
QUANTITATION OF HIV-1 IN HUMAN SALIVA AND BLOOD

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STATEMENT OF AUTHORSHIP

The contents of this thesis consist of original work carried out by the author (unless otherwise stated and duly acknowledged). No part of this thesis has been submitted in whole or in part for any other degree.


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Date: 20/4/94
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SUMMARY

The human immunodeficiency virus (HIV) has been infrequently detected in the saliva of infected subjects in the past. This may be due to technical problems associated with the assay used rather than the lack of viral particles actually present. A sensitive saliva based assay for the detection of HIV may have wide ranging applications in the screening of individuals suspected of having been infected and in anti-retroviral drug trials. Recently a new technique, the Polymerase Chain Reaction (PCR), has been successfully used in the detection of specific regions of genetic material at nano-molar concentration. This assay has since been applied to the detection and quantitation of intracellular proviral sequences of the HIV genome in blood specimens taken from humans infected with the virus.

The present study aimed to develop a PCR based assay for the detection and quantitation of HIV genetic material in human saliva and to correlate the concentration of viral DNA with the degree of gingival inflammation and systemic viral load. The assay conditions were first optimised for magnesium ion concentration and annealing temperatures using known concentrations of HIV DNA. Later, the assay was applied to 31 humans saliva samples from known seropositive subjects and 4 samples from seronegative controls. Blood specimens taken from the

seropositive subjects at the same time were assayed concurrently. Conventional virus isolation using co-culture techniques was performed on a subgroup of these specimens to determine the comparative sensitivity of the new assay.

The results showed that the PCR assay was able to detect as few as 4 copies of HIV DNA in 400,000 copies of human genomic DNA with a 99.5% confidence interval. The assay also proved to be more sensitive than virus isolation at detecting the presence of the virus in blood samples. However, HIV genetic material was not detected by either virus isolation or the PCR assay in the saliva of any subject. It was concluded that even in the presence of high blood concentrations of HIV DNA, the saliva normally contains very low concentrations of this genetic material. Several reasons for this finding are discussed and suggestions for future research in this field are made.

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DEDICATION

To my parents for their courage, love and support.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
ANUG	Acute Necrotising Ulcerative Gingivitis
ARC	AIDS Related Complex
AZT	Azidothioprine
bp	base pairs
CD ₄ , T ₄	Cluster of Differentiation (4). Denotes T Helper lymphocytes
CDC	Centre for Disease Control
cDNA	copy DNA
CO ₂	Carbon Dioxide
CPE	Cytopathic Effect
cpm	counts per minute
ddI	didanosine
DNA	Deoxyribonucleic Acid
dNTP	deoxy Nucleic Triphosphates
DS	Dextran Sulfate
dsDNA	double stranded DNA
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbant Assay
ENV	Envelope
GAG	Glycoprotein
GI	Gingival Index
GITC	Guanidium Isothiocyanate
gp	glycoprotein
GPA	Global Program on AIDS

HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HIV-1	HIV type 1
HIV-2	HIV type 2
HIV-G	HIV associated Gingivitis
HIV-P	HIV associated Periodontitis
HTTP	Hydroxythymidine Tetrasodium Triphosphate
IgG, IgM	Immunoglobulins G and M
IL-2	Interleukin 2
LAV	Lymphadenopathy Associated Virus
LTR	Long Terminal Repeat
MgCl ₂	Magnesium Chloride
p	protein
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGL	Persistent Generalised Lymphadenopathy
PHA	Phytohemagglutinin
POL	Polymerase
r	Pearson's correlation coefficient
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcriptase
ssDNA	single strand DNA
Taq	<i>Thermus aquaticus</i>
WHO	World Health Organisation
WR	Walter-Reed classification

CHAPTER ONE

REVIEW OF THE LITERATURE

1.1 INTRODUCTION

The Human Immunodeficiency Virus (HIV) has been identified as the causative agent of the Acquired Immunodeficiency Syndrome (AIDS) (1). Exposure of a previously uninfected host to the virus results in the slow and progressive reduction of the affected individual's ability to resist opportunistic infections and some types of malignant neoplasms. The oral cavity often presents manifestations of these complications.

Preventive endeavours have focussed on reducing the incidence of infection by the Human Immunodeficiency Virus Type I (HIV-1) through public health education campaigns directed at the most common routes of transmission and the members of the community most likely to be exposed to the virus through these routes. Direct blood to blood contact and sexual contact are the best documented routes through which HIV-1 has been transmitted (2,3). However, blood and semen are

not the only body fluids where the virus has been isolated (4). Viral antigens and viable virus have been detected in samples of cerebro-spinal fluid (5), colostrum and breast milk (6), vaginal secretions (7), tears (8), and saliva (9). Its presence in these fluids implies an infection risk which to date has not been supported by epidemiologic data (10).

Bacterially induced inflammation of the periodontal tissues in HIV-1 seropositive people has been classified as a distinct entity by Pindborg and others (11,12). A recent study of 54 HIV-1 infected but asymptomatic patients demonstrated a significant increase in attachment loss compared to controls over the same period (13). Despite the increased gingival exudate which would be expected in the mouths of patients with gingivitis and periodontitis, and the occasional macroscopic observation of blood in the saliva sample, they were not able to show a correlation between the presence of virus in the saliva and disease staging or degree of periodontal inflammation (14). This may be a reflection on the methods used to detect the virus, which required it to remain viable in the salivary environment, rather than a true description of viral titres in human saliva in the presence or absence of periodontal diseases. A knowledge of the prevalence and concentration of HIV-1 in the saliva may facilitate a better appreciation of the infection risk to clinicians as well as the influence oral disease may have on this infection risk. In addition, if the viral load can be quantitated, a salivary assay may be of value in future drug trials. Clearly, a new method is required which is capable of detecting the presence and concentration of HIV-1 in human saliva despite the actions of agents which may degrade or inhibit the virus. Due to the

limited volume of saliva obtainable in a single donation and the low concentration of blood products within saliva (15), a highly sensitive test for HIV-1 is required to investigate the potential infectivity of saliva from HIV- 1 seropositive subjects.

1.2 HIV DISEASE

1.2.1 Background

In 1981 the Centre for Disease Control (CDC) reported on a group of homosexual men in Los Angeles and San Francisco dying from infection by *Pneumocystis carinii*, a pathogen which had previously been considered very rare and often confined to heavily immunosuppressed individuals (16). Shortly after, further reports of a similar condition were made by the CDC on a group of persons with haemophilia A (17). It became apparent following blood analysis that these patients were deficient in the helper subgroup of T lymphocytes (18). From investigation into the social background of the affected individuals it also became apparent that this condition was horizontally transmitted from patient to patient either by blood transfusion or by the interchange of body fluids by other means (19). The condition had, by now, become known as the Acquired Immune Deficiency Syndrome (AIDS) and was characterised by the development of either an opportunistic infection or Kaposi's sarcoma (an unusual neoplasm that had previously been associated with certain immunosuppressed states) (20). Within two years a new retrovirus of the Lentivirus family had been identified and strongly associated with the disease by a research group in France (1). After several pseudonyms, an international agreement was reached on the name "Human Immunodeficiency Virus (HIV)" (21). As the detailed polypeptide and nucleotide structure of the virus became elucidated at least two distinct variants of the HIV became recognised (22,23). These have become known as HIV type 1 (HIV-1),

which is most prevalent in western societies, and HIV type 2 (HIV-2), which is generally found on the African continent.

1.2.2 Epidemiology

In just over 10 years since the aetiologic agent of HIV disease has been identified, the virus has been transported to almost every continent in the world. Data from the *WHO Weekly Epidemiological Record* and similar publications, based on figures derived from cases of HIV and AIDS in the United States, shows that the disease continues to spread (24,25,26). In August 1988 the U.S. Public Health Service estimated that between 1.0 and 1.5 million Americans were infected with HIV and 70,208 cases of AIDS were reported to the CDC (27). In January 1989 over 82,000 AIDS cases had been reported and 46,000 people had died in the U.S. alone (26). By the mid 1990s, the CDC estimates the cumulative number of diagnosed AIDS cases in the U.S. will be between 330,000 and 405,000 (28). The numbers of infected, and as yet undiagnosed cases will be far higher than this. However, current legislation and testing methods do not allow accurate estimates of the number of people infected (29).

World-wide, over 288,000 AIDS cases had been reported by October 1990 (24). Australia has the highest cumulative rate of AIDS per million of population [118 cases per million] for the Oceania region (30). In comparison to other nations, this figure is only exceeded by countries in North America, Central Europe and Africa. Asian incidence rates are very low (<4 per million on average). This fact may be explained by the delayed appearance of the epidemic on this continent. Reports

from Thailand now indicate a dramatic increase in the number of HIV-infected intravenous drug users. Many of these will develop AIDS within the next five years (31).

The Global Program on AIDS (GPA) of the World Health Organisation (WHO) has made several predictions for the global AIDS epidemic for the year 2000 using data derived from the Delphi questionnaire survey method (32). They have predicted that the world-wide cumulative total of AIDS cases in adults might reach 5-6 million by the year 2000 and 18 million people will have been infected by HIV (33). These figures are based on the current rate of reporting of the disease which still includes people who became infected prior to the world-wide education and prevention drives. It has been reported that the rate of new cases of HIV infection is decreasing in some high risk groups due to preventive measures, blood screening and needle exchange programs (34).

1.2.3 Clinical and Immunological Aspects

HIV disease, in its terminal stages, presents in the form of AIDS. However, patients infected with HIV may remain asymptomatic for many years (35). In 1986 the CDC published a classification of four clinical stages of HIV disease which, despite some modifications, is still universally accepted (36). These stages are summarised below:

Stage 1: The acute viraemic stage begins approximately two weeks after primary exposure to the virus (37). Its usual expression is of a mononucleosis (glandular fever) like fever and rash lasting several weeks before spontaneously resolving, though on occasion no symptoms have been reported by some patients. During this period and beyond, the virus continues to replicate, thereby increasing the host's infectivity. Detection of the virus in the blood is possible within several weeks. Current commercial blood tests for HIV infection are designed to detect antibodies to the virus which do not become apparent until seroconversion several months after the primary exposure (see Figure 1.1).

Stage 2: The asymptomatic stage corresponds with the dormant phase of the virus within the infected cells and high blood antibody titres to the viral antigen. This period may last 10 years or more and is usually the period during which the patient sero-converts (38). The high antibody titres to certain components of the virus result in a reduced infectivity since there is very little cell free virus in the circulation. However, the genetic code for HIV is incorporated into the DNA of many lymphocytes and macrophages and is therefore safe from the hosts immune defences (39).

Stage 3: The Persistent Generalised Lymphadenopathy (PGL) stage, where a number of sites demonstrate enlarged lymph nodes greater than 1cm in diameter and persist for periods exceeding three months. As with stage two, the infected individual is only marginally infectious despite a progressive reduction in the number of immune competent cells. Patients may fluctuate between stages two

and three for some time but once stage three has been reached it is considered a prelude to the impending onset of stage four.

Stage 4: Represents the end of the quiescent phase of HIV disease and the beginning of AIDS. This stage is further subdivided into several categories which are defined clinically.

AIDS related complexes (ARCs) or constitutional diseases (subgroup A) such as sudden weight loss > 10%, night sweats, diarrhoea, and malaise which persist longer than 3 months are often the first symptoms the infected individual reports when seeking medical advice. Further symptoms such as neurologic disorders (subgroup B), secondary infections such as candidiasis or pneumonia (subgroup C), and neoplasms such as Kaposi's sarcoma (subgroup D) are indicators of full blown AIDS and represent a poor prognosis beyond twelve months. The immune system is depleted to such an extent that antibody titres against the virus are reduced and as a result the concentration of cell free virus in the circulation rises causing increased infectivity of the affected individual.

An alternative classification system has been adopted by the United States Defence forces. Known as the Walter-Reed (WR) classification, it incorporates a variety of clinical, laboratory and social factors. The stages range from WR 0 through to WR 7. A detailed description of this classification was published by Redfield, Wright and Tarmont in 1986 (40).

The oral cavity, is often a site of early clinical signs of the onset of AIDS (41). Oral lesions such as hairy leukoplakia, oral Kaposi's sarcoma and candidiasis frequently pre-empt systemic signs and should always be investigated further.

