.76	0.26	0.11	42.75	0.19	0.11	60.43
KPDR_198 (A1)	0.59	0.48	80.65	0.58	0.62	106.86	0.58	0.33	56.34
KPDR_199(D)	0.68	0.60	88.37	0.44	0.35	80.96	0.66	0.41	62.40
KPDR_200 (A1)	0.43	0.26	60.66	0.36	0.26	70.44	0.38	0.18	47.62
KPDR_202 (A1)	0.53	0.28	52.18	0.45	0.41	93.03	0.47	0.20	42.22
KPDR_205 (A1)	0.84	0.87	103.59	0.59	0.47	79.86	0.80	0.68	85.82
KPDR_212 (C)	0.57	0.47	81.15	0.48	0.41	85.42	0.42	0.31	74.05
KPDR_216 (A1)	0.21	0.08	37.62	0.26	0.19	73.00	0.16	0.07	42.58
KPDR_223 (A1)	0.16	0.22	138.71	0.34	0.25	72.49	0.11	0.12	104.50
KPDR_224 (A1)	0.30	0.25	84.23	0.46	0.38	81.74	0.24	0.14	58.16
KPDR_230 (A1)	0.40	0.17	43.47	0.29	0.09	30.58	0.34	0.14	40.48

KPDR_235_26_09_GP46F.ab1	0.12	0.06	53.78	0.41	0.10	23.54	0.14	0.07	48.53
KPDR_236 (A1)	0.15	0.10	66.00	0.13	0.10	75.19	0.14	0.09	60.28
KPDR_237 (A1)	0.68	0.67	99.56	0.64	0.73	114.22	0.60	0.50	82.78
KPDR_88 (A1)	0.47	0.57	120.90	0.33	0.50	152.58	0.40	0.36	90.10
KPDR_96 (A1)	0.27	0.29	106.67	0.26	0.28	107.28	0.21	0.19	92.82
KPDR_95 (A1)	0.55	0.66	119.35	0.38	0.61	162.67	0.54	0.47	87.13
KPDR_110 (A1)	0.27	0.30	111.24	0.41	0.54	132.35	0.20	0.29	143.63
KPDR_146 (A1)	0.65	0.69	105.20	0.48	0.53	108.70	0.52	0.49	94.59
KPDR_151(A1)	0.10	0.07	71.00	0.12	0.10	84.35	0.07	0.07	91.67
KPDR_177 (C)	0.07	0.07	101.43	0.06	0.08	127.12	0.05	0.08	147.06
KPDR_199 (D)	0.39	0.48	121.17	0.37	0.51	136.02	0.38	0.30	78.93
KPDR_202 (A1)	0.67	0.46	68.86	0.65	0.39	60.12	0.59	0.30	51.27
KPDR_207 (D)	0.40	0.25	62.06	0.29	0.25	86.16	0.34	0.17	48.84
KPDR_216 (C)	0.56	0.35	62.61	0.48	0.53	110.14	0.50	0.21	41.85
KPDR_237b (A1)	0.12	0.06	45.08	0.09	0.13	152.27	0.18	0.05	30.34
KPBM_258 (A1)	0.52	0.34	65.50	0.41	0.48	117.52	0.45	0.23	51.01
KPBM_262 (A1)	0.58	0.57	98.62	0.39	0.37	94.92	0.53	0.37	70.29
KPBM_263 (A1)	0.35	0.15	42.61	0.43	0.26	60.84	0.32	0.11	34.91
KPBM_264 (A1)	0.14	0.07	50.00	0.15	0.15	99.35	0.14	0.07	46.48
KPBM_310 (A1)	0.47	0.63	133.55	0.45	0.62	138.62	0.35	0.39	110.89
KPBM_314 (A1)	0.37	0.34	90.57	0.38	0.43	113.05	0.29	0.21	74.56
KPBM_324_GP46F.ab1 (A1)	0.49	0.64	130.39	0.50	0.61	122.11	0.37	0.37	99.46
KPD_710 (A1)	0.39	0.17	44.94	0.64	0.53	83.41	0.31	0.13	40.26
KPD_721 (A1)	0.54	0.28	52.70	0.41	0.58	140.63	0.49	0.15	31.16
KPD_724 (A1)	0.34	0.22	65.07	0.22	0.31	137.22	0.28	0.16	56.99
KPD_738 (A1)	0.59	0.52	88.23	0.57	0.62	109.91	0.58	0.35	61.18
KPD_764 (A1)	0.72	0.70	98.18	0.53	0.55	103.80	0.65	0.49	75.34
KPD_830 (A1)	0.74	0.70	93.93	0.55	0.64	115.55	0.65	0.51	77.68
KPD_846_GP46F.ab1(A1)	0.43	0.26	59.35	0.26	0.22	81.44	0.40	0.22	54.61

