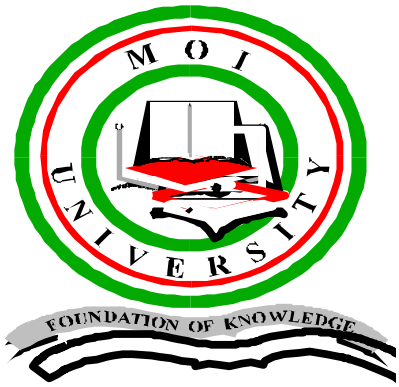


# INFLAMMATORY CYTOKINE RESPONSES IN WOMEN WITH HIV, HPV AND CERVICAL DYSPLASIA AT MOI TEACHING AND REFERRAL HOSPITAL, ELDORET KENYA

**BY**

**SETH KIRUI CHEBWEK**

**2015**

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**SETH KIRUI CHEBWEK**

**SM/PGI/04/09**

**A Research Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Immunology of the Department of Immunology, Moi University**

**2015**

# DECLARATION

## Declaration by the Candidate

This Thesis is my original work and has not been presented for a degree in any other University. No part of this thesis may be reproduced without the prior written permission of the author and/or Moi University.

Seth Kirui Chebwek Sign…… Date: ……

## Declaration by Supervisors

This thesis has been submitted with our approval as University Supervisors.

Dr. Kirtika Patel Sign…… Date: ……
Dept. of Immunology

Dr. Omenge Orang'o Sign…… Date: ……
Dept. of Reproductive Health

Moi University,

## ABSTRACT

**Background** Cervical cancer is the second commonest cancers among women and is caused by Human Papillomavirus (HPV). HIV+ women are at a higher risk of acquiring HPV increasing their odds of developing pre-cervical cancer lesions (dysplasia) and cervical cancer. In HPV infection and cervical dysplasia there is increased production of inflammatory cytokines. Early diagnosis remains key in prevention of cervical cancer but reduced sensitivities and specificities of available screening methods pose challenges. Interrogating alternative approaches to improve diagnosis is important. Screening women for inflammatory cytokines and comparing it with HIV, HPV and cervical dysplasia has potential diagnostic value.

**Broad Objective** To compare inflammatory cytokine responses in women with HIV, HPV and cervical dysplasia.

**Methodology** This was a cross sectional study design targeting women above 18 years attending cervical cancers screening clinics at AMPATH. Stratified sampling technique was employed to select HIV positive and negative respondents. A sample size of 88 was determined using a formula for calculating difference in proportions in two populations. Cervicovaginal lavage samples were collected from women. This was assayed for inflammatory cytokines using flow cytometry (bead assay) and Human papillomavirus (HPV) using hybrid capture technique. Cervical dysplasia was determined by doing Visual inspection with acetic acid (VIA). Ethical approval was obtained from IREC. Analysis was done using student t-test, chi square and Mann Whitney U test. P values < 0.05 were considered significant.

**Results** The mean age of participants was 37.4 years. A significantly higher proportion of HIV- women were married. HIV+ women also had significantly higher number of sexual partners. There was no difference in levels of inflammatory cytokine levels between HIV+ and HIV- women. Interleukin (IL) 1β and IL8 were significantly higher in HPV+ women. IL1β and IL8 were also significantly higher in women with dysplasia (VIA+). The prevalence of HPV was 59.1% in HIV+ women and 43.2% in HIV- women.

**Study Limitations:** The study was limited by lack of correction for dilution effect in CVL and the determination of HIV negativity status by self- reporting. Other genital tract infections were not ruled out.

**Conclusion and Recommendations** HIV+ women have similar inflammatory cytokine responses to HIV- women. There is relative higher production of inflammatory cytokines IL1β and IL8 in women with dysplasia (VIA+), and women infected with HPV compared to their respective negative counterparts. Further investigation on the relationship between IL1β; IL8 and VIA is needed and the potential of utilizing these cytokines in cervical cancer diagnosis should be explored.

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## LIST OF ABBREVIATIONS

AMPATH-Academic Model Providing Access to Healthcare
ASCUS-Atypical Squamous Cells of Undetermined Significance
BD® – Beckton Dickenson
CBA-Cytometric Bead Array
CD-Cluster of Differentiation
CIN-Cervical Intraepithelial Neoplasia
CVL-Cervicovaginal Lavage
DNA-Deoxyribonucleic Acid
EDTA-Ethylene Diamine Tetra-acetic Acid
FACS-Flow Activated Cell Sorter
FSW-Female Sexual Worker
HAART-Highly Active Antiretroviral Therapy
HIV-Human Immunodeficiency Virus
HPV- Human Papillomavirus
HR-High Risk
HRP-Horse Radish Peroxidase
HSIL-High grade Squamous Intraepithelial Lesion
IARC-International Agency for Research on Cancer
ICO-International Cancer Organisation
IFN-Interferon
IL-Interleukin
IP-Inflammatory Protein
LR-Low Risk
LSIL-Low grade Squamous Intraepithelial Lesion
MHC-Major Histocompatibility Complex
MIP-Macrophage Inhibitory Protein
MTRH-Moi Teaching and Referral Hospital
PBMC-Peripheral Blood Mononuclear cells
PCR-Polymerase Chain Reaction
PE-Phycoerythrin
RNA-Ribonucleic acid
SPSS-Statistical Package for Social Sciences
STI-Sexually Transmitted Infections
TMB-Tetra Methyl Benzidine
TNF-Tumor Necrosis factor
VIA- Visual Inspection with Acetic acid
VIAM- Visual Inspection with Acetic acid and microscopy
VILI- Visual Inspection with Lugols Iodine
WHO- World Health Organisation

## ACKNOWLEDGEMENT

I wish to extend my sincere gratitude to all my colleagues, lecturers, supervisors and staff of AMPATH reference laboratory for the help extended to me during the development of my research proposal and final thesis. My gratitude goes to Dr. Kirtika Patel and Dr. Omenge Orang'o for their constant guidance and supervision, and to Dr. Wilfred Emonyi and all AMPATH reference laboratory for their technical advice.

# 1.0. INTRODUCTION

## Background

Cervical cancer is one of the killer cancers that affect women and is a malignancy associated with HIV infection. Over 2540 million women worldwide are at risk of developing cervical cancer and approximately 15.3% of them eventually develop the disease. Majority of this burden (86%) occurs in developing countries (1). Human papillomavirus (HPV) infection is an established cause of cervical pre-cancerous and cancerous lesions and has been found to be present in 99% of cervical cancer lesions. Most HPV infections are transient (2, 3). There are more than 100 types of HPV that have been isolated; about 40 types infect the ano-genital human tracts (2-4). The International Agency for Research on Cancer (IARC) has classified HPV into high risk (HR)/ oncogenic types e.g. 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and low-risk (LR) types e.g. 6, 11, 42, 43, 44, 54, 61, 70, 72, 81(5). HPV types 16 and 18 are responsible for about 70% of all cervical cancer cases worldwide (1, 6)

The incidence of HPV infection among HIV infected is higher although studies have had differing conclusions on HIV and cervical cancer association (7, 8). The odds of developing Cervical Intraepithelial Neoplasia (CIN) or Squamous Intraepithelial Lesion (SIL) dysplasia in HPV infected women has been found to be higher (up to 8 times) among HIV+ women than their HIV- counterparts (up to 5 times) indicating that the interaction of HPV and HIV infections increases the risk of cervical dysplasia (9).

The role of inflammation in development of various cancers has been discussed before. Tissue injury whether physical, chemical or through infectious agents triggers an inflammatory response which is supposed to eliminate the injury. However in certain instances, e.g. chronic inflammation, this doesn't occur and there is loss of cellular

control thus further tissue injury. Such inflammatory responses have been linked to several cancers including cervical cancer (10).

HPV infection elicits both cellular and humoral responses. Th1 cytokines play a key role in HPV clearance, while Th2 cytokines promote persistence of HPV (11). A shift to Th2 responses and decreased Th1 cytokines levels is associated with increased cervical dysplasia and severity of cervical lesion (12). Several cytokines including IL10, IL8, IL6 have been found to be increased in the cervical or vaginal fluids of women with differing CIN grades of cervical dysplasia as compared to their controls (13), and their production has been attributed to an inflammatory reaction to injury and also constitutively by both malignant and normal cells (10, 14, 15).

Cervical cancer prevention and treatment depends heavily on mass screening using tests which detect early changes on the cervical epithelium. The performance of these tests varies with reports of low sensitivities or specificities, especially because the interpretations of some of these tests are very subjective and depend on personal factors for example experience (16, 17). The influence of the immune responses to the cellular and histologic tissue manifestation and the potential use of immunological parameters in cervical cancer diagnosis have not been exhaustively investigated.

This study sought to determine HPV infection among HIV positive and negative women undergoing cervical cancer screening using Visual Inspection with Acetic acid (VIA) method. Their cervical-vaginal lavage (CVL) was also assayed for levels of Interleukins (IL) 12, IL10, TNF$\alpha$, IL1$\beta$, IL6 and IL8.

**Problem Statement**

Kenya and other developing nations have experienced a surging increase in cases of cervical cancer (18). The problem is prominent in HIV infection and other immune-compromised conditions because of susceptibility to HPV and other sexually transmitted infections (STIs) (7, 19, 20). While vaccination is available its cost is prohibitive making it inaccessible to most in developing countries (1, 6, 18). Early detection and management of cervical cancer lesions therefore remains a key strategy in increasing the chances of remission and complete resolution of such tumors. Therefore improved diagnosis is particularly important among at risk persons such as HIV+ individuals.

**Research Questions**

1. Do cytokine responses differ between HIV positive and HIV negative women?
2. Do cytokine responses differ between VIA positive and VIA negative women?
3. Do cytokine responses differ between HPV positive and HPV negative women?

**General Objective**

To determine and compare cytokine responses in women with HIV, HPV and cervical dysplasia.

**Specific Objectives**

1. To determine and compare cytokine responses between HIV positive and HIV negative women.

2. To determine and compare cytokine responses between VIA positive (cervical dysplasia) and VIA negative women.

3. To detect HPV and compare cytokine responses between HPV positive and HPV negative women

**Null Hypothesis**

There is no difference in levels of inflammatory cytokines in women with HIV/HPV and cervical dysplasia and those without HIV/HPV and cervical dysplasia.

**Rationale and Justification**

Studies evaluating immune responses to HPV infection indicate an initial increase in Th1 response. A shift to Th2 cytokines and increased inflammatory cytokines is associated with increased CIN cervical dysplasia (21, 22). However, none of these cytokines have been studied in relation to VIA or their contribution to its interpretation despite the increased use of VIA for cervical cancer screening by Kenya and other developing countries. The study therefore sought to understand the relationship between inflammatory cytokines and VIA interpretation in the presence or absence of HIV and HPV viruses.