1.2.4 Natural History of HIV Disease

i) Infection with HIV

HIV is transmitted through three primary routes (2,3,42):

1. Sexual contact with an infected individual
2. Exposure to infected blood or blood products
3. Perinatally from an infected mother to her child

Laboratory and epidemiological data have indicated that HIV is not transmitted through casual contact, immunoglobulin preparations, the hepatitis B vaccine, or contact with insects (43,44).

After the acquisition of infection, persistent antibodies to HIV usually develop within three months. The average duration from infection to seroconversion has been estimated to be 2.6 months in one study (45), although in some cases seroconversion does not occur for six months or more (46). Although rare, some reports have indicated that persons who initially demonstrate antibodies to HIV antigens may later lose seropositivity (47). The significance of this finding is not yet established.

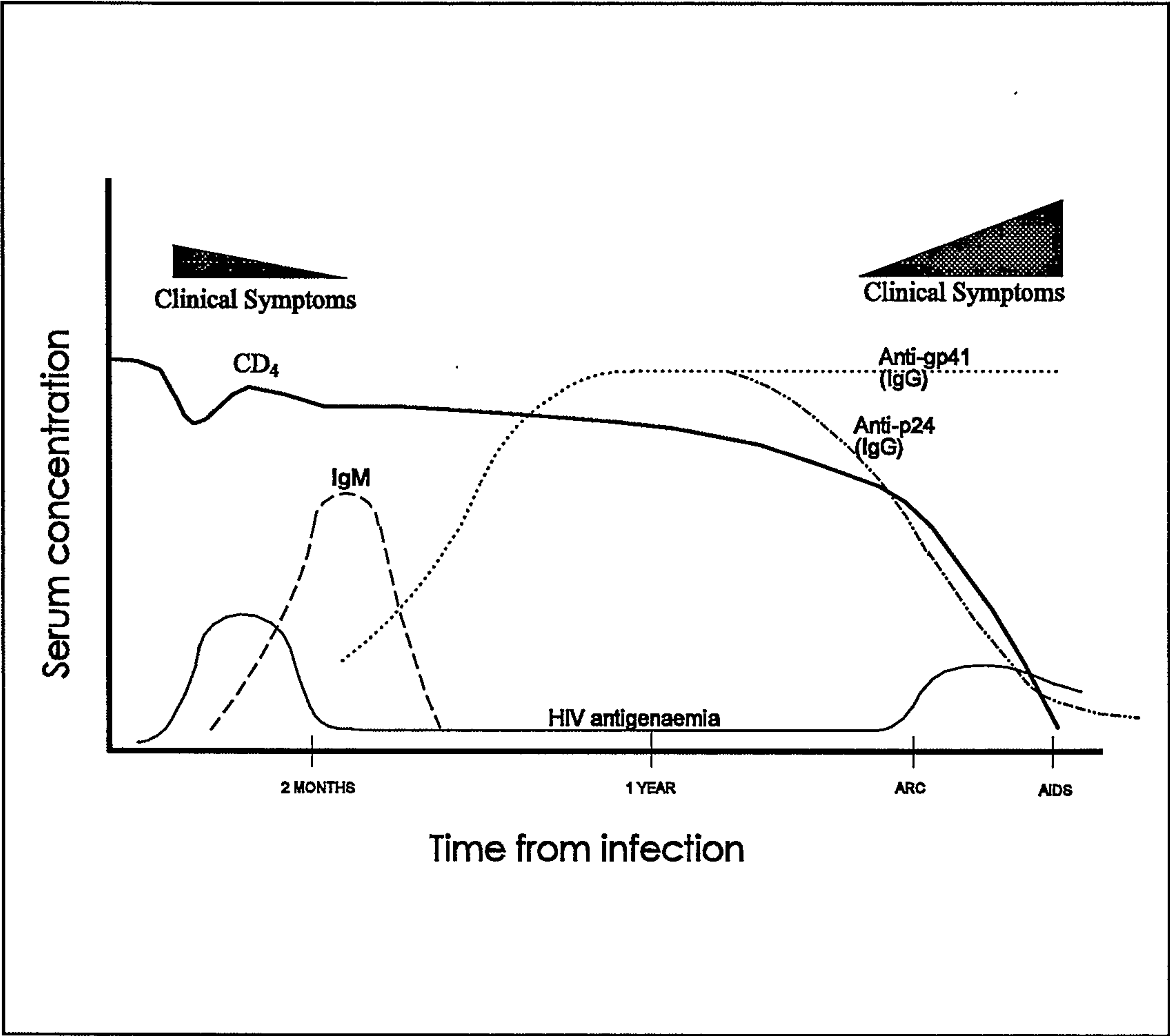


Figure 1.1: The changing concentrations of blood borne antibodies (IgM and IgG), antigen and CD₄ cells over the course of HIV disease from time of primary infection to AIDS.

ii) Progression to AIDS

The route of infection may effect the rate at which the disease progresses to AIDS. Prospective studies in homosexual males in whom the date of seroconversion was well characterised showed that no case developed into AIDS in the first year of HIV infection, but 2% of men developed AIDS within 2 years of infection, 5% within 3 years, 10% within 4 years, 23% within 6 years, 37% within 8 years and 48% within 10 years (48).

Recipients of blood and blood products through transfusions were shown in similar studies to progress to AIDS faster than the male homosexuals (49). It is presumed that this is a result of the greater inoculum of virus received, the older average age of transfusion recipients or the immunologic status of the infected recipients.

Selwyn and co-workers (1988) studied the rate of progression of HIV to AIDS in a group of intravenous drug abusers (50). This study also found a greater percentage of cases progressing to AIDS in 24 months (12%) than that seen in the male homosexuals (48). The authors noted, however, that the patients in their study group could not be relied on for an accurate history and many HIV-infected intravenous drug abusers also had substantial morbidity from illnesses other than those meeting the AIDS case definitions such as bacterial pneumonia, pulmonary tuberculosis, endocarditis, and sepsis.

iii) Incubation Time/ Latency Period

The average incubation time (defined as the time between acquisition of HIV infection and onset of AIDS) varies considerably in respect to the mode of acquisition. More recent analysis of CDC AIDS surveillance data have suggested average times of approximately 8-11 years (35,51). Early diagnosis, anti-retroviral therapy and prophylactic antibiotic regimens have been instrumental in delaying death from AIDS in HIV infected individuals (52). In addition, reports of some cases of HIV infection which have not yet converted to AIDS after 12 years or more have combined to extend the average time to 11 or longer (53). A recent finding from a cohort of patients infected with the same viral strain by blood transfusion appears to indicate that this latency period may also be determined by the strain of the infecting agent (54).

iv) AIDS Survival Time

The median survival time for patients once a diagnosis of AIDS (CDC category IV) has been made is approximately 12 months. Since the diagnosis of AIDS is based on any of a number of clinical findings, it is prudent to examine the average survival time for some of the most common conditions diagnostic of AIDS. For instance, Rothenberg *et al* (1987) studied 5833 cases of AIDS in New York and found that survival time for patients presenting with *Pneumocystis carinii* pneumonia was approximately 11 months while those who presented with other opportunistic infections other than candidiasis survived only approximately six months. On the other hand, patients presenting with Kaposi's sarcoma had a survival time of approximately 2 years (55).

v) Serology

After primary HIV-1 infection, specific antibodies directed against the major structural gene products of HIV-1 develop (56). In a study of 190 individuals comprised of AIDS patients, ARC sufferers, healthy homosexuals and laboratory staff the major antigenic determinants of HIV-1 were the surface glycoproteins gp160 and gp120 (57). Cooper *et al* (1987) found specific HIV-1 antibodies in the circulation within 2 weeks of onset of the acute illness (38). Immunoglobulin M (IgM) is typically the first antibody type to appear in response to the proteins coded by the *gag* and *env* regions. It appears within two weeks of onset of acute illness and is in the circulation for approximately 3 months before rapidly disappearing (58). IgM antibody decreases concomitantly with an increase in HIV-1 specific IgG. Therefore, the detection of HIV-1 specific IgM antibodies is a sign of recent infection (59).

HIV-1 p24 antigen can be detected by Enzyme Linked Immunosorbant Assay (ELISA) in serum prior to seroconversion (60). Von Sydow *et al* (1988) were able to detect HIV-1 p24 antigen in all of 13 subjects for whom serum samples were available during the first 18 days after the onset of the acute illness, suggesting that HIV-1 antigenaemia occurs consistently during this period of infection (61). In most persons a rise in the serum HIV-1 antibody titre is accompanied by a decrease in the serum HIV-1 p24 antigen concentration. Therefore, throughout stages 2 and 3 of HIV disease, very few viral particles can be found in circulation. With the progression of the disease to stage 4 there is a re-appearance of the antigenaemia which is considered an indicator of poor prognosis (62,63).

1.3 VIROLOGY

1.3.1 Viral Structure

The virus associated with HIV disease is a member of the non-transforming and cytopathic lentivirus family of retroviruses. It is therefore closely related to the visna virus of sheep (64), the equine infectious anaemia virus (65) and the feline immunodeficiency virus (66). The structure of the virus was first elucidated using immuno-electron microscopy by Gelderblom *et al* in 1987 (67). Briefly, the virus is an enveloped spherical agent approximately 90nm in diameter containing a protein core (p24) within which lie the enzymes reverse transcriptase (RT), integrase and two short lengths of a single-stranded RNA genome (Figure 1.2).

The outermost structural component is a glycolipid bilayer derived from the host cell from which it arose. Traversing this bi-layer is a transmembrane glycoprotein (gp41) (68) which provides a loose attachment for the major envelope glycoprotein, gp120, located on the external "spikes" of the viron (69). The combined structure of these two glycoproteins was initially isolated and termed gp160.

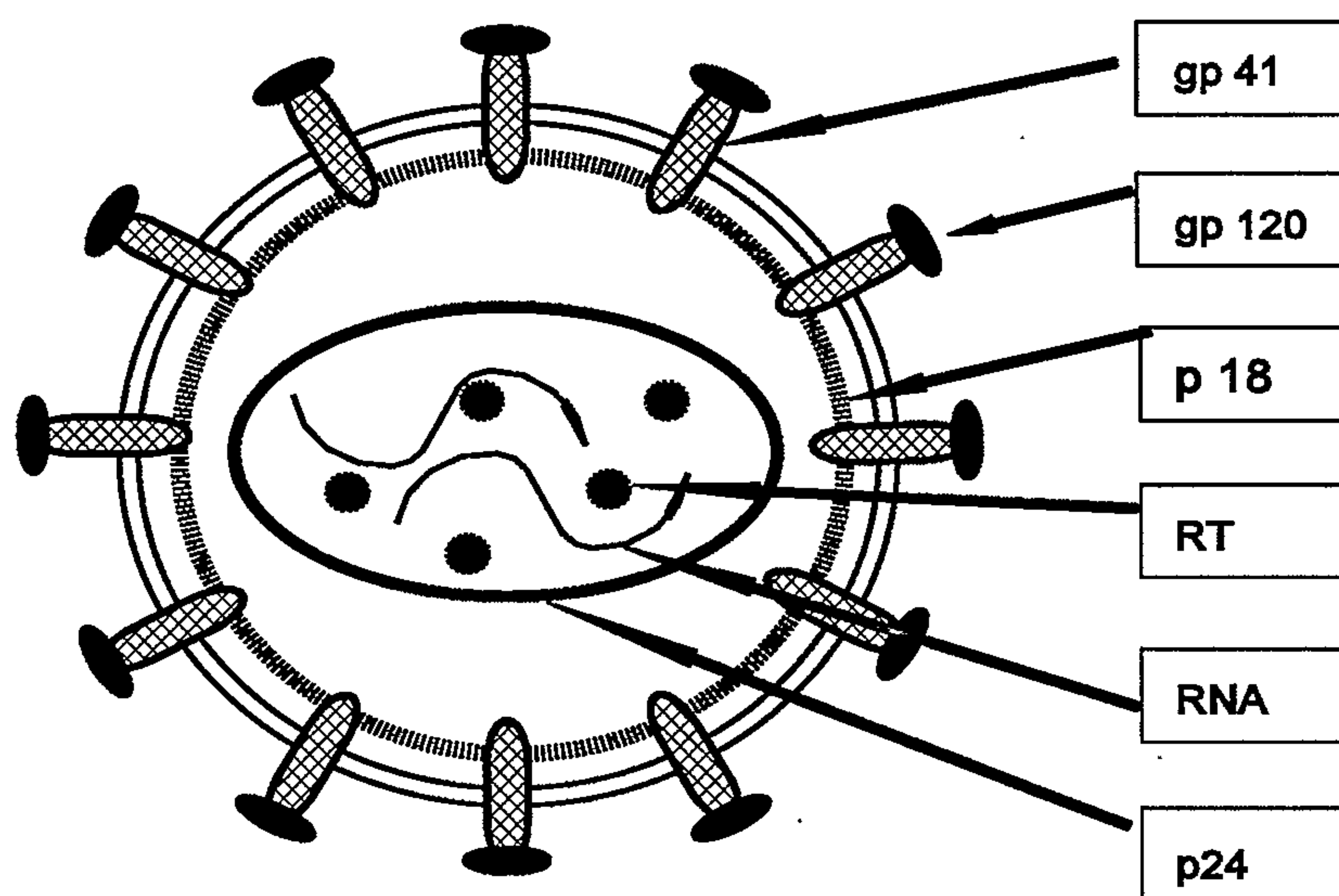


Figure 1.2: The ultrastructure of the HIV-1 particle. Glycoproteins (gp) and Proteins (p) are structural and antigenic. Ribonucleic Acid (RNA) carries the genetic information for the virus which is transcribed into DNA by the Reverse Transcriptase (RT) enzyme.