KPD_860 (A1)	0.35	0.26	74.50	0.31	0.35	114.43	0.30	0.19	62.54
KPD_982 (A1)	0.17	0.08	47.67	0.25	0.11	43.09	0.18	0.07	40.22
KPD_1023 (D)	0.67	0.21	30.70	0.24	0.10	41.18	0.56	0.14	24.69
KPD_1034 (A1)	0.24	0.09	37.45	0.43	0.26	59.12	0.19	0.07	35.11
KPD_1156 (A1)	0.08	0.06	83.12	0.19	0.06	32.47	0.06	0.06	101.61
KPD_1168 (C)	0.27	0.28	103.65	0.41	0.06	15.44	0.28	0.17	62.18
KPD_1178 (A1)	0.33	0.47	141.39	0.80	0.54	67.80	0.28	0.33	118.02
KPD_1216 (A1)	0.66	0.52	79.45	0.48	0.62	128.57	0.66	0.38	57.53
KPDR_151 (A1)	0.74	0.09	11.92	0.52	0.12	22.56	0.66	0.07	10.21
KPDR_194 (A1)	0.51	0.28	54.69	0.65	0.53	81.45	0.46	0.22	47.63
KPDR_199 (D)	0.64	0.63	97.52	0.71	0.77	108.32	0.59	0.43	73.59
KPDR_202 (A1)	0.41	0.19	46.94	0.31	0.24	76.11	0.36	0.11	32.11
KPDR_288 (A1)	0.30	0.26	86.67	0.50	0.51	102.19	0.25	0.21	83.67
KPDR_289 (A1)	0.48	0.19	39.08	0.57	0.26	46.19	0.44	0.15	33.33
KPDR_290 (A1)	0.70	0.64	91.52	0.41	0.41	100.00	0.54	0.42	77.88

Note:

OD Control-KE = Optical density of control wells using Consensus *HIV Env* gp41 peptide (Kenya)

OD Control-G = Optical density of control wells using Consensus *HIV Env* gp41 peptide (Global)

OD Control-rIDR = Optical density of control wells using Consensus *HIV Env* gp41 peptide (rIDR-M)

OD PH3 –KE = Optical density of control wells using Consensus *HIV Env* gp41 peptide (Kenya) and treatment with Citrate Buffer at pH 3

OD PH3 –G = Optical density of control wells using Consensus *HIV Env* gp41 peptide (Global) and treatment with Citrate Buffer at pH 3

OD PH3 –rIDR-M = Optical density of control wells using Consensus *HIV Env* gp41 peptide (rIDR-M) and treatment with Citrate Buffer at pH 3

A.1-KE = Avidity Index of control wells using Consensus *HIV Env* gp41 peptide (Kenya)