## Study Algorithm

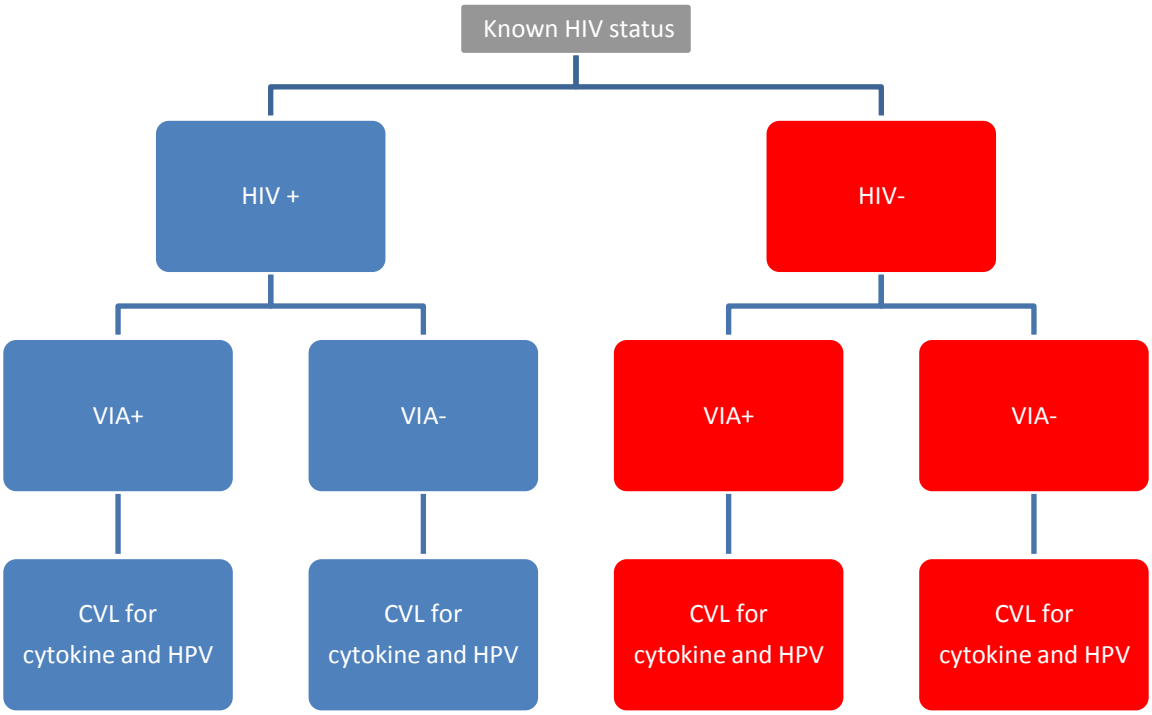


**Figure 1-1 Study Algorithm**

## 2.0. LITERATURE REVIEW

Cervical cancer is a malignancy of the cervix which occurs as a result of the integration of Human Papilloma Virus (HPV) viral genome into the host cellular genome resulting in the formation of cervical neoplastic cells. The proliferation of these neoplastic cells leads to various grades of Cervical Intraepithelial Neoplasia (CIN), otherwise referred to as dysplasia or squamous intraepithelial lesions (SIL), which may progress to invasive cervical cancer (23). HPV has been isolated in 99% of cervical cancers and it is now a consensus that it is the cause of cervical cancer (2, 4, 5).

Cervical cancer is imposes a heavy health burden on the world population. Worldwide 2540 million women are at risk of developing cervical cancer; 527,624 women were diagnosed with cervical cancer ; 265,653 died from the disease making it rank third among female cancers worldwide by 2013 (1). In Kenya 12.92 million are at risk of developing cervical cancer; 4, 802 Kenyan women were diagnosed with cervical cancer and 2451 died from it in 2013 (18). Cervical cancer is amongst the few preventable cancers. This is achieved through STI control, early screening and HPV vaccination. HPV vaccination, though a key preventive strategy, remains largely inaccessible to most women in developing countries where cervical cancer burden is greatest, due to its prohibitive costs (1, 6, 18)

HPV belong to a class of viruses called *papovaviridae*. They are non-enveloped double stranded DNA viruses housed in icosahedral capsids 55nm in diameter. The genome contains about 8000 nucleotide base pairs, which encode for six early (E1,E2, E4-E7) and two late phase (L1 and L2) genes (3, 4, 24-26), which encode for proteins involved in a wide range of functions like transcription and translation of viral components, host cell

degradation for viral escape and oncogenic transformation of host cells (25, 26). Transmission of HPV can be sexual or non-sexual (vertical transmission or through gynaecological instruments) (2, 27).

The most common oncogenic (high risk) HPV types include 16 and 18, while the most frequent low risk type is HPV 6. High risk HPV infection is associated with increased cytological abnormalities, and a greater percentage of those infected are diagnosed with high grade or low grade squamous intraepithelial lesions (L /H SIL) and invasive cancer (7, 19, 28-30).

Eleven percent of women worldwide and 21.3% of African women in the general population harbour cervical HPV infection at a given point in time. HPV prevalence in Africa varies from country to country. In Kenya 39.6% of women with normal cytology in the general population harbour cervical HPV infection (18). In Guinea the overall HPV prevalence was found to be 50.8% among the general population (31), Gambia 13% (28), Zambia 68% for high risk (HR) HPV and 36% low risk (LR) HPV (32), Kenya 55.6% for HR HPV and 25% for LR HPV among Female Sex Workers (FSW)(33), Uganda 42.6% (34), Nigeria 26.3% (35) and 26% in South Africa (36) .

The relationship between HIV and HPV has been a subject of interest. HR HPV types have been found to be more common among cervical cancer patients and HIV+ respondents (8). A hospital based study in Uganda found HPV prevalence of 46.2% in the general population, 59.5% in HIV+ and 39.1% in HIV-. Eighty eight percent (88.9%) of HIV- and 100% of HIV+ had high risk HPV types (34). HPV infection in Zambia was 80% among HIV+ and 55% among HIV-. Among HIV+ women 70% had HR HPV

genotypes compared to 35% of HIV– women (32). A study conducted among FSW in Kenya found a HR HPV prevalence of 55.6%. HR HPV was in 74% of HIV+ compared to 45% of HIV– women (7). The above studies also revealed multiple HPV infection occurs more commonly in HIV infection, agreeing with observations of a study in Gambia in which the low multiple infection by HPV was attributed to low prevalence of HIV in Gambia at that time (28). Increased cervical cytological abnormalities have also been observed in HIV+ women (34). Younger HIV+ women have been associated with cervical cancer compared to their HIV negative counterparts even after adjusting for number of sexual partners (8).

HIV promotes HPV prevalence by interfering with the immune system. One mechanism of inducing immune suppression is by depleting or redistributing Langerhans cells from mucosal surfaces or interfering with their Major Histocompatibility Complex (MHC) presenting ability. HIV also promotes Th2 cytokines which down regulate cytotoxic T lymphocytes (CTL) responses (37). HIV *tat* protein also up regulates E6 and E7 HPV oncoproteins (37-39). HIV+ individuals, especially those with low CD4 counts have been associated with increased HPV viral loads and slower clearance of HPV (7, 40).

Cytokine responses for HPV infection may include T-helper (Th)1 (pro-inflammatory), Th2 (anti-inflammatory) cytokine responses. Th1 cytokines play a key role in HPV clearance, while Th2 cytokines promote persistence of HPV (11). Decreased Th1 cytokine level is associated with increased severity of cervical lesions (12). Cytokines found to be increased in HPV infection include GM-CSF, IFN $\gamma$, and IL2 for Th1 and IL

4, -5, -10 and -13 for Th2 responses. Responses have also been observed for IL 1α, -6, -8, -17, MIP1α and TNFα (41).

Bais and colleagues found significant increase in plasma concentrations of IL-2 of HR-HPV negative compared to HR-HPV positive individuals. They also found women with cervical dysplasia had increased IFNγ levels than their controls; however, significant differences were observed for TNFα between study group and controls (42). Later Bais and colleagues found HPV infected women had decreasing plasma levels of Th1 cytokines (IFNγ, TNFα and IL 2) with increasing CIN dysplasia grades, but IFNγ increased again from CIN III to carcinoma while IL12 increased gradually to CIN II then decreased to carcinoma (43).

Local inflammatory cytokines play a very important role in HPV infection and cervical dysplasia. Zara and colleagues found higher IL1β concentration in CVL of HIV+ women with SIL, while both IL 1β and IL6 were lower in women with vaginal infection in comparison to respective controls (44). Behbakht and co-workers also observed increased IL1β and IL 6 among women with cervical dysplasia than their controls (45). Infection with other lower genital tract bacteria which also has a role in inflammatory cytokine production has been found not to be associated with high grade cervical lesions in the presence of HPV (46). Increased concentration of IL1α and IL 6 have been demonstrated *in vitro* to significantly enhance the growth of both neoplastic and normal cells, and are not dependent on presence of HPV DNA (15), contradicting what others have suggested that the two cytokines only stimulate growth of neoplastic cells (47). IL-6 has also been observed to act in an autocrine fashion (15).

Traditionally cervical cancer is screened using pap-smear, followed by colposcopy directed biopsy to determine presence of dysplasia or cancer. Others methods of screening include detection of presence of HPV by PCR or hybrid capture technique(4, 17). However many developing nations including Kenya are increasingly using Visual inspection with iodine or acetic acid (VILI/ VIA) for cervical cancer screening because of its lower costs, simplicity, safety and acceptability(48), especially when performed by trained personnel (49). In VIA, acetic acid causes a reversible coagulation or precipitation of cellular proteins resulting in a momentary change of colour of the squamous epithelium from pink to white (23). The percentage of women in the general population in Uganda positive for VIA only and for both VIA/VILI was 11% and 16%, respectively. Similar VIA positive results of 12.7% have also been found in India (17, 49). Although VIA specificity and sensitivity increases with experience, its specificity is reported to be lower than cytology, leading to over diagnosis and treatment. When VIA/VILI was evaluated using colposcopy as a standard, 72% of those identified as positive for cervical lesions by VIA/VILI were false positives by colposcopy (50)

# 3.0. METHODOLOGY

## Study Area

AMPATH cervical cancer screening and prevention program (ACCSPP) was initiated in 2008 with research funding from Fogarty International Center and later transformed to provide cervical cancer screening services specifically targeting AMPATH HIV positive clients due to their increased susceptibility for cervical cancer. Eventually the demand for the service increased to include HIV negative clients and even spread to AMPATH satellite clinics. Services have been up-scaled to include VIA screening, papsmear, colposcopy and biopsy and Loop electro surgical excision procedures (LEEP). The study was conducted in the AMPATH/MTRH cervical cancer screening clinics between the periods of November 2011/ Sept 2012.

## Study Design

The study design was cross sectional. Data was collected at a point in time from women attending cervical cancer screening.

## Study Population

The study population was all women attending cervical cancer screening clinics at AMPATH/MRTH. The target population was the women attending cervical cancer screening clinics.

**Inclusion criteria:** Only women aged above 18years participated in the study.

**Exclusion criteria:** Women who were currently or recently pregnant (3 months), those with active cervical or vaginal infection or those on active menses were excluded

## Sampling

Stratified sampling was used to select respondents meeting the participation criteria. HIV positive status was confirmed by ascertainment of enrollment into AMPATH program, a HIV care program where HIV positivity is determined VIA ELISA testing. HIV negative status was determined by participant report of no previously known HIV positive test. Upon determination of HIV status an equal number of HIV positive and HIV negative respondents was selected through convenient sampling.

EpiCalc® 2000 software was used to compute sample size by comparing proportions among the study groups. Prior data indicate HR-HPV positivity to be at 74% among HIV+ and 45% among FSW in Kenya (7). Therefore an expected proportion (p1) of 0.74 was adopted for the HIV+ group and p2 of 0.45 for the HIV- one at a significance level of 95% and power of 80% yielding a sample size of 44 per group and 88 overall. Comparable sample sizes have been utilized in previous studies of similar nature (44).

$$N = \frac{\{Za\sqrt{\bar{p}\,\bar{q}} + Zb\sqrt{p_1q_1 + p_2q_2}\}^{\,2}}{p_1p_2} = 44 \text{ x2 groups} = 88$$

$$where: \quad \bar{p} = p1 - p2 \qquad \bar{q} = q1 - q1$$

### Bias Minimization

Interviewer bias was minimized by use of uniformly structured questionnaires, mostly comprised of closed ended questions.

## Ethical Considerations

Ethical approval was sought from MOI UNIVERSITY/MTRH ethical review committee. All participants underwent a process of informed consent and the research objectives and clinical procedures were explained to the participants' prior obtaining consent. Participants were also made aware of all possible adverse effects that could arise as a result of the research procedures and made aware of all alternatives that they had apart from participation. All participants provided a signed informed consent form (see appendix) in addition to their verbal consent. Only qualified medical personnel were allowed to obtain samples from the participants. The researcher ensured maintenance of confidence of information provided by the respondents for the purposes of research. Privacy and confidentiality was maintained during the course of the interview sessions, and all interview sessions and clinical procedures were done in private. The respondents did not receive any direct benefit, apart from the health information that they received when attending the clinic even if they choose not to participate in the study. Patients who had suspicious lesions for cancer and those with confirmed cancerous lesions were referred to appropriate care providers within the hospital. Participants were not compensated for travel since they were recruited upon their own choice of attendance of cervical cancer clinic.