These glycoproteins play several pivotal roles in HIV disease. The gp 160 complex is the most antigenic region of the viral surface and thus serologic tests use antibodies to this glycoprotein as markers for clinical infection. Gp 120 is specifically recognised by the CD₄ molecule on helper T lymphocytes (T₄) and some cells of the monocytic lineage. This glycoprotein mediates specific adsorption to host cells containing the CD₄ receptor and thereby promotes access into the cells (70). These glycoproteins are also the sites of greatest variation due to changes in their genetic coding (up to 20% of the genetic sequence) and thus have frustrated conventional attempts to formulate a vaccine against HIV (71,72). The HIV-1 has a lipid envelope like the herpes and influenza viruses and is susceptible to inactivation by physical agents and disinfectants and HIV is no exception in this regard. Exposure to modest temperatures for brief periods is sufficient to inactivate the virus, while hypochlorite, gluteraldehyde, hydrogen peroxide, ethanol, phenolics, certain detergents (NP-40), and high or low pH have all been shown to be effective against the virus, explaining the non-incidence of fomite transmission.

Directly underneath and apposed to the viral membrane lies a membrane-associated protein (p18) which is believed to regulate virus morphogenesis and the egress from the host cell (73).

Within the protein core is the viral RNA and the enzyme reverse transcriptase (RT) (p64). This RNA dependant DNA polymerase transcribes the genetic information encoded on the viral RNA (vRNA) to double stranded DNA once the viral contents have entered into the cell (74). Transcription of genetic information from RNA to DNA is against the normal biologic flow of genetic information and unique to retroviruses. The single strand DNA spontaneously reassembles in the optimal conditions found within the cell nucleus, forming double stranded copy DNA (cDNA) which incorporates into the chromosomal DNA of the host cell using the enzyme Integrase (p31) (75). The expression of the incorporated DNA may not become evident for some time, remaining dormant until the host cell is activated by antigen or cytokines which increase cellular activity or induce terminal differentiation (38,76).

The HIV proviral genome is approximately 10 kilobases (10,000 bp) in length. Gene mapping techniques have identified eight major genes coded for by the viral RNA (77) (Figure 1.3). Three domains of the HIV-1 genome have been well characterised. The *gag* gene which codes for the core protein (p24), the *pol* gene coding for the reverse transcriptase-protease-endonuclease enzyme, and the *env* gene which codes for the internal and external envelope glycoproteins (gp120, gp41). HIV also has at least five additional genes; the *tat*, *tr�/art*, and *3' orf (nef)*, genes which serve regulatory functions such as up regulating or down regulating virus replication (78,79), and the *sor* and *R* genes whose complete functions have yet to be elucidated (80).

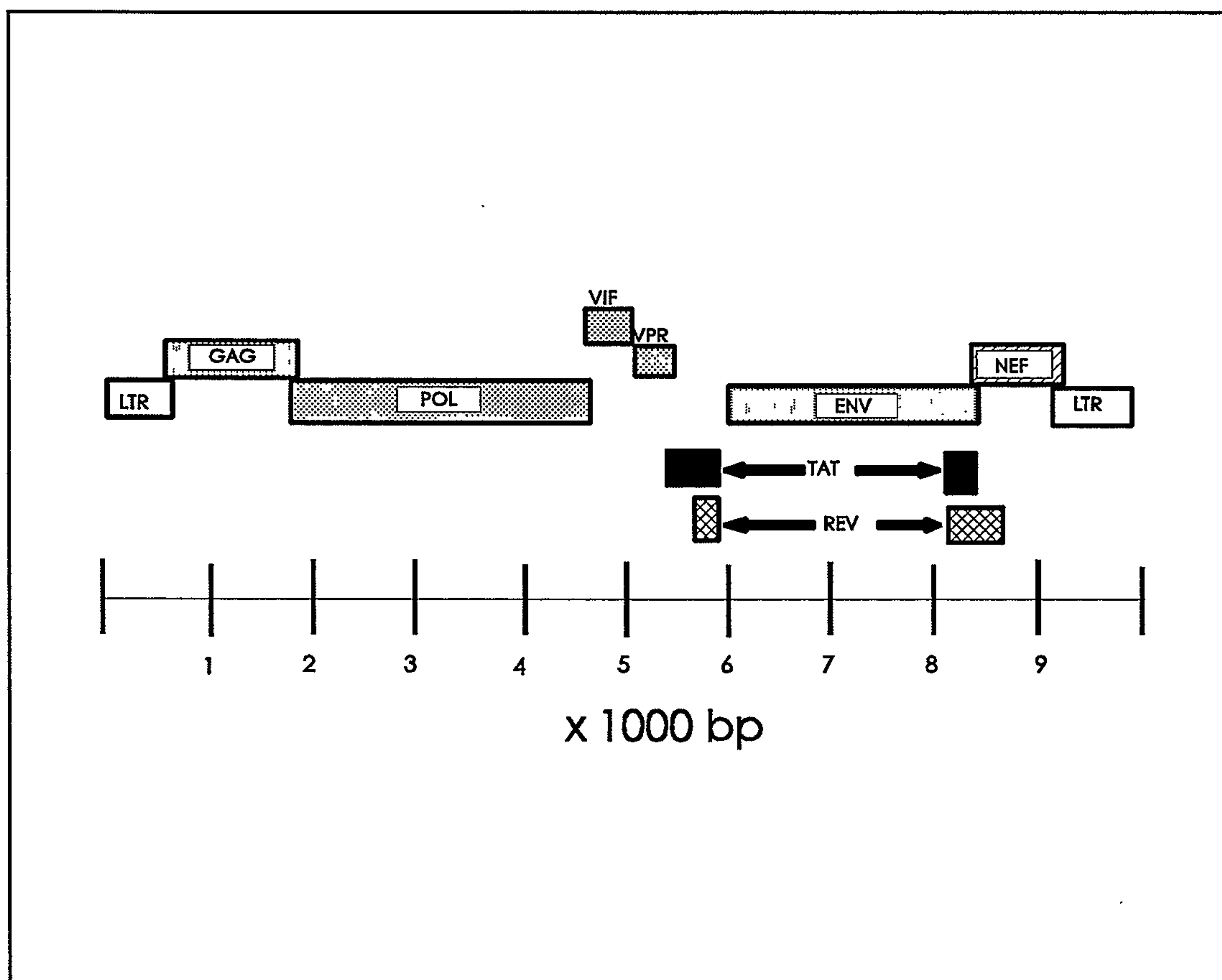


Figure 1.3: The HIV-1 genome (9740 bp in length). Each end contains Long Terminal Repeat (LTR) regions which have regulatory functions. The envelope region of the virus is encoded for by the ENV gene. The POL gene codes for the polymerase enzyme (reverse transcriptase) while the GAG gene codes for the Group Antigen. The other short regions provide various regulatory functions which are yet to be completely elucidated.

1.3.2 Entry into the Host Cell

HIV-1 preferentially replicates in CD₄ positive helper T cells (81). It has been suggested that the CD₄ antigen is the receptor for viral infection (82,83). Recent studies have now suggested that other cell surface moieties may be important co-factors for viral entry. CD₄ negative cells such as some brain derived cells and fibroblasts have been infected by HIV *in vitro* (83) while other cells which are CD₄ positive are resistant to HIV infection (84). It is suggested, therefore, that other cellular proteins are required for viral entry in combination to the CD₄ molecule.

1.3.3 Infectivity and Pathogenesis

The HIV is able to selectively infect and ultimately incapacitate the immune system, the function of which is to protect the body against non-self. The resultant increase in susceptibility to opportunistic infections and neoplasms leads to AIDS and finally death. The immune defect is progressive and irreversible according to current understanding.

Infection is believed to begin on the cell surface with the CD₄ and associated molecules acting as receptors for viral gp120. Through this union the virus binds to the cell surface and is internalised by endocytosis under an undetermined stimulus. Once within the cell, the virus is uncoated and the genomic RNA is transcribed into a proviral DNA by RT before being inserted into the host cell DNA by the integrase enzyme (75). A significant quantity of proviral DNA remains unintegrated within the cell as linear or circular DNA fragments which are believed to have bearing on the cytopathic effect on host cells by HIV (85).

Once integrated, the DNA of viral origin remains dormant in a latent phase, its expression only starting when the cell is itself activated by a subsequent infection (86). Upon entering into the cell cycle, the proviral DNA codes for the transcription of viral genomic RNA (which is later destined to be the material within the core of a new viron) as well as mRNA (also termed spliced RNA) which is destined to be translated for the production of the structural proteins of the virus such as core and envelope, as well as for the RT and integrase enzymes. Large quantities of virus and viral constituents are thus produced, some of which are released through pinocytosis into the circulation and thereby facilitating the spread of infection to other susceptible cells, while those which remain in the cell lead to the eventual death of the cell by any one of a number of postulated mechanisms (87,88,89).

The cells most at risk to infection by the HIV are those with CD4 molecules on their surface. However, some circulating blood cells (such as microglial cells of the brain, Langerhan's cells of the skin and mucous membranes, follicular dendritic cells of lymph nodes) which are sparsely coated with these surface markers are somewhat refractory to the cytopathic effects (CPE) of the virus and act as a continuous reservoir of new virus into the blood (87,88,90). In addition, Montagnier's group reported HIV-1 in Epstein Barr virus infected B cells (91). However, the subgroup of the thymus derived lymphocytes known as the 'Helper T cell' (T_4) are most affected by HIV infection resulting in a quantitative depletion of T_4 cells within the circulation from the normal values of 500-1400/mm³ to 200/mm³ or less.

1.4 PERIODONTAL CONSIDERATIONS IN HIV DISEASE

1.4.1 Introduction

Bacterially induced inflammation of the periodontal structures and nearby tissues in HIV seropositive people has been classified as a distinct entity (11,12). A broad name for this condition, AIDS-Related Necrotising Stomatitis, has been proposed to promote the notion that the various degrees of inflammation and tissue destruction reported in the literature are all along a single continuum encompassing the previously described HIV-gingivitis (HIV-G), HIV-periodontitis (HIV-P), and HIV-stomatitis (92). The progression of HIV-G to HIV-P was described by Winkler *et al* (1988) (93) and numerous accounts of a progressive ANUG like lesion extending beyond the usual boundaries of what would normally be considered periodontitis tends to support such a general classification (94). For the purpose of this paper the clinical appearance of these lesions will be described independently.

1.4.2 HIV-Gingivitis

Winkler *et al* (1988) (93) defined HIV-G as lesions confined to the soft tissues that show an intensely red linear band of erythema of the free and attached gingiva and sometimes even the alveolar mucosa. Some areas may show a punctate or diffuse erythema involving the entire attached gingiva, on occasion even coalescing. Both the band-like and the diffuse type of erythema are associated with spontaneous gingival bleeding resulting in patients complaining of blood on their pillows or waking with blood clots in their mouths. Typically, HIV-G responds poorly to

improved oral hygiene or intensive scaling and root planing (95). The inability to improve the erythema was considered to compromise the patient's long term periodontal condition (96).