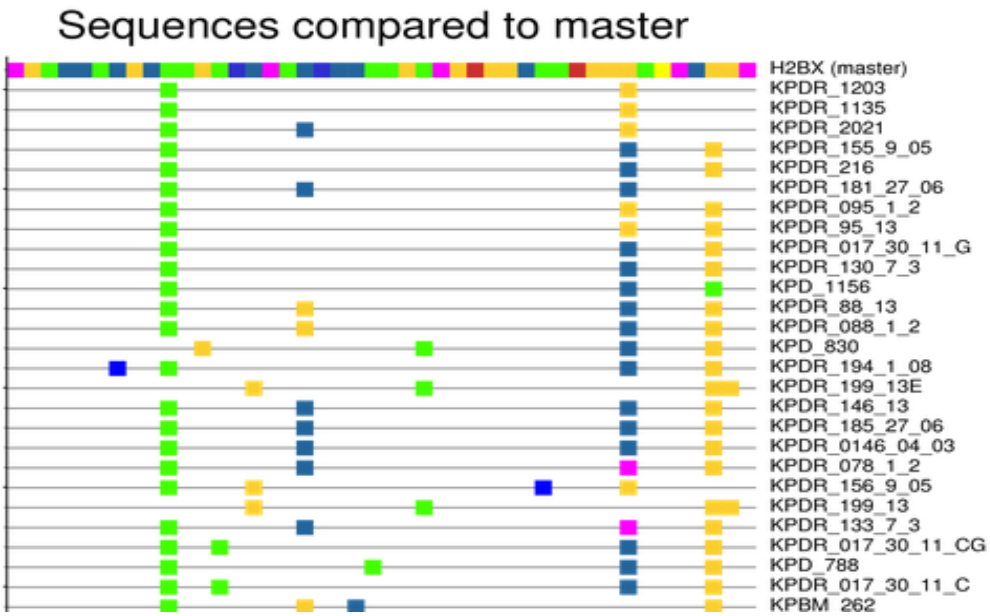
A.1-G = Avidity Index of control wells using Consensus *HIV Env* gp41 peptide (Global)

A.1- rIDR-M = Avidity Index of control wells using *HIV Env* gp41 peptide (rIDR-M)

Appendix 6: Amino acid substitutions /variation among 91 sequences in comparison with H2BX

Average similarity with master: 0.880
Number of sequences: 91
Master: H2BX
Color Legend (standard):

His (H)	Asp (D), Glu (E)
Lys (K), Asn (N), Gln (Q), Arg (R)	Met (M)
Ile (I), Leu (L), Val (V)	Phe (F), Trp (W), Tyr (Y)
Cys (C)	Ala (A), Gly (G), Ser (S), Thr (T)
Pro (P)	Gap
Other	