## Sample handling, collection and storage

### a. Collection and Processing of cervical specimen

A qualified trained nurse collected cervicovaginal lavage (CVL) by inserting 5ml sterile normal saline into the external cervical os and irrigating the endocervical region for approximately 1 minute using a sterile disposable plastic pasteur pipette. The CVL

pooled in the posterior fornix of the vagina was withdrawn using the same pipette (51). The fluid was transferred into a sterile tube and transported to the laboratory at 4ºC within 2 hours and processed by centrifugation at 1000 x g for 10 minutes at 4ºC. The supernatant was aliquoted and stored at -80ºC until further processing. The remaining cell pellet was re-suspended in normal saline and frozen at -80ºC.

**b. Visual Inspection with acetic acid**

The procedure was explained to the women participants and consent obtained prior to undertaking VIA. Two nurses trained in VIA examination conducted the screening as per IARC guidelines. In brief 5% acetic acid was gently applied using a cotton swab soaked in acetic acid after wiping off secretions. After 1 minute the cervix was observed for any white lesions, particularly in the transformation zone close to the squamocolumnar junction, or dense, non-removable aceto-white areas in the columnar epithelium. A positive VIA outcome was defined as a ‘‘sharp, distinct, well-defined, dense (opaque, dull, or, oyster white) aceto-white areas with or without raised margins, abutting the squamo-columnar junction in the transformation zone’’ or ‘‘strikingly dense aceto-white areas in the columnar epithelium’’ or ‘‘condyloma and leukoplakia occurring close to the squamo-columnar junction turning intensely white’’ 1 minute after the application of a 5% acetic acid solution (23).

## HPV and Cytokine Detection

### Cytokine Detection

The levels of IL12, TNFα, IL10, IL-1β, IL-6, IL-8 inflammatory cytokines were determined in cervical-vaginal lavage supernatant using BD® Human Cytometric Bead Array as per manufacturer's instructions (see appendix).

### Assay Procedure for supernatant

Mixed Capture Beads were added to the appropriate assay tubes followed by addition of Human Inflammatory PE Detection Reagent to the all assay tubes. Human Inflammatory Cytokine Standard dilutions were also added to the appropriate control assay tubes. An equal volume of each test sample was subsequently added to the test assay tubes. The tubes were incubated for 3 hours at room temperature and away from direct exposure to light. Wash buffer was added to each assay tube and centrifuged at 200 × *g* for 5 minutes. Supernatant was carefully aspirated and discarded from each assay tube. Wash Buffer was again added to each assay tube to re-suspend the bead pellet. The samples were then analyzed on a BD® FACS Callibur flow cytometer after brief vortexing of each sample.

### HPV DNA Detection

High Risk (HR) HPV DNA was detected using Hybrid capture 2 (HC2) method. This is a nucleic acid hybridization technique with signal amplification using microplate chemiluminescence that quantitatively detects 13 HR HPV DNA. Manufacturer's instructions were followed (see appendix).

In brief, specimens containing the target DNA were denatured then hybridized with a specific HPV RNA probe. The resultant RNA: DNA hybrids were captured onto the

surface of a microplate well coated with antibodies specific for RNA: DNA hybrids. Immobilized hybrids were then reacted with alkaline phosphatase conjugated antibodies specific for the RNA: DNA hybrids, and detected with a chemiluminescent substrate. Several alkaline phosphatase molecules were conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrid resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted which is measured as relative light units (RLUs) on a luminometer. The intensity of the light emitted denotes the presence or absence of target DNA in the specimen. An RLU measurement equal to or greater than the cutoff value indicated the presence of high-risk HPV DNA sequences in the specimen but those less than the cutoff value indicated the absence of the specific high-risk HPV-DNA levels below the detection limit of the assay.

## Data Management

### Data Collection Entry and Safety

Data for each respondent was collected by using a semi-structured questionnaire (see appendix). All respondents were given unique identification numbers during data collection and entry. All questionnaires, laboratory generated data and summarized data were stored in a safe lockable place accessible only to authorized individuals.

### Data analysis and Statistical Considerations

Data was entered using EpiData® software. SPSS® Ver.16 was used for statistical operations. Normally distributed variables were analysed using T-test for comparison while chi-square was used for categorical variables and Mann–Whitney U-test for skewed data. P-values $<0.05$ were considered significant

# 4.0. FINDINGS

**Socio-demographic characteristics**

The socio demographic characteristics for the study participants are shown in Table 4:1 below. The overall mean age of participants was 37.41 years; the mean age at first marriage and at first sexual intercourse lie between 16.6-18.8 years. Mean parity was 5 children. There were only a few statistically significant differences in demographic characteristics between the HIV+ and HIV- negative groups. Among the HIV positive women 50% were currently married. Their mean age at first marriage and age at first sexual intercourse was 18.8 (SD 3.3) and 16.6 (SD2.0) years, respectively. 72.7% of the HIV negative women were married. Their mean age, age at marriage and age at first sexual intercourse was 36.3 (SD 9.2), 18.7 (SD 2.7) and 16.6 (SD 3.1) years, respectively. Statistically significant differences between the HIV+ and HIV- group were only observed in the marital status where more of the HIV- group were married compared to the HIV+ group, most of whom were widowed /divorced/separated (45.5%). Difference in the number of active sexual partners between HIV+ and HIV- groups was also statistically significant with more HIV- group (88.6%) reported to have one sexual partner compared to their HIV+ counterparts (68.2%). Majority of the HIV- (85.4%) were also found to be or have been in monogamous marriages compared to their HIV+ counterparts (52.4%).

### Table 4:1 Socio Demographic Characteristics

| CHARACTERISTIC | TOTAL | HIV POSITIVE[1] | HIV NEGATIVE[2] | T/CHI-VALUE[3] | P-VALUE[4] |
|---|---|---|---|---|---|
| **Age(in years)** | **37.41 (SD 9.297)** | **38.6 (SD 9.4)** | **36.3 (SD 9.2)** | **1.172** | **0.244** |
| **Age at 1st marriage** | **18.8 (SD 3.0)** | **18.8 (SD 3.3)** | **18.7 (SD 2.7)** | **0.154** | **0.878** |
| **Age at 1st sexual Encounter** | **16.59 (SD 2.629)** | **16.6 (SD 2.0)** | **16.6 (SD 3.1)** | **0.161** | **0.872** |
| **Active Sex Partners** | | | | | |
| **None** | **18 (20.5%)** | **13 (29.5%)** | **5 (11.4%)** | **5.596** | **0.036** |
| **One** | **69 (78.4%)** | **30 (68.2%)** | **39 (88.6%)** | | |
| **More Than One** | **1 (1.1%)** | **1 (2.3%)** | **0 (0%)** | | |
| **Parity <mean, (range)>** | **5 (0, 20)** | **4 (0, 11)** | **5 (1, 20)** | **1.593** | **0.115** |
| **Marital status** | | | | | |
| **Single** | **5 (5.7%)** | **2 (4.5%)** | **3 (6.8%)** | | |
| **Married** | **54 (61.4%)** | **22 (50%)** | **32 (72.7%)** | **6.241** | **0.045** |
| **Widow/separated/ Divorced** | **29 (33.0%)** | **20 (45.5%)** | **9 (20.5%)** | | |
| **Kind of marriage** | | | | | |
| **Monogamous** | **57 (68.7%)** | **22 (52.4%)** | **35 (85.4%)** | **10.493** | **0.001** |
| **Polygamous** | **26 (31.3)** | **20 (47.6%)** | **6 (14.6%)** | | |

[3] &[4] = significance between [1] and [2], respectively

### Cytokine Responses

Three out of the six cytokines assayed i.e. IL12, IL10 and TNFα were not detected in majority of the samples and this was common for both HIV+ and HIV- groups (data not shown). Of the three remaining cytokines, IL6, IL1β and IL8, their overall distribution was skewed to the left. Majority of the respondents had IL6 levels below 100 pg/ml, IL1β below 400 pg/ml and IL8 below 2000 pg/ml as seen in Figure 4-1. A similar distribution was seen among HIV+ and HIV- categories of women. Comparison of cytokines was based on median cytokine levels and inter-quartile ranges.

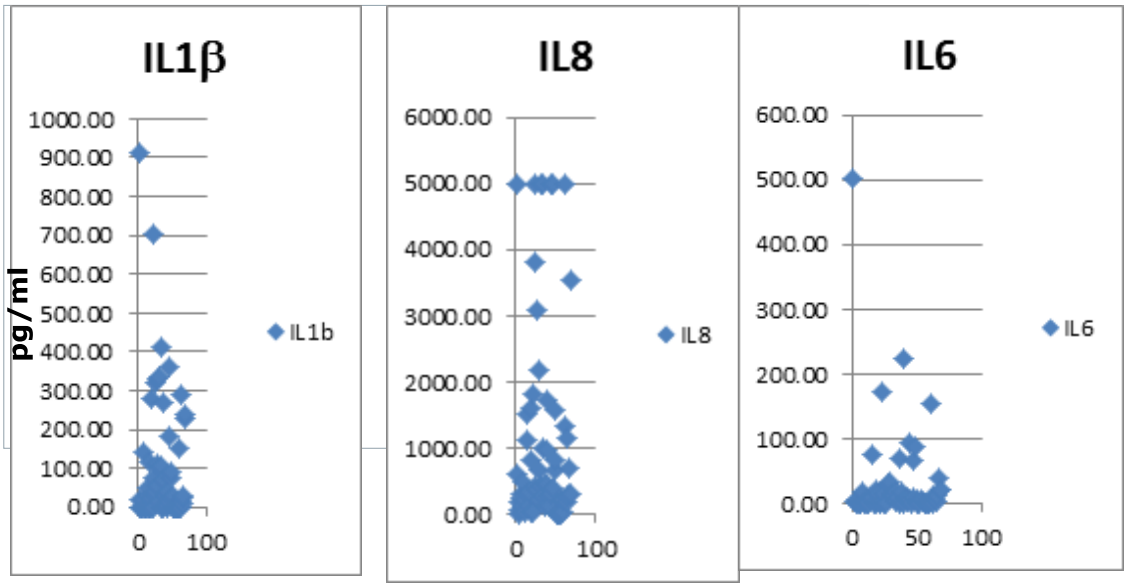


**Figure 4-1: Overall Cytokine Distribution**

### Comparison of cytokine levels according to HIV and VIA status

Overall HIV positive women had non-significant higher levels of cytokines than HIV negative women[ IL6 (p=0.055), IL1β(p=0.373) and IL8(p=0.326)]. Women with dysplasia (VIA+) had non-significant higher levels of IL 6 than VIA- women (p=0.160), but significantly higher levels of IL1β and IL8 (p value =0.001) (Figure 4-2).

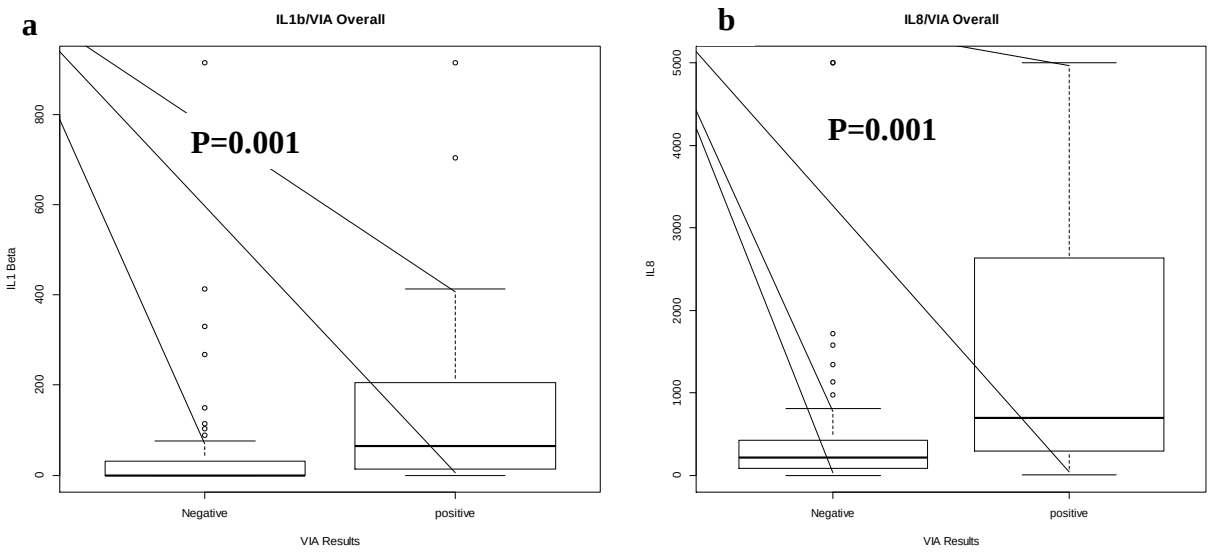


**Figure 4-2 a,b : Comparison of cytokines by VIA Status**

Among HIV+ women those with dysplasia (VIA+) had higher cytokine levels than VIA-, although none of the differences in the 3 cytokines attained significance [ IL6 (p=0.141), IL1β (p=0.081), IL8 (p=0.079)]. Among the HIV-, VIA+ women had higher IL6 levels than VIA- counterparts (p=0.401). However IL1β and IL8 levels were significantly higher in VIA+ than VIA- counterparts (p=0.008 and p=0.001 respectively) (Figure 4-3).