1.4.3 HIV-Periodontitis

HIV-P has been described as having all the characteristics of HIV-G but also involving a severe and rapidly progressing soft tissue necrosis and destruction of the attachment apparatus and bone (97). The appearance and course of the lesions has lead to their description as resembling an ANUG superimposed over a rapidly progressive periodontitis (94). Unlike HIV-G, affected areas are often very localised to the proximal region between two teeth and are characterised by ulceration, cratering, marked oedema and erythema. Periodontal pocketing is often not evident due to the simultaneous loss of crestal alveolar bone and necrosis of the marginal gingiva. Progression is extremely rapid (bone loss of 90% of root length being reported in as little as 3 months (98)) resulting in exposure and sequestration of alveolar bone (98,99). The surrounding tissues may have a normal appearance. Severe pain is another characteristic of HIV-P (97), and is often described as a dull ache of the entire jaw, a feature more in common with Paget's disease rather than ANUG which presents as an acute pain in the gingival margins. Symptoms of pain may precede clinical signs of periodontitis.

Tissue biopsy and labelling with monoclonal antibodies for T₄ and T₈ cells demonstrated a complete lack of these cells in the periodontal tissues of 7 out of 8 HIV-1 seropositive subjects with clinical signs of periodontitis (100). Negative controls showed normal quantities and ratios of these cell. This finding may suggest a possible mechanism for the characteristic rapid attachment loss associated with HIV-P.

The incidence of HIV-P has not been clearly determined. Masouredis *et al* (1992) found 8 cases of HIV-P in 136 (6%) HIV-1 seropositive patients presenting at an outpatients clinic (101). On the other hand, Riley *et al* (1992) (102) in a cohort of 200 HIV-1 seropositive dental patients with gingivitis, periodontitis and ANUG were unable to identify any patients with clear signs of HIV-P as described by Winkler *et al* (1986). A larger, controlled, clinical study would be required to determine the incidence of this relatively rare complication. Recently, Yeung *et al* (1993) reported on the periodontal changes in 30 HIV-1 seropositive patients in a prospective study over 18 months. They found greater attachment loss over time in the study group when compared to controls. It was concluded that HIV-1 contributes to the progression of a pre-existing periodontitis rather than increasing its incidence (13).

1.4.4 HIV-Stomatitis

ANUG and periodontitis in otherwise healthy individuals are restricted to the region of the teeth. Bacterial infection of the oral cavity originating at the periodontium of HIV seropositive people can extend well beyond such parameters to involve larger areas of the oral cavity. Soft tissue defects extending beyond the mucogingival junction or exposure and necrosis of palatal bone may occur (103).

Advanced cases of this noma-like condition are generally restricted to people in third world countries where early professional intervention is not readily available.

1.5 DETECTION OF HIV

Many tests are available to detect the presence of HIV-1 infection in an individual's blood. The demonstration of antibodies to a region of the viral envelope is sufficient to warrant a confirmatory second test. However, antibody tests give no indication of the viral load and infectivity of the body fluid being tested. The following discussion briefly outlines the common diagnostic tests for HIV infection but will mainly be concerned with those tests used to detect the virus itself rather than antibodies to it.

1.5.1 The Western Blot

The Western blot qualitatively detects the presence of antibodies to a number of virus associated proteins, envelope proteins, and polymerase gene products. In the Western blot assay, proteins from a lysate of HIV-1 are separated according to molecular size by polyacrylamide gel electrophoresis (PAGE). Following electrophoresis the protein fractions are transferred to nitrocellulose paper and allowed to bind with the patient's serum. The anti-HIV-1 antibody from the patient's serum is then detected by an antihuman IgG antibody conjugated with peroxidase. When the conjugate is exposed to a substrate, a coloured band will appear on the paper if serum antibody was bound to viral protein.

The Western blot immunoassay with positive bands at 24 and 41kD have been used as the "gold standard" by which other tests results are judged. However, this assay has some limitations in the assessment of HIV-1 seropositive patients.

Indeterminate results are occasionally seen in patients in the terminal stages of HIV disease due to the lack of antibody production (63). In infants, the serologic diagnosis of HIV-1 infection is confused by the presence of HIV-1 antibodies transplacentally acquired from the mother. Maternal antibodies will persist for almost a year in the majority of infants and over 15 months in 22% of cases (104). Western blot is therefore an unreliable indicator of HIV status in these neonates for up to 18 months. The Western blot test, though highly sensitive, has been used only as a confirmatory test due both to the technical complexity of the procedure and the requirement to handle whole virus (105). Finally, four out of a surveyed five laboratories using Western blot assays for HIV diagnosis in the United States returned false positive results (106). This study raised some doubt on the reliability of the Western blot assay for the routine diagnosis of HIV-1 infection.

1.5.2 Peripheral Blood Mononuclear Cell Co-culture

The traditional technique of infecting normal cells *in vitro* by the addition of blood products from a suspected HIV patient has been used for some time (107). This technique uses non-infected peripheral blood mononuclear cells (PBMC) from healthy donors cultured in RPMI (Roswell Park Memorial Institute), foetal bovine serum, antibiotics and antifungals, sodium bicarbonate buffer, and phytohemagglutinin (PHA) (108). The PHA activated cells are further stimulated by the addition of interleukin 2 (IL-2). PBMCs from HIV-1 infected patients are added to the cell culture and the mixture is incubated for several days. New PHA stimulated donor PBMCs are added on every third day (feeding of the cultures).

Once the virus has replicated and infected other cells in the cell culture, its presence can be detected by assaying for reverse transcriptase enzyme activity or HIV-p24 antigen. In addition, microscopic examination of the culture may reveal morphologic changes to infected cells. This cytopathic effect (CPE) is often characterised by giant cell formation with multiple nuclei arranged in a ring formation (107), although other presentations may also be seen. Early CPE appears as a ballooning of the cell cytoplasm along the outer edges of clusters of lymphocytes. Later, infected cells coalesce to form syncytia or large giant cells. Finally the cell cytoplasm begins to decrease, form granules which eventually disintegrate.

Cell co-culturing is a very sensitive and specific technique(109). Its limitations include the protracted time required for a result, the labour intensive nature of the procedure, and the requirement of large volumes of fresh, uninfected donor blood for feeding of the cultures.

1.5.3 The Reverse Transcriptase Assay

The reverse transcriptase (RT) assay is most commonly used to demonstrate RT activity in PBMC co-cultures inoculated with blood from suspected HIV-1 positive patients. RT activity is demonstrated by the ability of the culture supernatant to catalyse the incorporation of tritium-labelled thymidine 5'-triphosphate tetrasodium salt (HTTP) into a RNA template to form a heteroduplex. The heteroduplexes are then detected by their radioactivity in a β beta scintillation counter.

The RT assay lacks the specificity or sensitivity of other assays used to show the presence of HIV. It is therefore most commonly used to confirm the presence of viable virus in cell co-culture.

1.5.4 The Enzyme Immunoassay

The Enzyme immunoassay (EIA) antigen tests are more sensitive and specific and detect HIV-1 in cell culture earlier than the RT assay (60,110). A sample is added to a solid surface pre-coated with the antibody to a region of HIV-1. Following several hours of incubation the unbound antigen is eluted and a second antibody (goat IgG) added. This new antibody is specific for another region of the antigen bound to the anchored antibody. An antisppecies IgG, conjugated with the enzyme peroxidase, is added to react with the antigen-antibody complex anchored to the solid surface. Following a final wash the substrate to the peroxidase enzyme is added. A colour change is observed (if HIV-1 antigen is present in the original specimen) and read on an EIA plate reader at an absorbence of 405nm.

This test is time and cost effective. As a result it has become the standard assay for screening of blood donations. The ELISA differs from the above mentioned assay in two ways. Firstly, the test seeks antibodies to HIV-1 antigens. Secondly, it utilises latex beads coated with the HIV-1 derived antigen which are dropped into wells containing dilutions of sample serum. Serial washes and additions of enzyme conjugated probes and substrate follow the steps described above. This assay enables large numbers of samples to be processed at once and much of the procedure can be automated.

The sensitivity and specificity of the ELISA test has been widely scrutinised. Ozanne and Fauvel (1988) compared five commercial ELISA kits and found them to have sensitivities ranging from 96.51% to 97.67% and specificities ranging from 99.6% to 100% (111). Others found significant discrepancies between Western blot, ELISA, and immunoprecipitation assays (112). Thorn *et al* (1987) reported the use of a unique, synthetic, CBre3 antigen for the detection of HIV-1 *env* antibodies using ELISA techniques (113). The study reported a greater sensitivity and specificity with this assay than the Western blot. However, despite occasional false positive results, ELISA remains an initial screening test, with confirmation coming from Western blot or Immunofluorescence assays (114).

1.5.5 Immunoprecipitation

T lymphocytes infected with virus are incubated overnight in a culture medium containing S^{35} methionine. Cells are lysed with detergent, and a cytoplasmic extract is made. Labelled virus released in the supernatant is banded on a sucrose gradient. Immunoprecipitation is then carried out by the addition of antiserum to HIV-1 p24 protein derived from HIV-1 seropositive subjects. The immunocomplexes are then analysed by PAGE under denaturing conditions. Results are assessed by autoradiography using x-ray film and intensifying screens over several hours.

1.5.6 Polymerase Chain Reaction*

Unlike the preceding assays, the polymerase chain reaction (PCR) technique does not directly identify the presence or absence of HIV-1 but is rather a means by which short regions of the viral genome can be amplified to a concentration where less sensitive but highly specific DNA probing techniques can be applied. Briefly, a short region of DNA derived from the viral RNA is copied numerous times using the biologic enzyme DNA Polymerase. These copies are then recopied in a cyclical process which results in an exponential increase in the copy number. Within 30 such cycles the concentration of the specific DNA region can be increased approximately 1 million fold to a level where conventional DNA probe technology can identify the presence of the DNA region. The regions of DNA and the probe used are unique to the HIV-1 genome. Hence the high specificity of DNA probe technology is supplemented with the high sensitivity of the PCR technique.

*Details of the polymerase chain reaction (PCR) procedure is described in chapter 2.

1.6 HIV IN SALIVA

1.6.1 Introduction

Isolation of the hepatitis B virus, hepatitis C and the Epstein Barr viruses in saliva, and their transmission via this route has been well documented (115,116,117). In contrast, concern over the potential spread of HIV-1 through contaminated saliva has not been substantiated by epidemiologic data. Saviteer *et al* (1985) reported the lack of apparent transmission through oral contact in two nurses 9 months after giving an AIDS patient mouth to mouth resuscitation (118). Drummond *et al* (1986) reported an 18 month follow up on a female who had been deeply bitten on the finger by a HIV infected person. Although the wound resulted in profuse bleeding, the victim remained seronegative (119). These well documented reports were in contrast to those which cited cases of horizontal transmission of HIV through saliva by exclusion of other possible routes (120,121).

Following the isolation of HIV-1 in the serum of AIDS and ARC patients, several investigators isolated the virus in saliva. Groopman *et al* (1984) used a co-culture technique to assay for HIV-1 in twenty saliva samples taken from 4 patients with AIDS, 10 with ARC, 4 healthy seropositive and 2 seronegative individuals who were in high risk groups for the acquisition of the virus (9). The saliva was centrifuged and the supernatant filtered through 0.45µm filters prior to inoculation into a continuous cell culture of PHA stimulated PBMCs. The presence of virus was assessed by assays for reverse transcriptase or by direct immunofluorescence of the culture cells (122). Virus was isolated in 7 blood samples taken from the 18

seropositive patients. In contrast, only 8 saliva samples taken from the same cohort had detectable virus. The virus was detected in four patients with ARC and four patients who were in stage 2. Confirmation of this finding was made, in part, in a larger study by Ho *et al* in 1985 on 83 saliva samples taken from 71 patients sero-positive to HIV-1 antigen. This group comprised of patients in the various stages of HIV disease. Unlike the previous study, however, they found only 1 sample in 83 (1%) (from an AIDS sufferer) contained the virus using similar co-culture and detection techniques (122). Levy and Greenspan (1988) examined 71 saliva samples from 55 HIV-1 seropositive patients using cell co-culture. Four instances of HIV-1 in these samples were noted (123). Greenspan isolated HIV-1 in 1 patient from 10 with oral hairy leukoplakia while 5 of 14 showed antibodies to the virus (124). In contrast, others were unable to find virus in 21 samples taken from 7 patients (125). Using both co-cultivation and ELISA assaying for p24 antigen, Barr *et al* in 1992 found only 5 saliva samples from 218 were positive to p24 assay and only 2 samples were positive to co-cultivation. There was no correlation between the samples containing p24 and those infectious in co-culture (126). Most recently, researchers at the Department of Virology, ICPMR at Westmead Hospital were able to detect HIV in 6 of 28 saliva samples using cell co-culture (14). Taken together, these findings show that HIV-1 is infrequently isolated in the saliva of HIV infected persons using the traditional co-culture technique.