KPDR_236
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KPDR_194_13E
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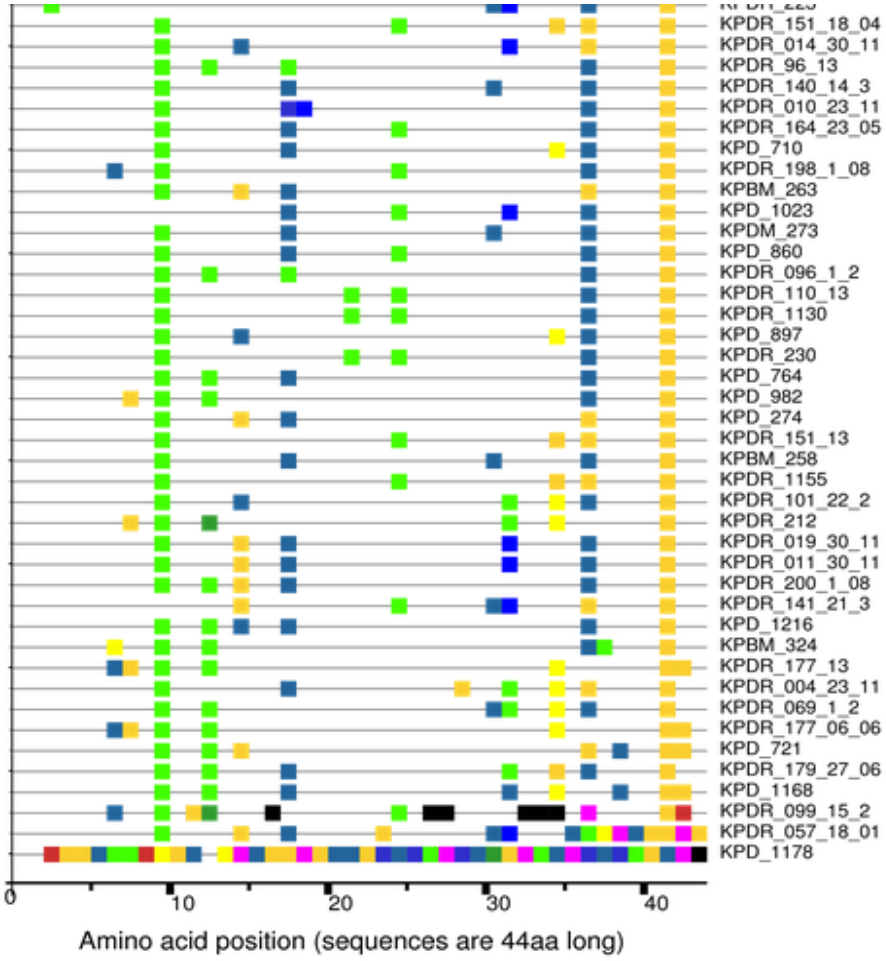
KPDR_901
KPBM_314
KPDR_063_25_01
KPDR_049_11_01
KPDR_188_11_07
KPD_1034
KPD_724
KPDR_199_1_08
KPDR_1750_1_08
KPBM_264
KPDR_46_11_01
KPDR_176_06_06
KPDR_125_7_3
KPDR_235
KPDR_237
KPDR_205
KPDR_151_13E
KPDR_223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KPDR_96_13
KPDR_140_14_3
KPDR_010_23_11
KPDR_164_23_05
KPD_710
KPDR_198_1_08
KPBM_263
KPD_1023
KPDM_273
KPD_860
KPDR_096_1_2
KPDR_110_13
KPDR_1130
KPD_897
KPDR_230
KPD_764
KPD_982
KPD_274
KPDR_151_13
KPBM_258
KPDR_1155
KPDR_101_22_2
KPDR_212
KPDR_019_30_11
KPDR_011_30_11
KPDR_200_1_08
KPDR_141_21_3
KPD_1216
KPBM_324
KPDR_177_13
KPDR_004_23_11
KPDR_069_1_2
KPDR_177_06_06
KPD_721
KPDR_179_27_06
KPD_1168
KPDR_099_15_2
KPDR_057_18_01
KPD_1178
0
10
20
30
40
Amino acid position (sequences are 44aa long)

Appendix 7: Papers published by James Kimotho

1. **Diversity within the Immunodominant Epitopes of Envelope** gp41 from HIV-1 in Kenya and Its Effects on Performance ofthe HIV-1 Antibody-Based Detection Kits

   American Journal of Internal Medicine, 2015; 3(1): 15-22.Published online February 9, 2015 (http://www.sciencepublishinggroup.com/j/ajim), doi: 10.11648/j.ajim.20150301.13

   ***James Kimotho**, Zipporah Ng'ang'a, Edna Nyairo, Missiani Ochwoto, Nicholas Nzioka, Francis Ogolla, Michael Kiptoo*

2. **Laboratory Evaluation of the Validity of the Current HIV Testing Algorithm in Kenya**

   American Journal of Internal Medicine, 2015; 3(1): 15-22.Published online February 13, 2015 (http://www.sciencepublishinggroup.com/j/ajim), doi: 10.11648/j.ajim.20150301.14

   ***James Kimotho**, Zipporah Ng'ang'a, Edna Nyairo, Missiani Ochwoto, Nicholas Nzioka, Francis Ogolla, Michael Kiptoo*

3. **ELISA Kit for Detection of Hepatitis B Surface Antigen in Plasma and Serum Based on Polyclonal Antibodies Produced by KEMRI, Kenya**. *Pharmaceutical Journal of Kenya*, (2009). 19 (3), 13-13.

   ***Kimotho J. H.,** Wamachi A, Gikunju J, Khamadi S.S, Muchiri S.*

4. **Concise Pharmacology & Pharmacotherapeutics**

   Vol.1, 2012,*East Africa Pharmaceutical Loci publishers*, Nairobi, Kenya.

   ***Kimotho J. H.,** Kibwage I.Wanjiku,M., Mshilla, M.*

5. **East African Pharmaceutical Loci; a Regional Drug Index for Health Care Practitioners**

   Vol.10, 2015. *East Africa Pharmaceutical Loci publishers*, Nairobi, Kenya.