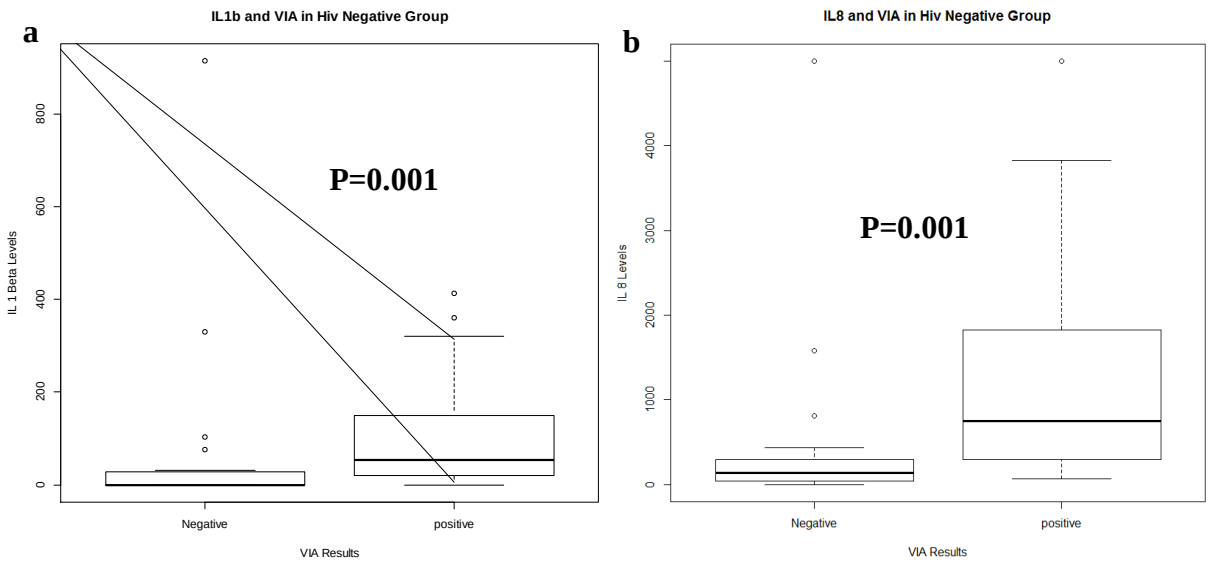


**Figure 4-3 a,b: Comparison of cytokines among HIV Negative**

## High Risk HPV Prevalence

Overall HPV prevalence was 51.1%. The prevalence of HR HPV amongst HIV + women was 59.1% and 43.2% among HIV- women as indicated in (Figure 4-4).

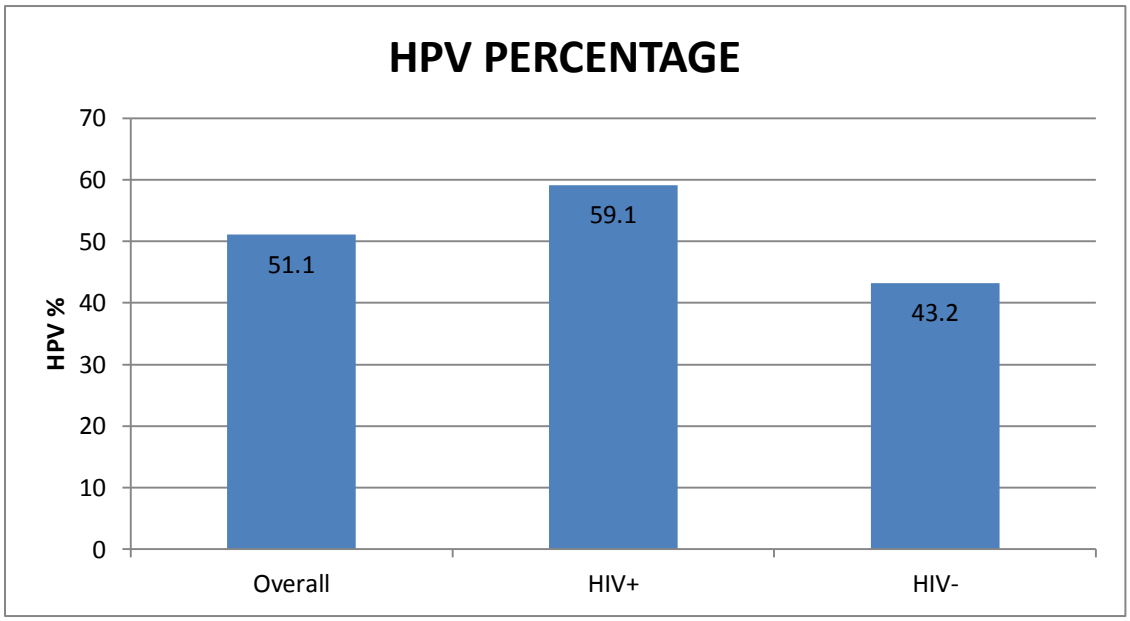


**Figure 4-4: HPV Prevalence**

Comparison of the levels of IL12, TNFα and IL10 was similar between HPV infected and uninfected women (data not shown). IL6 was higher in HPV positive than HPV negative though this was not significant (p=0.075). IL1β and IL8 were significantly higher among the HPV+ group than the HPV- group (p =0.001). (Figure 4-5).

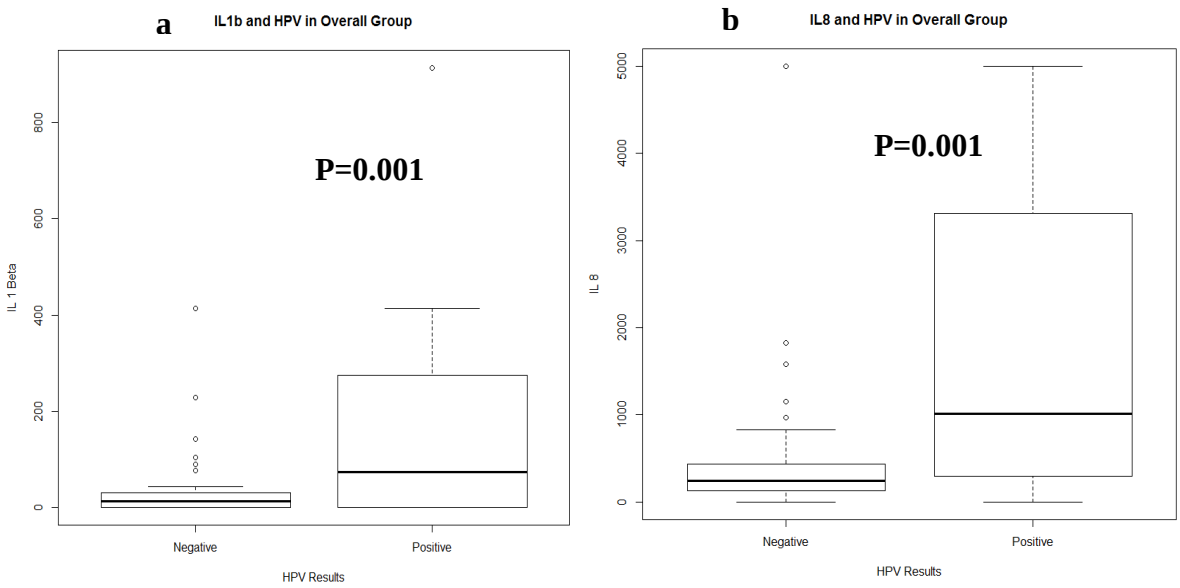


**Figure 4-5 a, b: Comparison of cytokines by HPV status**

Among HIV infected women comparison between those with HPV infection and those without HPV infection yielded no significant differences for IL6 (p=0.951), IL1β (p=0.244) and IL8 (p=0.101). However among the HIV negative those infected with HPV had significantly higher levels of IL6, IL1β and IL8 (Figure 4-6)

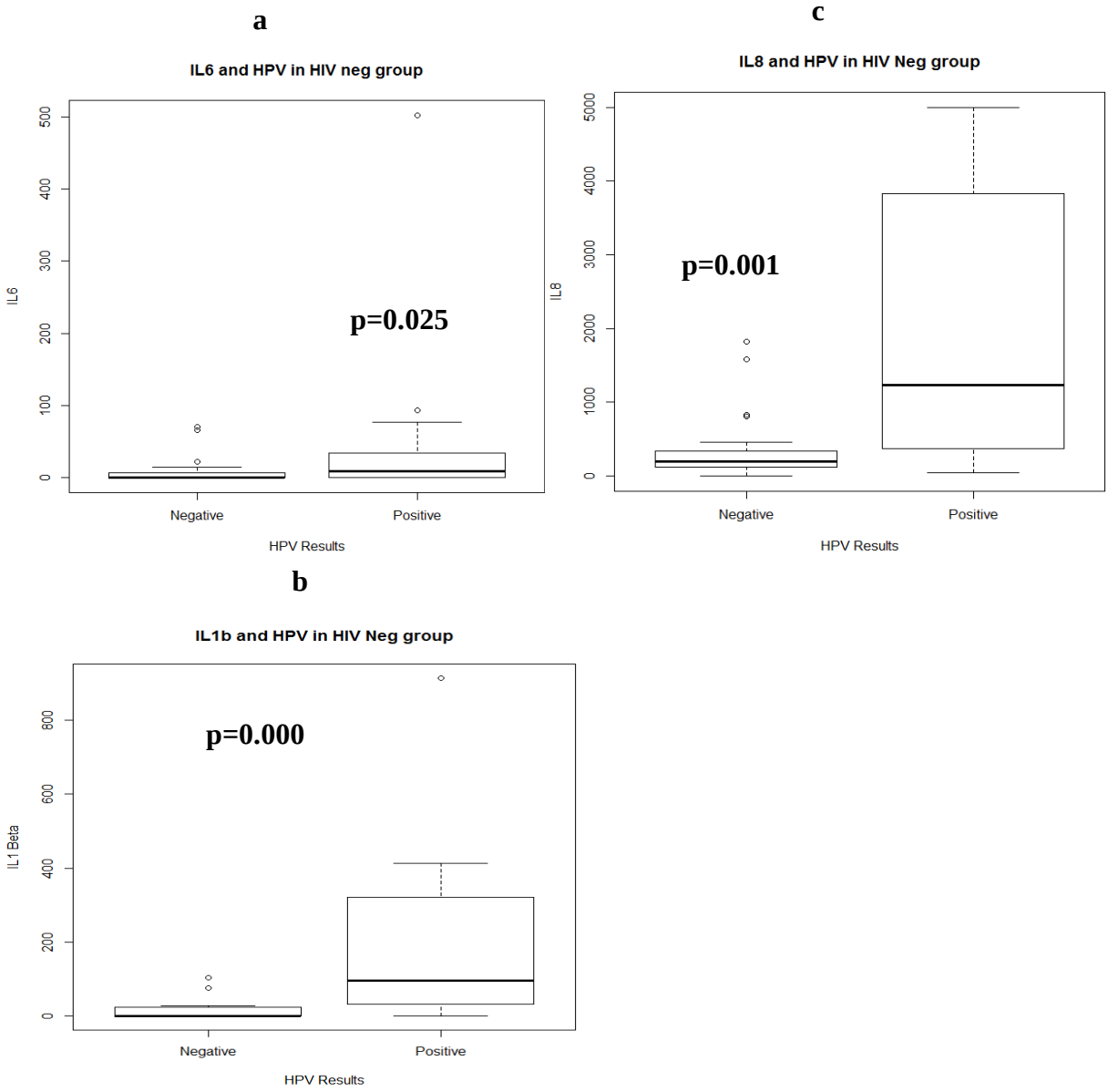


**Figure 4-6 a, b, c: Comparison of cytokines in HIV Negative Women**

## 5.0. DISCUSSION

In this study we compared the levels of pro- and anti-inflammatory cytokines in HIV infection status, HPV infection status and Dysplasia status (VIA). In general the pro inflammatory cytokines, IL6, IL1β and IL8 were found to be increased.