1.6.2 Antibodies to HIV in Saliva

Investigation into the occurrence and composition of antibodies to HIV-1 in the saliva of seropositive subjects has been led by the Baltimore group of Archibald and co-workers. They reported the presence of anti-HIV antibodies in 22 of 28 saliva samples (79%) taken from seropositive patients in Japan. In contrast, none of the 10 seronegative controls exhibited antibodies to the virus using the same assay (127). Later, this same group found secretory IgA specific for HIV-1 antigens in the saliva of a group of patients (128). Yeung *et al* (1993) detected HIV-1 antibodies in 46 of 50 seropositive patients using ELISA and confirmed this by Western blot in 36 cases (14). Thus, it is apparent that unlike the isolation of the virus itself, detection of the antibodies to the virus in saliva is relatively common using standard techniques. As a result, it has been suggested that saliva can be used as a screening test for suspected carriers of HIV-1 (129).

1.6.3 Salivary Inhibition of HIV

In vitro studies have shown that incubation of HIV-1 in the saliva of either HIV seropositive or seronegative persons led to a failure to infect PBMCs in cell culture. A comparison between the methodology of the Groopman *et al* (1984) (9) (in which a majority of patients were found to have HIV-1 in their saliva) and those that followed, such as Ho *et al* (1985) (122), showed that the most apparent difference between the two studies was the prior filtering of the saliva by the Groopman group. It may therefore be postulated that the virus becomes attached to a salivary macromolecule which does not precipitate during centrifugation but is too large to pass through a 0.45µm filter and that the adherence of HIV-1 to this

macromolecule prevents viral infection of cells in co-culture. This hypothesis is supported by the work of Fultz *et al* in 1986 and Fox *et al* in 1988 and 1989. Fultz's group found that the addition of Lymphadenopathy Associated Virus (LAV)** to samples of human or chimpanzee saliva and incubating for 30 minutes caused a complete inhibition of viral infectivity when the samples were added to cell culture. Filtration of the saliva prior to inoculation with LAV removed the inhibitory activity from the human but not the chimpanzee saliva (130). Therefore, it was concluded by Fultz that two components of the saliva had inhibitory activity. Since tears do not share this inhibitory ability (8), it is unlikely to be a result of enzymes such as lysozyme which is common to both fluids. Fox *et al* (1988 and 1989) further characterised the nature of the inhibitory activity through a series of experiments in which virus was introduced into saliva samples from 34 males, females, children, and seropositive homosexuals. Using co-culture methods, it was found that dilution of saliva was able to reduce the inhibitory activity of saliva, though not linearly. The activity was not affected by freeze thawing once only nor by heating to 56°C for 30 minutes. Inhibitory activity was found in both the cell pellet and the supernatant and was the same in all patient groups (131,132). Their conclusion, once again, was that a macromolecule was responsible for the observed effect but did not speculate on the nature of this molecule. In addition, since the inhibitory activity was the same in both infected and non-infected groups, it is unlikely to be singularly the result of a specific mechanisms such as secreted antibodies. In 1990, Archibald and Cole reported a series of *in-vitro* experiments to further elucidate the mechanism of HIV-1 inhibition by saliva. Filtration of

** LAV was an erstwhile name for HIV.

uninfected donor saliva through 0.45µm filters prior to the addition of HIV-1 resulted in a reduction in the anti-infective capacity of the saliva while filtration of the saliva following viral inoculation and incubation at 37°C for 45 minutes significantly reduced the occurrence of CPE in co-culture. It was therefore postulated that the inhibitory agent was a macromolecule which bound HIV-1 and formed complexes that could not pass through fine filters and thus prevent the binding of virus to cells expressing the CD₄ marker (133). This was supported by data from another study demonstrating whole saliva was capable of non-immune inhibition of the herpes simplex virus (134) and that sulfated polysaccharides, such as dextran sulfate (DS), had a demonstrable antiviral activity *in vitro* (135). Mitsuya *et al* (1988) demonstrated the inhibition of HIV-1 uptake by CD₄ cells in the presence of DS (136). Saliva contains high molecular weight mucins (derived mainly from the submandibular gland) which are highly sulfated and may have a similar activity. This hypothesis was consistent with data which showed the greater inhibitory activity found in whole saliva and submandibular saliva compared to parotid saliva alone (132).

1.7 CONCLUSION

The HIV has been infrequently found in the saliva of HIV-1 seropositive individuals using viral culture and Enzyme-Linked Immunosorbent Assay (ELISA) techniques. Although these assays have high sensitivity and specificity when the virus is present in high concentrations, such as in the plasma, their value has not been proven when the virus is diluted to levels beyond their detection limit. Thus clinical research into the effects of drug regimens on the shedding of virus through the oral cavity, the availability of virus in saliva during oral infection, or the effects of saliva on the virus is limited by the detection method used. A reliable, highly sensitive and discriminating method for the detection of virus in the saliva of HIV positive patients is therefore sought. The purpose of this research project is to further develop a quantitative PCR assay which not only detects HIV genetic material in the saliva of HIV positive patients, but also provides an the estimation of virus concentration in the oral fluid. The results of this assay will then be correlated to clinical data regarding the sampled patient's gingival and periodontal disease state. In addition, similar quantitative data derived from the sampled patient's blood will be compared to determine whether a correlation exists between viral load in blood and saliva.

CHAPTER TWO

THE POLYMERASE CHAIN REACTION

2.1 OVERVIEW

Relatively high antibody titres against HIV-1 surface or core antigens can be detected in the blood following seroconversion (38,56,137). In contrast, the concentration of free virus is low and infected cells may only demonstrate viral DNA and surface markers (76,86). In 1 ml of blood from HIV-1 infected individuals approximately 10^3 copies of HIV-1 DNA can be detected. This compares with about 10^{12} copies of the Hepatitis B virus (HBV) DNA found in the same volume of blood from an average HBV carrier (138). Direct demonstration of the presence of virus within a cell has been limited by the sensitivity of the tests used and, in the case of cell culture techniques, the requirement of viable virus (1,3-8,13-14,108,122-124). Other diagnostic methods such as serologic assays are unreliable prior to seroconversion and in the latter stages of AIDS due to the low HIV-1 antibody titre in the circulation during these stages (56).

Saiki *et al* in 1985 (139) were the first to describe the amplification of a specific DNA sequence of low concentrations such that it could be detected using conventional methods. The potential applications of this technique in the detection of other genomic sequences was immediately recognised (140). The polymerase chain reaction (PCR) method has since been adapted by Ou and co-workers (141) for the detection of HIV-1 nucleic acid sequences in body fluids at concentrations previously below detection limits.

2.2 DNA STRUCTURE

The three dimensional structure of Deoxyribonucleic acid (DNA) was first elucidated by Watson and Crick (1953) (142). DNA is a long, unbranching macromolecule containing genetic information encoded in a binary like series of purine and pyrimidine sequences. Two helical polynucleotide chains are coiled around a common axis to form a double helix. Sugar and phosphate groups perform a structural role for each chain of the double helix. This 'backbone' is constant throughout the molecule and consists of phosphodiester bridges joining the 3'-hydroxyl group of one sugar to the 5'-hydroxyl region of the next, thus giving each chain polarity, though in opposite directions. Any of the four bases may attach to either chain. The bases can be grouped into two types:

- a) purines; comprising Adenine and Guanine,
- b) pyrimidines; comprising Cytosine and Thymine.

The bases, being hydrophobic, are on the inside of the helix whereas the phosphate and sugar units are on the outside. The two chains of the helix are held together by hydrogen bonds between specific regions on complementary bases. Only two 'base pair' (bp) combinations exist, Adenine with Thymine and Guanine with Cytosine. It is the precise sequence of bases along the molecule which comprises the genetic information.

Since the bases unite specifically with only their pair, duplication of the DNA sequence is easily facilitated as either of the single chains can act as a template for the construction of the other. Duplication of a length of double stranded DNA requires its prior separation (denaturation) into two single strands either by raising the temperature of the DNA *in vitro* to over 85°C or through the action of specific enzymes *in vivo*. The enzyme DNA Polymerase, once attached to a single strand of DNA, uses it as a template for the construction of a complimentary strand. DNA polymerase acts in the 5' to 3' direction only and has a capacity for the correction of errors in duplication through its endonuclease ability. This ensures a high degree of replication fidelity (143).

2.3 PRINCIPLES OF PCR

The PCR is an exponentially amplifying, target specific, primer driven cyclical DNA polymerase reaction. Under the right *in vitro* conditions the PCR can copy a specific nucleic acid sequence of 100-2,000 bp one million fold or more in only a few hours (144). The reaction requires two oligonucleotide primers (primer pairs) each of which are complimentary to specific nucleotide sequences (primer sites) on opposite strands of the target DNA several hundred base pairs apart (Figure 2.1). The length of DNA between the two primer sites will be the region amplified by the reaction (target sequence). The DNA polymerase enzyme requires the prior attachment of a primer to initiate the copying process. Each primer anneals to a primer site at the 5' end of the target sequence on their respective strand or to subsequent amplification products (copies) initiated by the other primer on previous rounds of the PCR. Therefore, one primer (the 'upstream' primer) attaches to one strand of the DNA and the other primer ('downstream') attaches to the other strand some distance away. To enable the annealing of each primer to their sites, the double-stranded DNA (dsDNA) must first be thermally denatured at 95°C for several minutes to form single strand DNA (ssDNA). Reduction of the temperature to approximately 50°C for 30 sec to 2 minutes allows annealing of the primers to the primer sites. The DNA polymerase catalyses the primer directed DNA synthesis, using the existing DNA strand as the template. Subsequent elevation of the temperature to 72°C for a period of approximately 2 minutes facilitates the extension (copying process). Extension is stopped by heating the reaction to the denaturation temperature once again. The

result is an approximate doubling of the amount of target sequence in the sample. Since the primer pairs are at the 5' ends of each DNA strand, extension from each primer proceeds in opposite and overlapping directions such that DNA synthesis initiated by one primer generates a new strand with a region complimentary for the other primer. Repeating the cycles of denaturation, annealing and synthesis results in an exponential growth in copy number. Mullis and Faloona (1987) expressed this growth by the formula 2^n where n =the number of cycles used. However, in practice, the amplification is more accurately described by the formula $Y=S \times (1+e)^n$ where Y is the amount of DNA after amplification, S is the starting number of target copies, n is the number of cycles performed, and e is the average efficiency of amplification per cycle (144).