   ***Kimotho J. H.,** Kibwage I.Wanjiku,M., Mshilla, M.*

6. **Development and Evaluation of an in-house IgM-capture ELISA for the Detection of Chikungunya and Application to a Dengue Outbreak Situation in Kenya in 2013.**

   Jpn J Infect Dis 2015 Apr 10. Epub 2015 Apr 10.

   *Caroline Wasonga, Shingo Inoue, **James Kimotho**, Kouichi Morita, Juliette Ongus, Rosemary Sang, Lillian Musila*

7. **Serological surveillance development for tropical infectious diseases using simultaneous microsphere-based multiplex assays and finite mixture models.**

   PLoS Negl Trop Dis 2014 31;8(7):e3040. Epub 2014 Jul 31.

   *Yoshito Fujii, Satoshi Kaneko, Samson Muuo Nzou, Matilu Mwau, Sammy M Njenga, Chihiro Tanigawa, **James Kimotho**, Anne Wanjiru Mwangi and Ibrahim Kiche.*

8. **Hepatitis B virus subgenotype A1, occurrence of subgenotype D4, and S gene mutations among voluntary blood donors in Kenya.**

   Virus Genes 2013 Dec 5;47(3):448-55. Epub 2013 Sep 5.

   *Simeon Owuor Kwange, Nancy L M Budambula, Michael Kibet Kiptoo, Fredrick Okoth, Missiani Ochwoto, Margaret Oduor**, James Hungo Kimotho.***

9. **Genotyping and molecular characterization of hepatitis B virus in liver disease patients in Kenya.** Infect Genet Evol. 2013 Dec;20:103-10. doi: 10.1016/j.meegid.2013.08.013. 23

   *Ochwoto M[1], Chauhan R, Gopalakrishnan D, Chen CY, Ng'ang'a Z, Okoth F, Kioko H, **Kimotho J**, Kaiguri P, Kramvis A.*

10. **Hepatitis B Virus Basal Core Promoter and Pre-core Region Mutations among Liver Disease Patients at Kenyatta National Kenyatta**

    Africa Journal of Health Science, Vol 60, 2011

    *Missiani Ochwoto, Fredrick Okoth, Henry Kioko, **James Kimotho**, Zipporah Ng'ang'a, Deepark K, Ranjit Chauhan, and Anna Kramvis*

Appendix 8: Presentations of the findings of this study in major conferences

1. Annual Pharmaceutical Society of Kenya Scientific Symposium, Whitesands Beach and Spa Resort, 28th June to 1st July 2015

2. Third International Conference for College of Health Sciences, University of Nairobi and Kenyatta National Referral and Teaching Hospital , 10th June 2015

Appendix 9: KEMRI Ethical Review Committee Approval of the Protocol

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
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

KEMRI/RES/7/3/1 **April 27, 2012**

TO: **DR. JAMES H. KIMOTHO (PRINCIPAL INVESTIGATOR)**

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

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

Dear Sir,

RE: **SSC PROTOCOL No. 2170- REVISED (*RE-SUBMISSION*): DEVELOPMENT AND VALIDATION OF ENZYME-LINKED IMMUNOSOBENT ASSAY AND IMMUNOCHROMATOGRAPHIC DIAGNOSTIC KITS FOR HUMAN IMMUNODEFICIENCY VIRUS, GUIDED BY THE PREVAILING MUTANTS OF HIV *ENV* GENE IN KENYA (*VERSION 2170-002 DATED 25TH APRIL 2012*)**

The ERC Secretariat acknowledges receipt of the revised proposal on 26th April 2012.

This is to inform you that the Committee determines that the issues raised at the 199th ERC meeting of 13th March 2012 are adequately addressed. Consequently, the study is granted approval for implementation effective this **27th day of April 2012** for a period of one year. Please note that authorization to conduct this study will automatically expire on **April 26, 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 **March 15, 2013**. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

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
-3 MAY 2012
P.O. Box 54628 - NAIROBI