The comparably elevated IL6, IL1β and IL8 levels found in HIV+ in relation to HIV- women though not significant suggests a lesser role, if any, of the HIV in the production of these cytokines.

Inflammation is a relatively non-specific response to tissue injury caused by exogenous substances. The relationship between pro-inflammatory cytokines and different grades of CIN dysplasia has been reported before (42, 45, 46, 52, 53). However the relationship between VIA, a form of visual dysplasia detection, and inflammation is not well established. In this study we found that pro-inflammatory cytokines IL6, IL1β andIL8 were elevated in VIA+ women compared to VIA- women. However, only IL1β and IL8 were significantly higher in the VIA+. This increase in VIA + women suggests a relationship between VIA positivity, and presence of these cytokines. The principle behind VIA method is the coagulation of protein substances found below the cervical mucosa, which is then reflected as whitish lesions on the cervical surface (23). The researcher postulates that an increase of IL1β and IL8 could result to VIA positivity interpretation due to the coagulation of these and other locally produced proteins. The role of these cytokines in inflammation has been described before. IL 8 is a pro-inflammatory chemokine which recruits inflammatory cells like neutrophils at inflamed sites, it has angiogenic potential and plays a role in neutrophil activation (54, 55). IL6 is a

regulatory cytokine and a pro-inflammatory cytokine involved in the modulation of immune responses and both acute and chronic inflammation. (55-58). It also has a role in promoting growth of neoplastic cells which results in dysplasia (15). Previously Behbakht and colleagues found increased levels of IL6 and IL1β among cervical dysplasia cases than controls but only IL1β was significantly elevated (45). Tjiong *et al* found increased levels of IL6 and IL8 in CVL of CIN cases and cervical cancer patients than the normal controls (52). Tjiong *et al* also found increased IL1β and other inflammatory cytokines in CIN and cervical cancer compared to control women (59).

Significant differences in IL1β and IL8 between VIA+ and VIA- women was lost among the HIV+ women but retained in HIV- women. We attributed this loss to a probable immune suppression or dysregulation among HIV positive women leading to loss of significance. However our results concur with those reported by Zara *et al* who also found increased IL1β levels among HIV+ women with SIL, while both IL 1β and IL6 were lower in women with vaginal infection in comparison to respective controls (44).

HIV+ women had a higher prevalence of HR HPV (59.1%) as compared to the HIV- women (43.2%) and this could be as a result of their greater susceptibility to HPV virus infection and persistence due to a suppressed immune system as has been found before (37, 60). Comparable HPV prevalence have been observed before in Zambia where HR HPV prevalence among HIV+ was 70% and 35% in HIV- women (32) and also 74% in HIV+ Kenyan female sex workers compared to 45% in HIV- (7).

The significantly higher levels of IL1β and IL8 levels in HR HPV+ women compared to the HR HPV- women could be attributed to a response to the HPV infection. The

production of IL6 and IL8 is known to be stimulated by viral infection and bacterial components (14, 54, 61, 62). Similar relationship between different cytokine levels and HPV virus has been described by various authors before. Campos *et al* detected higher levels of IL6 among those infected with HRHPV and also among those with LRHPV (in the presence of other infections), but the differences were not significant when compared to the control group (63). In a paper review, Nicol and colleagues noted that some investigators had found increased IL 6 in HPV infection. The review also highlighted reports of increased IL-8 in cervico-vaginal lavage samples of HIV+ women with cervical dysplasia related to HPV infection than women without HPV (37). However some authors did not find differences in the level of IL6 and IL8 between HPV infected and non-infected women (52). Contradicting results among those with HPV infection have also been found by Lieberman and colleagues who found no significant differences in cytokine levels between HPV infected and uninfected women, but noted a trend of depressed IL 1β, IL6, IL12, IL13 among those with incident HPV infection and a similar trend in IL 1β and IL13 among those with persistent HPV infection. They found inflammatory cytokines to be generally elevated compared to regulatory cytokines (64). It would be difficult to directly compare those results with ours given that we cannot determine the period of HPV infection in our study.

In this study we were able to demonstrate a general trend of increase in IL6, IL1β and IL8 in HIV positive women, HPV positive women and VIA positive women. We also demonstrated a significant increase in IL1β and IL8 women with dysplasia as reported by VIA, especially among the HIV negative women, which to the best of our knowledge has not been reported before. This is finding is significant should be consideration in the

design of potential diagnostic methods that could employ the use of cytokines for cervical cancer screening/diagnosis.

**Study Limitations**

The study was limited by lack of correction for dilution effect in CVL since this was not possible.

The determination of HIV negativity status was not robust as this was not confirmed by testing but by self- reporting.

We were unable to rule out other genital tract infections which could influence cytokine production. However the effect of this is expected to be minimal as discussed in the literature.

# 6. CONCLUSION AND RECOMMENDATIONS

## Conclusion

Cytokine responses are an important factor that needs to be considered in the diagnosis of cervical cancer, especially HIV+ women. The following conclusions were drawn:

1. There is no significant difference in inflammatory cytokine responses between HIV positive and HIV negative women even though IL1β and IL8 levels are higher in HIV positive women.
2. IL1 β and IL8 are increased in VIA positive women in comparison to VIA negative women, especially among HIV negative women.
3. HPV infection is higher in HIV positive women and HPV positive women have significantly increased levels of IL1 β and IL8 than HPV negative women.

## Recommendations

1. Further studies evaluating the relationship between IL1β, IL8 and VIA be done to determine whether the presence of these two pro-inflammatory cytokines influences or determines the VIA outcome.
2. The potential role of utilizing cytokine detection in conjunction with other tests like HPV detection and VIA should be investigated as a method of screening for cervical cancer.

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# APPENDICES
## Appendix I Consent Form

**Title of Study**

**Inflammatory cytokine responses in women with HIV, HPV and cervical dysplasia at Moi Teaching and Referral Hospital, Eldoret Kenya**

**Investigator**

**Seth Kirui**
**Idara of Immunology**
**Moi University- School of Medicine**
**P.O.Box 4606**
**Eldoret.**

**Purpose of Study**

This study is seeks to compare the cytokine responses among HIV/HPV co-infected women at diagnosed with cervical dysplasia and cancer ( using VIA) in an effort to underscore important differences between the two groups with the aim of improving cervical cancer diagnosis, prognosis and management**.**

**Procedures**

The researcher has explained to me in detail about the objectives and purposes of this research in detail. The procedures involved in this research include the collection of cervical secretions and cells for analysis and the screening using a visible dye (VIA).

**Risks**

I understand that these procedures generally harmless but minimal potential for harm exists. I may experience discomfort, slight pain and bleeding and irritation during collection of cervical cells. However only qualified personnel will be allowed to handle me to minimize on these risks.

**Benefits**

I understand that there will be no direct benefits to me, including transport reimbursement, as an individual apart from health information that I will receive and free screening for cervical cancer. This is because I will all samples will be collected during my routine visit to the screening clinic. However the results of this study will contribute to the body of knowledge on cervical cancer and its management.

**Privacy and Confidentiality**

I understand that I am entitled to utmost privacy during all the steps that will be undertaken during this research study. Only authorized research staff will be privy to private information I divulged as a result of this study and such information can only be shared with another third party after my express written consent.

**Right to refuse participation/withdraw**

I understand that it is within my right to freely to withdraw from this study or any of its procedures at any time that I feel I cannot go on without a feeling of guilt or obligation to anyone.

All questions and clarifications about this study can be addressed to the following contact persons:

**Seth Kirui** **0721-759004**

**Dr. Kirtika Patel** **0726215800**

**Dr. Omenge Orang'o** **0722609132**

**Statement of consent**

**I _____ have carefully read this**

**document and freely and willingly consent to participate in this study.**

| | **Name** | **Signature/Thumb print** | **Date** |
|---|---|---|---|
| **Respondent** | | | |
| **Witness (applicable for those who can't read)** | | | |
| **Study Staff** | | | |

**Mada ya Utafiti**
**Viwango vya kinga ya mwili ya cytokines kati ya wanawake walio na virusi vya Ukimwi, HPV na dalili ya saratani ya mlango wa uzazi katika Hospitali ya rufaa yaMoi, Eldoret Kenya**
**Mtafiti**

**Seth Kirui**
**Department of Immunology**
**Moi University- Chuo cha Udaktari**
**SLP 4606**
**Eldoret.**

**Kusudi ya Utafiti**

Utafiti huu unakusudia kulinganisha viwango vya kinga ya mwili ya cytokines baina ya walioambukizwa virusi vya ukimwi, HPV na waliopatikana na dalili za ugonjwa wa saratani ya mlango wa uzazi kwa kutumia mbinu ya kupimia ya VIA kwa madhumuni ya kubaini tofauti zilizo baina ya vikundi hivi ili kuchangia katika mikakati ya kuboresha uchunguzi, kupimwa na matibabu ya saratani ya mlango wa uzazi

**Utaratibu**

Mtafiti amenieleza kwa umakini kuhusu madhumuni na makusudio ya utafiti huu. Utatatibu wa utafiti huu unajumulisha ukusanaji wa majimaji ya mlango wa uzazi na chebechembe za mwili ili kuchunguzwa, na kupimwa kutumia mbinu ya VIA

**Hatari**

Naelewa kwamba njia hizi kwa ujumla hazina hatari yoyote ila kuna uwezekano mdogo wa madhara kutokea. Ninaweza kukerwa, kuskia uchungu mdogo, kutokwa na damu kidogo na kuwashwa wakati wa kukusanya chembechembe za mlango wa uzazi. Ili kupunguwa madhara yoyote ni wahudumu waliohitimu tu ambao wataruhusiwa kuwahudumia wateja.

**Manufaa**

Naelewa kwamba hakutakuwa na manufaa ya moja kwa moja kwangu binafsi ila kujua matokeo ya kupimwa kwangu ugonjwa wa saratani ya mlango wa uzazi. Matokeo ya utafiti huu yatachangia ujuzi na maarifa kuhusu saratani ya mlango wa uzazi na matibabu yake.

**Usiri**

Naelewa kwamba nina haki ya usiri na faragha kudumishwa wakati wa utaratibu wowote wa utafiti huu. Ni wahudumu wa utafiti huu walioidhinishwa ambao wataweza kupata ujumbe wa siri ambao nitatoa katika utafiti huu, na ujumbe huu hauwezi kupeanwa kwa mtu mwingine pasipo na idhini yangu.

**Uwezo wa kukataa kushiriki/kujiondoa**

**Right to refuse participation/withdraw**

Naelewa kwamba nina uhuru na haki ya kujiondoa kwenye utafiti huu ama utaratibu wowote wakati wowote najihisi siwezi kuendelea pasipo na kihisi kuwajibika kwa yeyote..