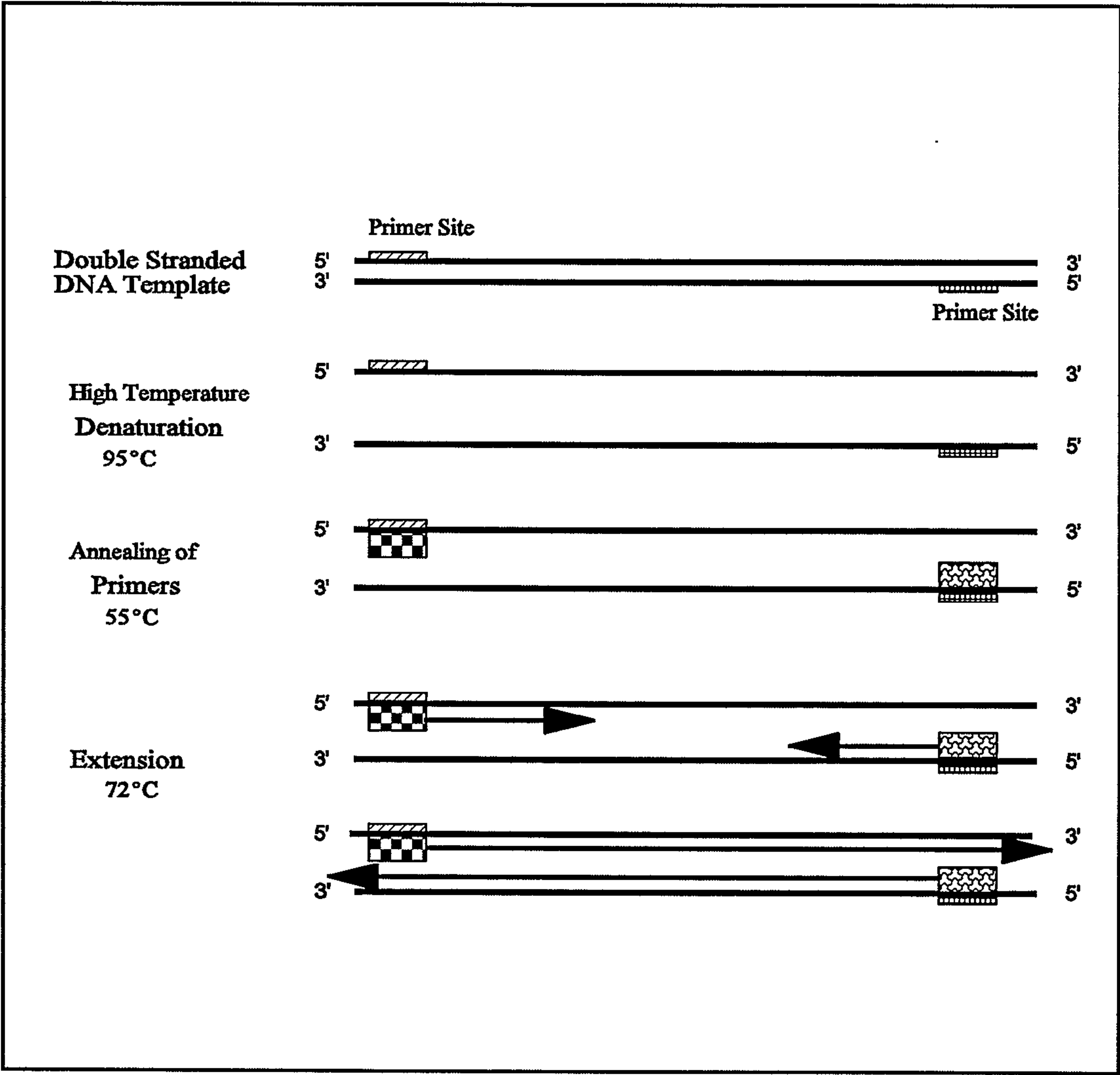


Figure 2.1: Principle of PCR amplification of a region of double stranded DNA.

2.4 IDENTIFICATION OF PCR PRODUCTS

The products of the PCR are DNA molecules of specific molecular weight corresponding to the portion of the target DNA bracketed by the primer pairs. At the completion of the PCR reaction, aliquots from each reaction well can be hybridised with a radioisotope labelled probe (usually containing P^{32}). The probe consists of a short length of DNA complementary to a region uniquely found on one chain of the expected product DNA. The PCR products, all identical in length, molecular weight, and charge, move through a polyacrylamide gel at the same rate when an electrical current is applied, concentrating the product into a discrete band. An autoradiogram can be created by placing the gel into a x-ray film cassette overnight. Discrete bands corresponding to the sites of probe labelled PCR products can be seen on the developed film. Alternatively, the banded regions can be excised and placed into a scintillation vial. The amount of radiation emitted in the vial can be enumerated in a scintillation counter to give quantitative data.

2.5 HIV PCR

Ou *et al* (1988) applied the PCR technique to multiple regions of the HIV-1 genome in samples taken from 3 groups of virus-culture positive and/or seropositive homosexual men (n=22) as well as seronegative controls (n=13). They found HIV-1 sequences in 100% of DNA specimens from seropositive and virus-culture positive patients as well as in 64% of seropositive but virus-culture negative patient specimens (141). The advantages of speed and sensitivity in detecting viral genomes using this method has been applied to many aspects of HIV-1 research. Laure *et al* (1988) using PCR detected HIV-1 DNA in six of fourteen newborn infants of seropositive mothers. Only one of those children had detectable viraemia using conventional co-culture methods. This result was available within days of birth rather than serologic assays which are undiagnostic in neonates for up to 18 months (145).

The marked heterogeneity of the HIV-1 genome has resulted in the use of primer pairs bracketing the most conserved regions (146). The long terminal repeat (LTR) regions of the HIV-1 proviral genome, gag, and env regions are often chosen (147). These regions are bracketed by the primer pairs: SK 29/SK 30, SK 38/SK 39 and SK 68/SK 69 respectively (Figure 2.2).

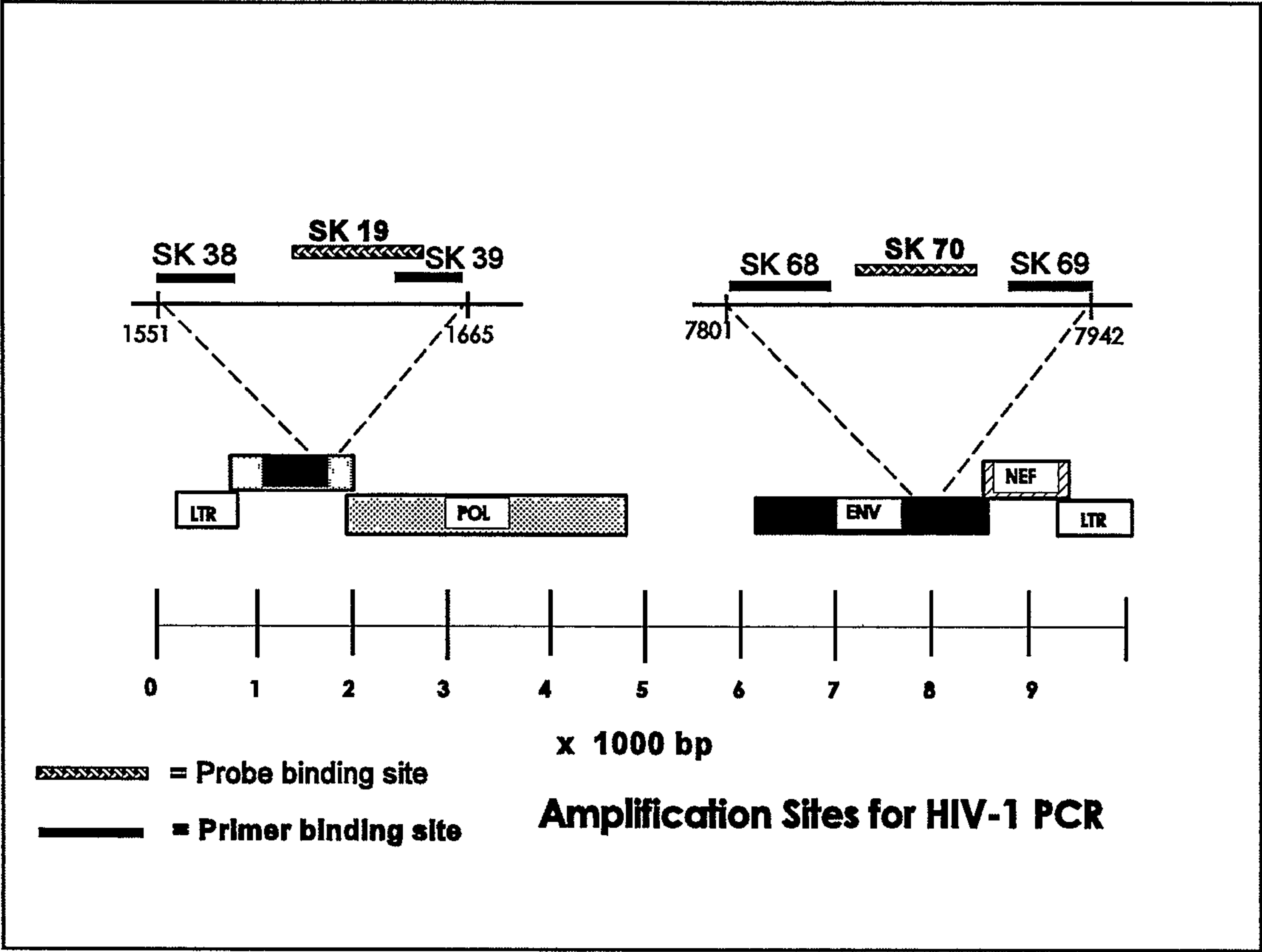


Figure 2.2: Two principle primer binding sites on the HIV-1 genome and the region recognised by the probes used for the detection of amplified regions.

2.6 OPTIMISING THE PCR

2.6.1 Polymerases

Initial PCR work was carried out using a DNA polymerase-I derived from *Escherchia coli* (E.coli) (139). This polymerase, known as the 'Klenow fragment', had a low error rate but was heat labile at the denaturation temperatures. Therefore, each cycle of the PCR required the addition of new polymerase (139). Saiki *et al* (1988) reported the use of a new polymerase derived from a thermophilic bacterium, *Thermus aquaticus* (Taq) which could survive extended incubation at 95°C. This heat-resistant polymerase is relatively unaffected by the denaturation step, and therefore can be used in repeated cycles. The new enzyme not only simplifies the PCR procedure, making it amenable to automation, it also substantially improves the overall performance of the reaction by increasing the specificity, yield, sensitivity, and the length of the targets that can be amplified (148,149). The polymerisation rate of Taq is 35-100 bases per second, depending on the target DNA sequence and reaction conditions such as enzyme concentration and the temperature of the extension phase (144). The optimal concentration of Taq is between 1.25 and 10 U per 100µl (144,149,150). Excessive polymerase will result in an increased rate of amplification of non-specific DNA regions (144).

2.6.2 The Primers

A primer will anneal to the complimentary region of the target DNA. At lower temperatures, the primers may anneal to regions which are only partly complementary, resulting in non-specific amplification products. The length of the primer is also critical. Primers shorter than 14 bases will tend to bind non-specifically and form unstable duplexes. Bugawan *et al* (1988) described the use of 10-mer primers with success although they advocated the use of longer primers (151). However, long primers require more time to anneal and this may result in a reduction of the amplification efficiency (152). In addition, during the annealing phase, product DNA will tend to anneal to complementary strands. Rapid cooling, shorter primers and the use of non-ionic detergents such as Triton x-100 will aid in the preferential annealing of primers over product. Finally, since G-C base pairs have three hydrogen bonds while A-T pairs have two, primers with higher G-C content at their 3' ends will be more stable.

2.6.3 Other Components of the Reaction Mix

i) Magnesium Chloride (MgCl_2)

DNA polymerases require a small amount of the divalent ion Mg^{++} for its action. The required concentration ranges from 1.5-10mM. Varying the Mg^{++} concentration will vary the specificity of amplification. It is recommended that the optimal concentration of Mg^{++} be assessed for each application (138, 144).

ii) Nucleic Acids (dNTP)

The four nucleic acids from which DNA is comprised are essential in the reaction mix. An equal 2.5mmol/l concentration of dATP, dCTP, dGTP, dTTP provides structural components for nascent DNA production.

2.6.4 PCR Efficiency

Several variables affecting the efficiency of PCR amplification have been described (138):

i) The number of cycles. With each cycle, the concentration of the product increases according to the formula described above, $Y = S \times (1 + e)^n$. As the concentration of the product increases, the chances of product strands reannealing to each other, rather than to the primer, increases. Consequently, the latter cycles of the reaction are less efficient (139).

ii) The amount of target. An overabundance of target sequence in relation to the available polymerase will result in reduced efficiency. Mullins and Faloona (1987) achieved only a 62% efficiency when amplifying the a 110-bp fragment at 1×10^{-13} mol concentration (1449) while Saiki *et al* (1985) achieved an 85% efficiency when they amplified the same gene at a concentration of 5×10^{-19} mol (139). It is likely that when the target DNA is present in high concentrations, rehybridisation of the amplified fragments occurs more readily than their hybridisation to primer molecules.

iii) The length of the target sequence. Target sequences 100-300-bp were amplified more efficiently than those which were 2000-bp (139). The inverse relationship between target length and efficiency is believed to be the result of incomplete copying of long targets in the time allowed during the extension phase of the cycle. This may lead to the primer site (the last sequence to be copied during each extension) not being created prior to denaturation, and thus the new strand will not be involved in further amplification cycles.

iv) The temperature of primer annealing and extension. As described above, the temperature determines the specificity of primer-target combination. Saiki *et al* (1988) found an increase of two orders of magnitude in the detectable concentration of the β globin gene when annealing concentration was increased from 40°C to 55°C (148).