Ikiwa una maswali yoyote ama unahitaji kufafanuliwa jambo lolote kuhusu utafiti huu unaweza kuwasiliana na watu wafuatao:

**Seth Kirui** **0721-759004**

**Dr. Kirtika Patel** **0726215800**

**Dr. Omenge Orang'o** **0722609132**

**<u>Taarifa ya maridhiano</u>**

**Mimi_____ nimesoma kwa umakini**

**na kuelewa hati hii na bila kushutumiwa na yeyote nimekubali kushiriki katika**

**utafiti huu**

| | **Jina** | **Sahihi/ Alama ya kidole gumba** | **Tarehe** |
|---|---|---|---|
| **Mtafitiwa** | | | |
| **Shahidi( Kwa wasioweza kusoma)** | | | |
| **Mwakilishi wa Utafiti** | | | |

## Appendix II Questionnaire

| Questionnaire Serial | 0 | 0 | 1 |
|---|---|---|---|

**Part A Screening (please confirm eligibility by crosschecking with the following checklist)**

| | |
|---|---|
| **Above 18 years** | ☐ |
| **Not Pregnant or Recent Birth (3 months)** | ☐ |
| **Not on active menses (past 4 days)** | ☐ |
| **No active vaginal or cervical infection** | ☐ |

**Part B**

Date of interview ___/___/___ (dd/mm/yy):

1. Age (in years) _____
2. Do you know your HIV status: Positive [1] Negative [2] Do not know[3]
3. Marital Status: Single [1] Married [2] Widowed or Divorced[3]
4. If ever married above what was your age at first marriage in years
   15 years or below [1] 16-20 [2] 21-25 [3] above 25 [4]
5. If married what kind of marriage: Monogamous [1] Polygamous [2]
6. How many children have you given birth to? (both alive and dead)
   None [1] One [2] two to three [3] four or more [4]
7. What was your age at first sexual encounter? (in years).
   Never [1] Below 15 [2] 16-20 [3] 21-25 [4] above 25 [5]
8. How many active sexual partners do you currently have?
   None [1] One [2] More than one [3]

**Part C- To be filled by clinician**

9. VIA screening results
   Positive [1] Negative [2]

| Nambari ya fomu | 0 | 0 | 1 |
|---|---|---|---|

**Sehemu A Uchunguzi kubaini uhakiki wa kushiriki katika utafiti**

**Juu ya miaka 18** ☐

**Huna ujauzito ama kuzaa karibuni (miezi 3)** ☐

**hauko katika hedhi ( siku 4 zilizopita)** ☐

**Hakuna maambukizi ya uke au mlango wa uzazi** ☐

**Sehemu B**

Tarehe ya kuhoji ___/___/___

10. Miaka _____

11. Umewahi pimwa na kupatikana na virusi vya ukimwi:

Ndio [1] La [2] Sijui[3]

12. Umeolewa: Ndio [1] La [2] Talakiwa[3]

13. Kama umewahi kuolewa, ulikuwa na miaka ngapi kwa ndoa ya kwanza

Chini ya miaka 15 [1] 16-20 [2] 21-25 [3] zaidi ya miaka 25 [4]

14. Aina ya Ndoa: Mke mmoja [1] Wake zaidi ya mmoja [2]

15. Umewahi kuzaa watoto mara ngapi? (jumla ya walio hai na waliofariki)

Hamna [1] Moja [2] 2-3 [3] 4 au zaidi [4]

16. Ulikuwa na miaka ngapi ulipofanya tendo la ngono kwa mara ya kwanza?

Sijawahi [1] Chini ya miaka 15 [2 16-20 [3] 21-25 [4] zaidi ya miaka 25 [5]

17. Una wapenzi wangapi wa ngono kwa sasa?

Sina [1] Mmoja [2] Zaidi ya mmoja [3]

**Sehemu C- Sehemu ya C**

18. Matokeo ya uchunguzi wa VIA

Ndio [1] La [2]

## Appendix III Cytometric Bead Array Procedure

Inflammatory cytokines will be determined by use of BD® Cytometric Bead Array.

**Principle of Test**

Beads specific for 6 cytokines i.e. IL-1β, IL-6, IL-10, IL-12p70, TNF-α, proteins, with distinct fluorescence intensities are coated with capture antibodies specific for the respective cytokines. These are then detected by a suitable flow cytometer. These cytokine capture beads are mixed with the PE-conjugated monoclonal antibodies specific to the cytokines. Upon incubation with test samples (or standards) they form sandwich complexes, which are then read by a flow cytometer generating suitable graphs for interpretation.

**Standards, Beads and Sample Preparation**

Human cytokine standards will be reconstituted using provided diluents and subsequently serially diluted according to manufacturer's instructions. One Vial of standard is reconstituted with 2ml of assay diluents to make a top standard (1:1). 8 12x75 ml tubes are then labeled 1:2, 1:4……..1:256. 300µl of assay diluent is added to each of these tubes. 300µl of top standard is added to the 1:2 tube, subsequently 300 µl of this mixture is added to the 1:4 tube and this goes on until the 1:256 dilution is attained. One tube containing assay diluents only serves as the negative control.

During bead standard preparation the number of assay tubes (including standards and controls) that are required for the experiment (eg, 8 unknowns, 9 cytokine standard dilutions, and 1 negative control = 18 assay tubes) is determined. Each Capture Bead suspension is vigorously vortexed for a few seconds before mixing. This is followed by addition of 10 µl aliquot of each Capture Bead, for each assay tube to be analyzed, into a single tube labeled "mixed Capture Beads" (eg, 10 µl of IL-2 Capture Beads × 18 assay tubes = 180 µl of IL-2 Capture Beads required). The Bead mixture is vortexed thoroughly. Centrifuge the mixed Capture Beads at 200 × *g* for 5 minutes then carefully aspirate and discard the supernatant. Re-suspend the mixed Capture Beads pellet in Serum Enhancement Buffer (equal volume to amount previously aspirated) and vortex thoroughly. Incubate the mixed Capture Beads for 30 minutes at RT and protect from direct exposure to light.

If samples are known or assumed to contain high levels of a given cytokine they should be diluted by the desired dilution factor (ie, 1:2, 1:10, or 1:100) using the appropriate volume of assay diluent. They are then mixed thoroughly before transferring samples to the appropriate assay tubes containing mixed Capture Beads and PE Detection Reagent

**Supernatant Assay Procedure**

1. Vortex the mixed Capture Beads and add 50 µL to all assay tubes.
2. Add 50 µL of the Human Inflammatory Cytokine Standard dilutions to the control tubes as listed in the following table.
3. Add 50 µL of each unknown sample to the appropriately labeled sample tubes.
4. Add 50 µL of the Human Inflammatory Cytokine PE Detection Reagent to all assay tubes.
5. Incubate the assay tubes for 3 hours at room temperature, protected from light.
6. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200*g* for 5 minutes.
7. Carefully aspirate and discard the supernatant from each assay tube.
8. Add 300 µL of Wash Buffer to each assay tube to re-suspend the bead pellet.

# Appendix IV HPV DNA PCR Procedure

## MATERIALS REQUIRED BUT NOT SUPPLIED

### Hybrid Capture System *In Vitro* Diagnostic Equipment and Accessories[A]

- Digene Microplate Luminometer 2000 (DML 2000™) Instrument and PC System, Printer Cable, Printer, *DML 2000™ Instrument and Version 2 Software User Manual*, and the *Digene Hybrid Capture System Version 2 (DHCS v.2) Software Interactive Operator's Guide* (Version 4.01 or later DML 2000 Assay Protocols for HPV are required)
- Hybrid Capture System Rotary Shaker I
- Hybrid Capture System Microplate Heater I
- Hybrid Capture System Automated Plate Washer I
- Hybrid Capture System Multi-Specimen Tube (MST) Vortexer I or 2 (Optional)[B]
- Conversion Rack and Rack lid (optional)
- Digene Specimen Rack and Rack lid (optional)
- EXPAND-4™ Pipettor and Stand (optional)[C]
- Hybrid Capture Cervical Sampler or DNAPap Cervical Sampler[D]
- Tube Sealer Dispenser and cutting device (optional, used with the MST Vortexer I or 2)
- Rapid Capture® System (optional for high volume sample-throughput testing)[E]
- Wash Apparatus
- Hybridization Microplates
- Microplate Lids
- Empty Microplate Strips (available from Costar, Model #2581); optional for use with the Automated Plate Washer I
- Extra-Long Pipette Tips for removal of specimen
- Specimen Collection Tubes
- Specimen Collection Tube Rack
- Specimen Collection Tube Screw Caps
- Disposable Reagent Reservoirs
- DuraSeal® Tube Sealer Film
- Hybridization Microtubes
- Microtube Rack
- Plate Sealers

### General Laboratory Use Equipment and Accessories

- 65 ± 2°C water bath of sufficient size to hold either 1 Conversion Rack (36 x 21 x 9 cm) or specimen racks
- Microcentrifuge (optional for centrifuging probe vials to obtain maximum probe volume)
- Vortex mixer with cup attachment
- Single-channel Micropipettor; variable settings for 20-200 µl and 200-1000 µl volumes
- Repeating positive displacement Pipettor, such as Eppendorf Repeater® Pipette or equivalent
- 8-channel Pipettor: variable settings for 25-200 µl volumes
- Timer
- Sodium hypochlorite solution, 5% v/v (or household bleach)
- Parafilm® or equivalent
- Disposable aerosol-barrier Pipette Tips for single-channel pipettor (20 to 200 µl and 200-1000 µl)
- Disposable Tips for Eppendorf Repeater® Pipette (25 and 500 µl)
- Disposable Tips for 8-channel pipettor (25 to 200 µl)
- Kimtowels® Wipers or equivalent lint-free paper towels
- Disposable bench cover
- Powder-free gloves
- 5-ml and/or 15-ml snap-cap, round-bottom Polypropylene Tubes (for Probe dilution)
- 2.0-ml polypropylene microcentrifuge tubes with caps

### Additional Equipment and Accessories for PreservCyt Solution Specimen Processing

- Swinging Bucket Centrifuge capable of reaching 2900 ± 150 x *g* and holding 10-ml or 15-ml conical polypropylene centrifuge tubes
- 5-ml serological pipettes or transfer pipettes
- hc2 Sample Conversion Kit[A]
- Disposable tips for Eppendorf Repeater® Pipette (50 and 100 µl)

For Manual Vortex Procedure:

- hc2 Sample Conversion Tubes (15-ml conical)[F], Sarstedt 10-ml Conical tubes with Caps or VWR or Corning® brand 15-ml conical-bottom polypropylene centrifuge tubes with caps
- Tube rack to hold 10-ml or 15-ml conical tubes

For Multi-Specimen Tube Vortexer 2 Procedure

- hc2 Sample Conversion Tubes (15-ml conical)[F]
- Multi-Specimen Tube (MST) Vortexer 2
- Conversion Rack and Lid (specific for 15-ml conical tubes)
- Tube Sealer dispenser and cutting device
- DuraSeal® Tube Sealer Film (used with the MST Vortexer 2)

### Additional Equipment and Accessories for Surepath Preservative Fluid Specimen Processing

- Swinging Bucket Centrifuge capable of reaching 800 ± 15 x *g* and holding 15-ml conical polypropylene centrifuge tubes
- hc2 Sample Conversion Tubes (15-ml conical tubes)[F]
- 7-ml standard tipped transfer pipettes or equivalent
- QIAGEN Specimen Transport Media
- Disposable tips for Eppendorf Repeater® Pipette (100 µl)

[A] Only equipment and accessories validated with hc2 High-Risk HPV DNA Tests are available from QIAGEN.
[B] Also required for use when performing the Semi-automated RCS Application.
[C] Custom item. Other custom expandable multi-channel pipettes can be used, provided tip spacing of 3.2 cm is achievable when expanded. Alternatively, a single-channel pipette capable of pipetting 75 µl may be used.
[D] The performance characteristics of the hc2 High-Risk HPV DNA Test were established only with the collection kits indicated.
[E] Refer to the *Rapid Capture System User Application Manual* for instructions specific to the use of that system for high volume sample-throughput testing with this assay.
[F] The hc2 Sample Conversion Tubes (VWR or Corning® brand) available from QIAGEN must be used to assure proper assay performance when using the Multi-Specimen Tube Vortexer 2 procedure.

## REAGENT PREPARATION AND STORAGE

1. Upon receipt, store the kit at 2-8°C. The Wash Buffer Concentrate, Denaturation Reagent and Indicator Dye may be stored at 2-30°C, as desired.
2. Do not use after the expiration date indicated next to the symbol on the box label, or the expiration date of the prepared reagents (see below).
3. All reagents are provided ready to use, except Denaturation Reagent and High-Risk HPV Probe, and Wash Buffer Concentrate.