2.7 NESTED PCR

A further refinement of the PCR technique has recently been described (153). In a two stage PCR, the products of the first round act as templates for a second PCR using a primer pair designed to amplify a sequence internal to the target region of the first PCR. The use of this 'nested' set of primers results in increased sensitivity and overall yield and has enabled the use of direct visualisation of the amplified DNA sequence with ethidium bromide staining rather than a need for radioactive labelling. In addition to this greater sensitivity, the nested PCR increases the specificity of the amplification due to the requirement of four separate, highly specific, priming events to produce the product rather than two.

The nested PCR technique has been used to detect HIV-1 DNA in clinical specimens taken from 85 seropositive and 60 seronegative patients (154). Using three different of nested PCR primer sets detection rates were between 100% and 98% in the HIV-1 positive group while 0% in the HIV-1 negative group.

2.8 QUANTITATIVE PCR

Prior to the introduction of PCR techniques to the detection of HIV-1 plasma viraemia, it was considered very rare to find free virus in the blood of clinically asymptomatic subjects. Ved Brat *et al* (1992) reported no free virus in the blood of almost 100% of patients at Walter-Reed stages 1, 2, 3, and 4 using co-culture techniques and over 75% of cases in stages 5 and 6 (155). The success of PCR at detecting low concentrations of viral nucleic acids has encouraged many research groups to study the factors which change the effective concentration of the virus in the circulation or its nucleic acids within infected cells (156,157).

As mentioned previously, the PCR amplifies a target region of DNA exponentially according to the formula $Y=S \times (1+e)^n$. Thus, the final concentration of product is determined by the original concentration, the number of cycles and the efficiency of the reaction. When a high template concentration is used or occurs as a result of PCR amplifications, phenomena such as the substrate saturation of enzyme, product inhibition of the enzyme, incomplete strand separation, and product strand re-annealing can be limiting factors for efficient amplification. Accordingly, to obtain reliable quantitative evaluation of specific DNA the range of concentrations for the template should be such that the reaction remains within the exponential phase. The reaction efficiency is also determined by numerous other factors as described previously and is variable over time and conditions. In addition, the final number of

DNA copies cannot be enumerated accurately, although hybridisation with a radiolabelled probe and scintillation counting with a beta scanner can give a ratio which can be compared to a known quantity of labelled DNA. Therefore, determination of the original quantity of target DNA requires either knowledge of the other factors or a method to compare a product derived from a known quantity of DNA which has undergone identical processing at the same time. Two types of standards have been used to enable quantitation following a PCR amplification.

i) Internal Standards

Internal standards require the simultaneous amplification of two sets of DNA in the same well. Amplification of a different region of DNA whose concentration is known concurrently with the target DNA of unknown concentration has been described (141). This technique has been criticised due to the different primers used and the different lengths of DNA being amplified (158). An alternative is to use a DNA region which is of similar length and contains the same primer sites. Stieger *et al* (1991) reported the use of a competitive internal standard for the quantitation of HIV-1 genetic material within infected tissue culture cells. A mutant region the HIV-1 genome, which contained a restriction endonuclease site, was constructed. Known quantities of the mutant DNA were then added to an unknown concentration of cell culture DNA and PCR was performed. Since both DNA's shared the same primer sites and were in the same well, the amplification of both was the same. Following PCR, the mutant strains were cleaved using the BglII enzyme and the mix labelled with a radio-labelled probe. Electrophoresis separated the cleaved DNA from the intact target

DNA and scintillation counting allowed a comparison between the known original concentration of the mutant strain and the unknown concentration of the target (159).

An alternative technique using a synthetic region of DNA made from many primer sites in a linear arrangement has been described. An unknown concentration of target DNA, which shared primer sites with a known concentration of this synthetic DNA, were subject to the same PCR reaction. The two DNA regions were labelled with P^{32} probes and separated using PAGE. Their relative concentrations were compared using Cerenkov counting and thus the original concentration of the target DNA could be deduced (160).

The use of internal standards has several disadvantages for the quantitation of HIV-1 DNA. When HIV DNA and the internal standard are present in different amounts, they have different amplification efficiencies. This could be overcome by running a dilution series of internal standard for each sample, although this would be impractical if many samples were to be processed or if the amount of sample was low.

ii) External Standard

The use of known concentrations of HIV-1 DNA in separate wells undergoing PCR amplification concurrently with the target samples which contain similar genetic sequences is the characteristic of external standards. The DNA used in the external standard is exposed to conditions of primer concentration, temperature, cycle number, buffer concentrations, and DNTP concentrations identical to those experienced by the target DNA. A dilution series of the known concentration DNA is used and a standard curve plotted following PCR. Scintillation or densitometric values from the target DNA can then be compared to regions on the standard curve to derive an estimate of the number of DNA copies in the sample prior to PCR.

Oka *et al* (1990) used a dilution series of one copy per cell NY-M10 cells to construct a standard curve. Nine asymptomatic patients were then sampled and the concentration of HIV-1 nucleic acids in their PBMCs was analysed by comparing the PCR product concentration with the standard curve. In contrast, HIV p24 antigen was not detected in any of the samples using ELISA (161). Aoki *et al* (1990) used both an external and internal standard in assessing the changes of viral burden of a group of 13 HIV-1 positive men starting didanosine (ddI) therapy (162).

2.9 CONCLUSION

The PCR technique has been developed into a highly sensitive and specific method for the detection of DNA fragments at picomolar concentrations. The technique has subsequently been applied to clinical blood samples in HIV-1 infected patients to demonstrate the presence of viral genetic material in the circulation. Standardisation of the reaction using known concentrations of comparable genetic material has enabled a degree of quantitation and, hence, information regarding viral load in infected patients. This technology may have particular applications to other body fluids, such as saliva, where the concentration of virus appears to be below the detection limit of other assays. Alternatively, if high concentrations of HIV-1 are found in the saliva it would lend support to the hypothesis that a salivary constituent inhibits viral infectivity.

CHAPTER THREE

MATERIALS AND METHODS

3.1 RESEARCH PLAN

The aim of the present study was to develop a sensitive PCR assay to estimate the number of HIV-1 infected cells in biologic samples from known HIV-1 seropositive subjects. For this purpose an assay, using PCR technology, was developed with the technical support of the Department of Virology at Westmead Hospital. The assay aimed to provide quantitative data for the number of viral DNA copies detected in a saliva sample. The data was then compared to that derived from a blood specimen taken from the same subject (of which a cell count was made) and processed in a similar manner. The number of HIV-1 DNA copies in a standard aliquot was indicative of the number of virally infected host cells. Quantitation was facilitated by comparing assay results from the subject to comparable data derived from a standard of known copy number. Hence the number of infectious cells in the saliva and blood sample could be estimated.

Once established, the assay was to be applied to paired samples of saliva and blood taken from a group of HIV-1 seropositive patients. An oral examination was carried out to ascertain the presence of open wounds and the degree of gingival inflammation. These data were to be analysed for a correlation between viral load in blood and saliva as well as for the role of dental disease and gingivitis in the presence of HIV-1 infected cells in saliva.

Conventional and quantitative viral co-cultures were performed on a selection of clinical samples which had been quantitated using the new PCR assay. This data was used to validate the PCR assay as well as to compare the sensitivity of the new assay with the traditional means of virus detection in saliva.

3.2 ESTABLISHMENT AND OPTIMISATION OF THE ASSAY

3.2.1 Assay Conditions

A PCR assay was prepared according to previous reports (163) to test the efficacy of the reagents. HIV-1 DNA, derived from the stock 8E5 cell line*, and the primer pairs SK 38/SK 39 were used to establish a working assay. A second primer pair (SK 68/SK 69) was also used in the development of the assay (Table 3.1). This alternative primer pair was used to assess the influence a change in assay conditions may have on the resulting data as well as to confirm results derived from the use of the previous primer pair.

HIV-1 DNA was extracted from 8E5 cells using 125µl of extraction buffer (see Appendix A) for 1½ hours at 56°C. The extraction buffer activity was arrested by raising the temperature to 95°C for 10 minutes. The lysed cells were stored at -20°C until the following morning. DNA was extracted from the lysed cells using the Phenol-Chloroform method (see Appendix B). The precipitated DNA was finally resuspended in water and stored at -70°C until required.

* Positive control (NY-M10 and 8E5) cells were donated by Dr Yamamoto, Tokyo Medical and Dental University, Tokyo Japan.

Table 3.1: Sequences of oligonucleotide primer pairs and probes used in the present study and their locations on the HIV-1 genome.

Primer or Probe		Sequence (5' - 3')	Location
SK 38	Primer	ATAATCCACCTATCCCAGTAGGAGAAAT	<i>gag</i> 1551-1578
SK 39	Primer	TTTGGTCCTTGTCTTATGTCCAGAATGC	<i>gag</i> 1638-1665
SK 19	Probe	ATCCTGGGATTAAATAAAATAGTAAGAATGT ATAGCCCTAC	<i>gag</i> 1595-1635
SK 68	Primer	AGCAGCAGGAAGCACTATGG	<i>env</i> 7801-7820
SK 69	Primer	CCAGACTGTGAGTTGCAACAG	<i>env</i> 7922-7942
SK 70	Probe	ACGGTACAGGCCAGACAATTATTGTCTGGTA TAGT	<i>env</i> 7841-7875

3.2.2 The PCR Method

(i) Reaction Mix

To 10µl of cellular DNA solution 40µl of PCR reaction mix was combined in 150µl Eppendorf tubes to make a total volume of 50µl. The reaction mix contained the following:

Taq Polymerase (Promega, USA)	0.25µl
Sense Primer	5.0 µl (SK 38 or SK 68 5pmol/µl)
Antisense Primer	5.0 µl (SK 39 or SK 69 5pmol/µl)
Nucleotide Mix	4.0 µl (10 mM each dNTP)
10 x PCR Buffer*	5.0 µl
Sterile Water	20.75 µl
Total	40.00µl

* See Appendix A

The mixture was overlayed by two drops of paraffin oil before the eppendorf tubes were sealed and transferred to a thermocycler (Corbett FTS-1, USA) (Figure 3.1).

(ii) Thermal Cycling

The HIV-1 DNA in the tubes, containing the PCR reaction mixture, underwent amplification according to the procedure described in Section 2.3. First, the tubes were heated to 94°C for 7 minutes to ensure complete separation of the double stranded DNA, and then 20-25 cycles of denaturation, annealing and extension were carried out in the following manner:

Denaturation:	94°C , 30 seconds
Annealing:	55°C, 30 seconds
Extension:	72°C, 1 minute

The final extension step was allowed to continue for 7 minutes to ensure all strands had completely extended. The mix was then allowed to cool to room temperature.

3.2.3 Detection of Product

Confirmation of the successful amplification of a 114 bp region (corresponding to the region bounded by the SK 38/SK 39 primer pair) was first made by separation using poly-acrylamide gel electrophoresis (PAGE). Fifteen microlitres of the reaction product and 5µl of marker dye were transferred to a single well of the 10% PAGE gel (Figure 3.2). Under 150 mV and 10 mA, DNA strands of different sizes were separated over 1 hour. The gel was stained in a bath of ethidium bromide for 5 minutes and viewed under ultra-violet light. Banding on the gel in a region corresponding to a length of the HIV-1 *gag* gene (HIV-1 DNA segments) of approximately 110 bp in size represented successful amplification. Similarly, banding in the 140 bp region indicated successful amplification of the 141 bp region of the *env* gene by the alternative primer pair.