**For high volume sample-throughput testing, refer to the *Rapid Capture System User Application Manual* for the preparation of the High-Risk HPV Probe Mix, the Wash Buffer, Detection Reagent 1, and Detection Reagent 2 as those instructions are specific to the use of that system for high-volume sample throughput testing.**

<table>
<tr><th>REAGENT</th><th>PREPARATION METHOD</th></tr>
<tr><td>Denaturation Reagent</td><td>Prepare First:<br>Add 5 drops of Indicator Dye to the bottle of Denaturation Reagent and mix thoroughly. The Denaturation Reagent should be a uniform, dark purple color.<br><br>Once prepared, the Denaturation Reagent is stable for three months when stored at 2-8°C. Label it with the new expiration date. If the color fades, add 3 additional drops of Indicator Dye and mix thoroughly before using.<br>Warning: Denaturation Reagent is corrosive. Wear suitable protective clothing, glove, eye/face protection. Use care when handling.</td></tr>
<tr><td>High-Risk HPV Probe Mix (Prepared from High-Risk HPV Probe and Probe Diluent reagents)</td><td>Prepare during specimen denaturation incubation:<br><br>Important: Sometimes probe gets trapped in the vial lid.<br><br>Note: Extreme care should be taken at this step to prevent RNase contamination of Probe and Probe Mix. Use aerosol-barrier pipette tips for pipetting probe. Probe Diluent is viscous.<br>Care should be taken to ensure thorough mixing when preparing High-Risk HPV Probe Mix. A visible vortex must form in the liquid during the mixing step. Incomplete mixing may result in reduced signal.<br><br>• Centrifuge the vial of High-Risk HPV Probe briefly to bring liquid to bottom of vial. Tap gently to mix.<br>• Determine the amount of Probe Mix required (25 µl/test). It is recommended that extra Probe Mix be made to account for the volume that may be lost in the pipette tips or on the side of the vial. Refer to suggested volumes listed below. The smallest number of wells recommended for each use is 24. If fewer than 24 wells per assay are desired, the total number of tests per kit may be reduced due to limited Probe and Probe Diluent volumes.<br>• Transfer the required amount of Probe Diluent to a new disposable container. Depending on the number of tests, either a 5 ml or 15 ml snap-cap, round bottom, polypropylene tube is recommended. Make a 1:25 dilution of High-Risk HPV Probe in Probe Diluent to prepare Probe Mix.<br><br>
<table>
<tr><th>No. of Tests/Strips</th><th>Volume Probe Diluent*</th><th>Volume Probe*</th></tr>
<tr><td>96/12</td><td>4.0 ml</td><td>160.0 µl</td></tr>
<tr><td>72/9</td><td>3.0 ml</td><td>120.0 µl</td></tr>
<tr><td>48/6</td><td>2.0 ml</td><td>80.0 µl</td></tr>
<tr><td>24/3</td><td>1.0 ml</td><td>40.0 µl</td></tr>
<tr><td>Per Well</td><td>0.045 ml</td><td>1.8 µl</td></tr>
</table>
*These values include the recommended extra volume.<br>• Pipette High-Risk HPV Probe into Probe Diluent by placing the pipette tip against the inner wall of the tube just above the meniscus and expelling the contents. Do not immerse the tip into Probe Diluent.<br>• Vortex for at least 5 seconds at maximum speed to mix thoroughly. A visible vortex must be produced. Label as High-Risk HPV Probe Mix and keep in a clean, closed container until ready for use. Unused Probe Mix should be discarded.</td></tr>
</table>

| | |
|---|---|
| Wash Buffer | **PREPARE DURING CAPTURE STEP:**<br>**For the Hybrid Capture System Automated Plate Washer I**, the Wash Buffer can be prepared as described below and stored in a covered container or prepared 1 L at a time and placed in the Automated Plate Washer I Wash Reservoir. See the Table below for mixing volumes:<br><br>See Automated Plate Washer I Operation and Maintenance Manual for Care and Maintenance Instructions.<br><br>**Warning**: Wash Buffer Concentrate is toxic by ingestion. Wear suitable protective clothing, gloves, eye/face protection. To minimize exposure, add water to Wash Buffer Concentrate when preparing. |

| Amount of Wash Buffer Concentrate | Amount of Distilled or Deionized Water | Final Volume of Wash Buffer |
|---|---|---|
| 33.3 ml | 966.7 ml | 1L |
| 66.6 ml | 1,933.4 ml | 2L |
| 100 ml | 2,900 ml | 3L |

**Note: It is very important to leave the power to the Automated Plate Washer I on at all times. This allows the maintenance rinse to be performed after eight hours of non-use.**

**Prior to each assay, make sure the Automated Plate Washer I Waste Reservoir is empty and the Rinse Reservoir is filled with distilled or deionized water.**

**For the manual plate washing method**:

- Mix wash buffer concentrate well.
- Dilute 100 ml Wash Buffer Concentrate with 2.9 L of distilled or deionized water in Wash Apparatus and mix well (final volume should be 3 L).
- Seal the container to prevent contamination or evaporation.

Once prepared, the Wash Buffer is stable for three months at 2-30°C. Label it with the new expiration date. If Wash Buffer has been refrigerated, equilibrate to 20-25°C before using.

It is recommended that the Wash Apparatus and tubing be cleaned with 0.5% sodium hypochlorite solution and rinsed thoroughly with distilled or deionized water once every three months to prevent possible contamination from alkaline phosphatase present in bacteria and molds.

**VOLUMES FOR READY-TO-USE REAGENTS**

Detection Reagent 1 & Detection Reagent 2

**IMMEDIATELY PRIOR TO USE:**

Mix reagent thoroughly, then carefully measure the appropriate volume of Detection Reagent 1 or Detection Reagent 2 into a clean disposable reagent reservoir following the guidelines shown below. To avoid contamination, these Reagents **MUST NOT** be returned to the original bottles: **Discard unused material after use**. If an 8-channel pipettor is not being used, an appropriate repeating pipettor may be substituted. In this case, aliquots of the Reagent should be made into a polypropylene tube of sufficient size to hold the required volume as indicated below.

| No. of Tests/Strips | Volume Detection Reagent 1 or 2 |
|---|---|
| 96/12 | contents of bottle |
| 72/9 | 7.0 ml |
| 48/6 | 5.0 ml |
| 24/3 | 3.0 ml |
| 1 test | 0.125 ml |

## TEST PROCEDURE

**Specimens may contain infectious agents and should be handled accordingly.** The hc2 High-Risk HPV DNA Test can be performed manually as instructed in the package insert or using the Rapid Capture System instrument for high volume sample-throughput testing.

### HIGH VOLUME SAMPLE-THROUGHPUT TESTING USING THE RAPID CAPTURE SYSTEM

The Rapid Capture System is a general use automated pipetting and dilution system that can be used with the hc2 High-Risk HPV DNA Test for high volume sample-throughput testing. This system handles up to 352 specimens in eight hours, including a 3.5-hour period during which user intervention is not required; up to 704 specimen results can be generated in 13 hours. Denaturation of the specimens in preparation for testing is performed independently of the RCS, in the primary collection tube, as with the manual method of the hc2 High-Risk HPV DNA Test described below, prior to placing the specimens on the RCS platform. In addition, chemiluminescent signal detection and result reporting are performed using the offline luminometer system [Digene Microplate Luminometer (DML 2000™) Instrument] common to both the manual and RCS methods. Each of the hc2 High-Risk HPV DNA Test's procedural steps is performed in the exact sequence as the manual test procedure. The RCS Application allows for the staggered processing of up to 4 microplates, containing specimens and the required assay Calibrators and Quality Controls.

**When using the Rapid Capture System, refer to the *Rapid Capture System User Application Manual* provided with the instrument, in addition to this package insert, for necessary procedural and descriptive information.**

SETUP

1. If using the Microplate Heater I, **allow it at least 60 minutes to equilibrate to 65 ± 2°C from a cold start**. See *Microplate Heater I Operator's Manual* for details.
2. Confirm the water bath is at 65°C and the water level is high enough to immerse the entire volume in the specimen tubes.
3. Remove the specimens and **all** required Reagents from the refrigerator **prior to beginning the assay**. Allow them to reach 20-25°C for 15 to 30 minutes.

   **Note:** Prepare PreservCyt Solution and SurePath specimens prior to equilibrating any previously denatured specimens and kit reagents to room temperature.
4. Use the Digene Hybrid Capture System Version 2 (DHCS v.2) Software or the Digene Qualitative Software to create the assay plate layout. Refer to the respective software's User's Manuals for instructions on creating a plate layout.
5. Place Calibrators, Quality Controls, and specimens to be tested in a test tube rack, in the same order in which they will be tested. **The Negative Calibrator and High-Risk HPV Calibrator must be tested FIRST**. Run Negative Calibrator (NC), High-Risk HPV Calibrator (HRC), Low-Risk Quality Control (QC1-LR), High-Risk Quality Control (QC2-HR), and specimens in an 8-microplate well column configuration. See *Example Layout* below.

| Example Layout for a Run of 24 Microplate wells: | | | |
|---|---|---|---|
| Row | Column | | |
| | 1 | 2 | 3 |
| A | NC | Spec. 1 | Spec. 9 |
| B | NC | Spec. 2 | Spec. 10 |
| C | NC | Spec. 3 | Spec. 11 |
| D | HRC | Spec. 4 | Spec. 12 |
| E | HRC | Spec. 5 | Spec. 13 |
| F | HRC | Spec. 6 | Spec. 14 |
| G | QC1-LR | Spec. 7 | Spec. 15 |
| H | QC2-HR | Spec. 8 | Spec. 16 |

6. NC and HRC are tested in triplicate and QC1-LR and QC2-HR are tested singly with High-Risk HPV Probe Mix. Digene software determines the Calibrator and Quality Control positions in the microplate. Refer to the DML 2000 Instrument and Version 2 Software User Manual and the

Digene Hybrid Capture System Version 2 (DHCS V.2) Software Interactive Operator's Guide or Digene Qualitative Software User Manual for proper Calibrator/Quality Control/specimen setup in the software.

**DENATURATION**

**Notes:**

- **Warning:** Denaturation Reagent is corrosive. Use care and wear powder-free gloves when handling.
- **Important:** Some cervical specimens may contain blood or other biological material, which may mask the color changes upon addition of Denaturation Reagent. Specimens that exhibit a dark color prior to the addition of Denaturation Reagent may not give the proper color change at this step. In these cases, failure to exhibit the proper color change will not affect the results of the assay. Proper mixing can be verified by observing the color change of the Calibrators and Quality Controls.
- During the denaturation and hybridization steps, be sure that the water level in the water bath is adequate to immerse the entire volume of specimen in the tube.
- Calibrators, Controls, and specimens may be prepared up through the denaturation step and stored at 2-8°C overnight, or at -20°C for up to 3 months. A maximum of 3 freeze/thaw cycles may be performed with a maximum of 2 hours at room temperature during each thaw cycle. Mix well before using.
- Following denaturation and incubation, the specimens are no longer considered infectious.[26] However, lab personnel should still adhere to national/local precautions.
- Do not remove specimen collection device prior to denaturation.
- To avoid false-positive results, it is critical that all Calibrator, Quality Control, and STM specimen material come into contact with Denaturation Reagent. Mixing after Denaturation Reagent addition is a critical step: **Make sure the Multi-Specimen Tube Vortexer is set to 100 (maximum speed) and a visible vortex of liquid is observed during mixing such that the liquid washes the entire inner surface of the tube. If performing manual vortexing, make sure that each Calibrator, Quality Control, and specimen is mixed individually by vortexing each for at least 5 seconds at full speed such that the liquid vortex washes the entire inner surface of the tube followed by inverting the tube one time.**

**Calibrators, Quality Controls, and STM Specimen Preparation Procedure**

1. Remove and discard caps from Calibrators, Quality Controls, and STM specimens.

   **Note:** Caps removed from the specimen tubes are considered potentially infectious. Dispose of in accordance with national/local regulations.

2. Pipette Denaturation Reagent with Indicator Dye into each Calibrator, Control, or STM specimen using a repeating or adjustable pipettor. Take care not to touch the sides of the tube or cross-contamination of specimens could occur. The volume of Denaturation Reagent needed is equivalent to half the specimen volume. The exact volume for each type of Calibrator, Quality Control, and specimen is listed in the table below.

   **Dilute remaining Denaturation Reagent in bottle prior to disposing according to national/local laboratory procedures.**

| Calibrator, Quality Control, or Specimen | Volumes of Denaturation Reagent Required |
|---|---|
| Negative Calibrator | 1000 µl |
| High-Risk HPV Calibrator | 500 µl |
| Low-Risk or High-Risk Quality Controls | 500 µl |
| Cervical Specimen | 500 µl |

3. Mix the specimens using one of the two methods below.

   **Note:** MST Vortexer I can be used only with specimens collected with the DNAPap Cervical Sampler or the HC Cervical Sampler in Specimen Transport Medium (STM). Specimens

Manual/Individual Tube Vortexing Method

a) Recap the Calibrators, Quality Controls, and STM specimen tubes with clean Specimen Collection Tube Screw Caps.
b) Mix each tube thoroughly by vortexing individually, at high speed, for 5 seconds.
c) Invert each specimen tube one time to wash the inside of the tube, cap and rim.
d) Return the tube to the rack.

Independent of the vortexing method utilized, **there must be a visible vortex of liquid inside each tube during mixing such that the liquid washes the entire inner surface of the tube**. The Calibrators, Quality Controls, and specimens should turn purple.

4. Incubate the tubes in the rack in a 65 ± 2°C water bath for 45 ± 5 minutes (denatured Calibrators, Quality Controls, and specimens may be tested immediately, or stored as described in Notes above). Prepare High-Risk HPV Probe Mix during this incubation. See *Reagent Preparation and Storage* section.

**Hybridization Method Using Microtubes and Water Bath**

**Notes:**

- The processing of specimens collected with the HC Cervical Sampler in Specimen Transport Medium (STM) using the MST Vortexer method for mixing and the water bath method for hybridization **has not been validated**. Specimens collected with the DNAPap or the HC Cervical Sampler in STM and processed using the MST Vortexer method can be Hybridized utilizing the Microplate Heater I method **only**.
- If the denatured specimen has been stored at -20°C, allow the specimen to thaw to 20-25°C, and thoroughly vortex the specimen before proceeding with hybridization.

1. Label and Place the required number of clean hybridization microtubes into the microtube rack.
2. Remove Calibrators, Quality Controls, and specimens from the water bath after incubation. Vortex each tube individually for at least 5 seconds just prior to removing aliquots.
3. Pipette 75 µl of each Calibrator, Quality Control, or specimen into the **bottom** of appropriate hybridization microtube following the plate layout created under *Setup*. Avoid touching the sides of the microtubes and limit formation of air bubbles. Use a clean Extra-Long Pipette Tip for each transfer to avoid cross-contamination of Calibrators, Quality Controls, or specimens. It is not

necessary to remove the specimen collection device from the specimen transport tube. Denatured specimens may be capped with Specimen Collection Tube Screw Caps and stored with specimen collection devices remaining in the tubes.

4. After transferring the last specimen, **incubate the hybridization microtubes for 10 minutes at 20-25°C**.

5. Aliquot the prepared and thoroughly vortexed Probe Mix into a Disposable Reagent Reservoir. Carefully pipette 25 µl of the Probe Mix into each microtube containing Calibrators, Quality Controls, and specimens using an 8-channel pipettor and fresh tips for each row. Dispense the volume of Probe Mix into each hybridization microtube, preventing back splashing. Avoid touching the sides of the tubes. Inspect the rack from underneath to verify that all tubes have received the appropriate amount of Probe Mix.

6. Cover the microtubes with a plate sealer. Place rack cover on top of rack. Shake the microtube rack on Rotary Shaker I set at 1100 ± 100 rpm for 3 ± 2 minutes. *The Calibrators, Quality Controls, and specimens should turn yellow after shaking.* Tubes that remain purple may not have received the proper amount of Probe Mix. Add an additional 25 µl of Probe Mix to specimens that remain purple and shake again. If tubes remain purple after following this procedure, specimens should be retested.

   **Note:** After shaking, PreservCyt Solution specimens should turn pink instead of yellow.

7. Incubate the Microtube Rack in a 65 ± 2°C water bath for 60 ± 5 minutes. Ensure that the water level in the water bath is sufficient to cover the entire volume of hybridization mixture. The microtube rack will float in the water bath.

   **Note:** Create a plate layout file using the DHCS v.2 Software or Digene Qualitative Software if this has not been completed earlier.

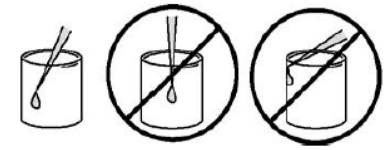
**HYBRID CAPTURE**

1. Remove all but the required number of Capture Microplate wells from the plate frame. Return the unused microplate wells to the original bag and reseal. With a marker, number each column 1, 2, 3. . . . and label the microplate with an appropriate identifier. The specimens will be added to the wells according to the example layout prepared under Setup.

2. Carefully remove Hybridization Microplate containing Calibrators, Quality Controls, and specimens from the Microplate Heater I. Immediately remove Plate Lid and place it on clean surface. Alternatively, remove Microtube Rack from the water bath. Immediately remove the rack lid and slowly pull the plate sealer up and across the rack.

3. Transfer the entire contents (approximately 100 µl) of the Calibrators, Quality Controls, and specimens from Hybridization Microplate wells or Microtubes to the bottom of the corresponding Capture Microplate well using an 8-channel pipettor. Use new pipette tips on the 8-channel pipettor for each column transferred and allow each pipette tip to drain well to ensure complete specimen transfer. If desired, the pipettor may be steadied by resting the middle of the pipette tips on the top edge of the capture microplate wells (see *Diagram 1*).

**DIAGRAM 1: CORRECT PIPETTING**




4. Cover microplate with the plate lid or new plate sealer and shake on the Rotary Shaker I at 1100 ± 100 rpm, at 20-25°C for 60 ± 5 minutes.

5. Prepare Wash Buffer. If using the Automated Plate Washer I, check Rinse and Waste reservoirs during this incubation. See Reagent Preparation section.
6. When the capture step is complete, remove the Capture Microplate from the Rotary Shaker I and carefully remove the plate lid or plate sealer. Remove the liquid from the wells by discarding into a sink; fully invert plate over sink and shake hard with a downward motion being careful not to cause a backsplash by decanting too closely to the bottom of the sink. **Do not reinvert plate**; blot by tapping firmly 2-3 times on clean Kimtowels® Wipers or equivalent lint-free paper towels. Ensure that all liquid is removed from the wells and the top of the plate is dry.

**HYBRID DETECTION**

**Notes:**

- Make additions across the plate in a left-to-right direction using an 8-channel pipettor.
- It is recommended that the reverse pipetting technique be utilized to improve consistency of reagent delivery. With this technique, the pipette tips are initially over-filled by using the second stop on the pipettor's aspirate/dispense control (plunger). See procedure below. Wipe tips on disposable reagent reservoir to remove excess reagent before delivery to plate.
- If desired, the pipettor may be steadied by resting the middle of the pipette tips on the top edge of the microplate wells. Take care not to touch the sides of the microplate wells or cross-contamination of specimens could occur. Refer to Diagram 1 shown earlier.

1. Aliquot the appropriate volume of Detection Reagent 1 into a disposable reagent reservoir (see *Reagent Preparation* Section for instructions). Carefully pipette 75 µl of Detection Reagent 1 into each well of the Capture Microplate using an 8-channel pipettor and the reverse pipetting technique.

   Reverse Pipetting Procedure:

   a) Attach tips onto an 8-channel pipettor; ensure all tips are firmly seated.
   b) Push the plunger of the pipettor past the first stop to the second stop.
   c) Immerse tips into the Detection Reagent 1 solution.
   d) Release plunger slowly and allow solution to fill the tips.
   e) Dispense 75 µl of solution into microplate wells by depressing plunger to the first stop. Do not release plunger until pipette tips have been reimmersed into the Detection Reagent 1 solution.
   f) Refill tips and repeat until all wells are filled. Fill wells of microplate from left to right. *Verify that all wells have been filled by observing the intensity of the pink color. All wells should have similar intensity.*

2. Cover plates with plate lid or clean Parafilm (or equivalent) and incubate at 20-25°C for 30-45 minutes.

**WASHING**

Wash the capture plate using one of the two methods below.

**Automated Plate Washer I Method**

**Note:** Always keep the Automated Plate Washer I on. Ensure that the Rinse Reservoir is filled and the Waste Reservoir is empty. The Automated Plate Washer I will routinely rinse the system for cleaning. See the Automated Plate Washer I Operation and Maintenance Manual for further instructions as needed.

**BEFORE EACH USE:**

- Verify that the Wash Reservoir is filled at least to the 1L mark with Wash Buffer Solution. If not, prepare the Wash Buffer solution. See Reagent Preparation section.
- Verify the Rinse Reservoir is filled with deionized or distilled water.
- Verify that the Waste Reservoir is empty and the cap is securely fastened.
- The Automated Plate Washer I will automatically prime itself before each wash, and perform maintenance rinse after each wash.

1. Remove plate lid and place plate on Automated Plate Washer I platform.
2. Verify that the power is on, and that the display reads "Digene Wash Ready."
   **Note:** If only a partial strip of capture wells is being used, empty microplate wells will need to be placed in capture plate to complete the column prior to washing.
3. Select the number of strips to be washed by pressing the "Rows" key and then "+" or "-" to adjust. Press "Rows" key to return to "Digene Wash Ready."
4. Press "Start/Stop" to begin.
5. The washer will perform six fill and aspirate cycles taking approximately 10 minutes. There will be a brief pause during the program so be sure not to remove the plate prematurely. When the Automated Plate Washer I is finished washing, it will read "Digene Wash Ready."
6. Remove the microplate from the washer when the program is finished. Plate should appear white, and no residual pink liquid should remain in the microplate wells.

**Manual Washing Method**

1. Remove Detection Reagent 1 from the wells by placing clean Kimtowels Wipers or equivalent lint-free paper towels on top of the plate and carefully inverting. Before inverting, ensure that the paper is in contact with the entire surface area of the plate. Allow the plate to drain for 1-2 minutes. Blot well on clean Kimtowels Wipers or equivalent lint-free paper towels. Carefully discard the used paper towels, to avoid alkaline phosphatase contamination of later steps.
2. Using the Wash Apparatus, hand wash the plate 6 times. Each well is washed to overflowing to remove Detection Reagent 1 from the tops of the wells. Washing begins at well A1 and continues in a serpentine fashion to the right and downward. After all wells have been filled, decant liquid into sink with a strong downward motion. The second wash is started at well H12 moving in a serpentine motion to the left and upward. This sequence of 2 washes is repeated 2 more times for a total of 6 washes per well.
3. After washing, blot the plate by inverting on clean Kimtowels Wipers or equivalent lint-free paper towels and tapping firmly 3-4 times. Replace the paper towels and blot again. Leave plate inverted and allow to drain for 5 minutes. Blot the plate one more time.
4. Plate should appear white, and no pink residual liquid should remain in the microplate wells.

**SIGNAL AMPLIFICATION**

**Notes:**

- Use a new pair of gloves for handling Detection Reagent 2.
- Aliquot **only** the amount of reagent required to perform the assay into the disposable reagent reservoir in order to avoid contamination of Detection Reagent 2. See Reagent Preparation Section. **Do not return Detection Reagent 2 to the original bottle. Discard unused material after use**.
- Detection Reagent 2 addition should be made without interruption. The incubation time of all wells must be as close as possible.
- Take care not to touch the sides of the microplate well or splash reagent back onto tips because cross-contamination of specimens could occur (See *Diagram 1*).

1. Carefully pipette 75 µl of Detection Reagent 2 into each well of the Capture Microplate using an 8-channel pipettor as previously described. *All microplate wells should turn a yellow color.* Verify that all wells have been filled by observing the intensity of the yellow color. All wells should have similar intensity.

2. Cover microplate with a plate lid, clean Parafilm (or equivalent), and incubate at 20-25°C for 15 minutes. Avoid direct sunlight.
3. Read the microplate on the Digene Microplate Luminometer 2000 (DML 2000™) Instrument after 15 minutes of incubation (and no later than 30 minutes of incubation).
4. The DML 2000 Instrument assay specific software protocol will allow the entry of pertinent assay information directly into the software.
5. If a full microplate was not used, remove used microplate wells from the microplate holder, rinse the holder thoroughly with distilled or deionized water, dry and reserve for next assay.

## Appendix V Institutions Consent