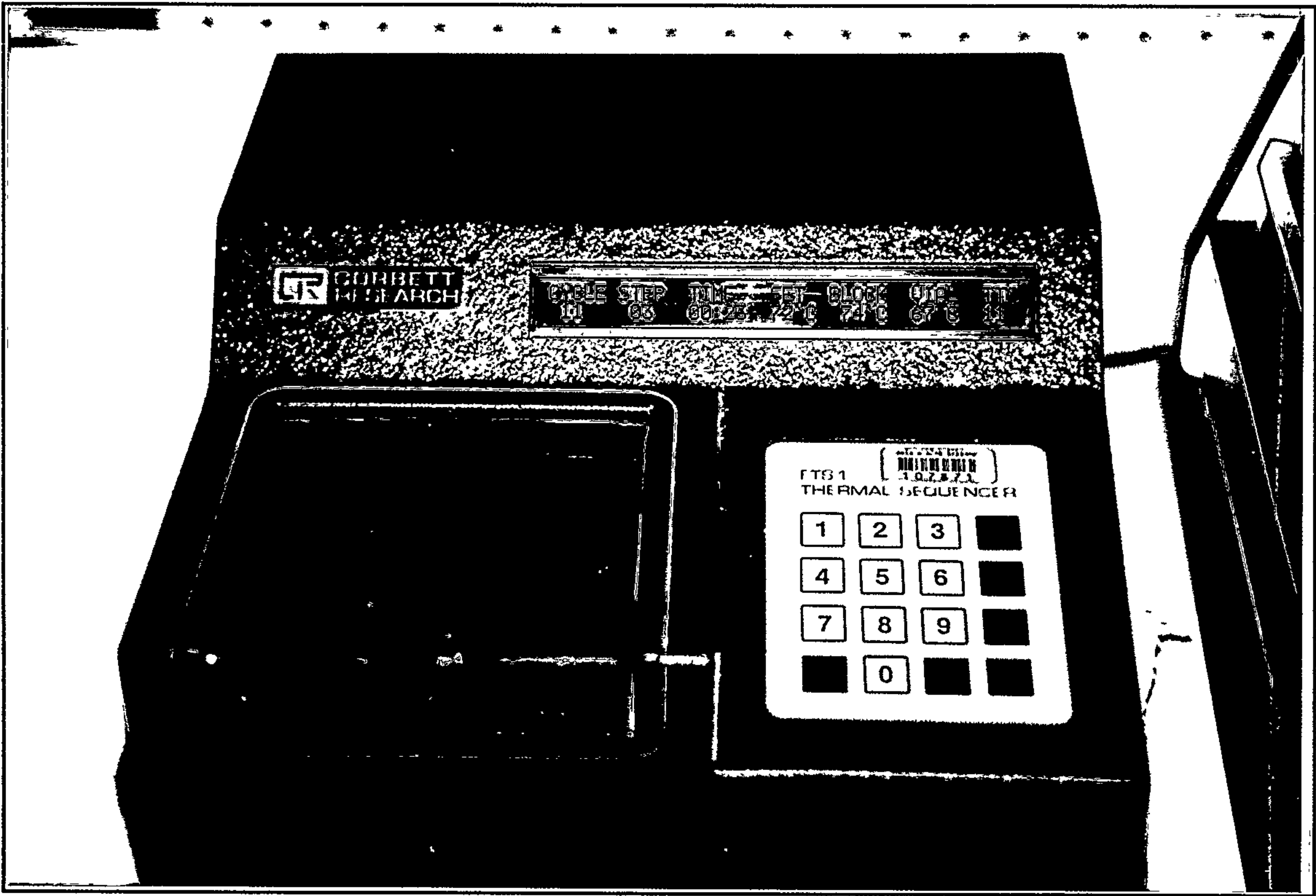


Figure 3.1: Corbett FTS-1 Thermal Cycler.

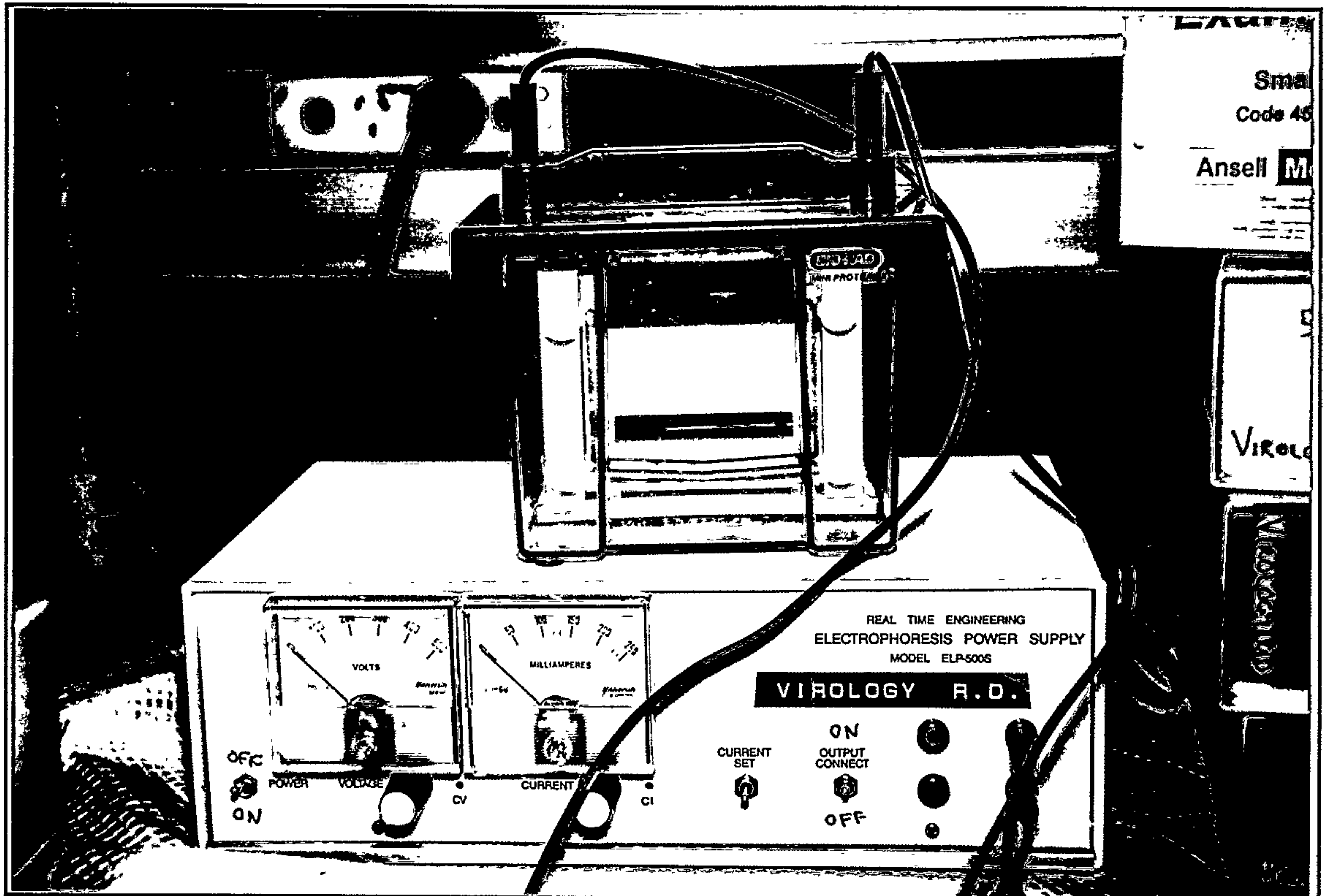


Figure 3.2: Bio-Rad Mini Protean B Electrophoresis Gel Chamber.

3.2.4 Optimisation of Assay Conditions

For each primer pair the optimum magnesium ion concentration and annealing temperature was determined. PCR buffers with MgCl_2 concentrations ranging from 0.5 mM to 3.0 mM (0.5 mM increments) were used to amplify 10 μl aliquots of the same positive control. All other reagents and experimental conditions were identical for each tube. The MgCl_2 concentration producing the strongest band was then used for all further PCR assays using that primer pair.

Similarly, the optimal annealing temperature was determined by repeating the assay with varying annealing temperatures with increments of 5 °C from 40 °C to 60 °C. The annealing temperature at which banding was most pronounced was used for all further PCR assays for each particular primer pair.

Further refinement of the assay was performed to determine the number of PCR cycles required to produce a standard curve with the optimal gradient in the concentration region expected for the clinical samples. This was determined by repeated amplifications of a dilution series (see later in section 3.2.7) of HIV-1 DNA with varying cycle numbers. The number of cycles at which the greater dilutions (lower concentrations) of HIV-1 DNA were being amplified exponentially (a steeper curve) without excessive plateauing of higher concentrations was recorded. The steepest portion of the standard curve was used to determine the approximate number of DNA copies in clinical samples. The preparation of these samples is described in section 3.4.

3.2.5 Labelling of Product

Radioisotopic labelling of the PCR product was substituted for ethidium bromide staining in later assay runs. This provided a greater sensitivity as well as a means by which the amount of product could be enumerated. A P^{32} labelled DNA sequence (probe) complimentary to a unique region of the sense strand of the product DNA segment was annealed in the following way.

Ten microlitres of the PCR product was heated to 95°C for 10 minutes to denature the DNA strands and placed on ice. Radioisotopic labelling was achieved by adding 100µl of an annealing mixture containing 25µl of a P^{32} labelled probe (SK 19 or SK 70) in 2475µl of PMA diluent (see Appendix A) to the tubes, sealed and incubated for 3 minutes at 60°C before being allowed to cool slowly to room temperature over 30 minutes. The reaction products were separated as described above (section 3.2.3). The product within the gel was electrophoretically transferred to a nitrocellulose membrane (Boehringer-Mannheim, Germany) which was then exposed to X-ray film in a cassette overnight (autoradiography). After developing, the film was inspected for evidence of discrete bands of radioactive decay products. The intensity of banding on the film was an estimate of the amount of product present.

3.2.6 Enumerating the Labelled Product

The regions of the membrane corresponding to the banding on the X-ray film were marked, excised with a scalpel and transferred to scintillation vials containing 10ml of scintillation fluid (Aquasol-2, Biotech Systems, USA). The radiation emitted from each vial over 60 seconds was enumerated by a β counter (Tri-Carb 1600CA, Packard, Switzerland) in counts per minute (cpm) and recorded. This value corresponds with the number of HIV-1 DNA segments produced by the PCR amplification and is proportional to the number of HIV-1 DNA copies in the original sample.

3.2.7 Quantitation

A standard curve was constructed as previously described (161). Peripheral blood mononuclear cells of a seronegative blood donor were separated by the method described in section 3.4. After cell counting, aliquots were adjusted to 400,000 PBMCs. The human genomic DNA from each aliquot was extracted by the phenol chloroform method (see Appendix B). To each aliquot containing DNA from 400,000 PBMCs a known quantity of HIV-1 DNA, derived from 8E5 or NY-M10 lymphocytes (containing one copy of HIV-1 DNA per cell) was serially diluted such that each reaction tube contained 1000, 333, 111, 37, 12, 4 or 0 (negative control) copies of HIV-1 DNA per 400,000 copies of cellular DNA. Sterile water was added to bring the final volume up to 10 μ l. All dilutions underwent simultaneous PCR amplification using equal quantities of reagents previously prepared as a stock reaction mix. The products were labelled and finally

enumerated by the method described in sections 3.2.5 and 3.2.6. For each dilution of DNA the resultant cpm was plotted on a logarithmic graph. A standard curve was thus produced with the axes being β emission (cpm) verses HIV-1 DNA copies.

3.2.8 Validation

The reproducibility of the standard curve was tested by repeating the assay using the same dilution series and reagents and, later, with a second primer pair (SK 68/SK 69) and probe (SK 70). The assay's sensitivity was investigated by statistical analysis of the background cpm as measured in the negative controls and comparison with the cpm's for the greater dilutions of target DNA. The HIV-1 DNA copy number (per 400,000 cells) at which the assay can distinguish the presence of viral DNA from negative controls was tested for by subjecting the mean cpm from repeated assays of the same dilution to the Student's *t* test. The sensitivity of the assay was determined as the number of DNA copies giving an average cpm significantly different to that of the negative controls.

3.3 COLLECTION OF CLINICAL SPECIMENS

3.3.1 Patient Selection

Patients at all stages of HIV infection were selected from the general patient pool attending the Infectious Diseases Clinic Westmead Hospital. After a brief discussion of the nature of the project and the patient's role in the sample collection, informed consent was obtained. Criteria used to select the patients were based on their ability to provide a minimum volume of saliva and their willingness to co-operate with an oral examination after the sample collection. Where possible patients not currently on anti-retroviral therapy were selected in order to maximise the potential for the detection of viral particles in the saliva (162).

3.3.2 Patient Examination

(i) History

Prior to saliva sampling the patient was briefly interviewed regarding their recent medical and dental history. The information sought included such things as current medications in the form of anti-retrovirals, antibiotics, and other drugs which may be causing a degree of xerostomia. In addition, relevant dental histories regarding recent dental treatment (including recent prophylaxis), current dental problems, and oral hygiene practices were also recorded. Specific questions about oral lesions and awareness of bleeding in the mouth following brushing were also asked.

(ii) Oral Examination

Immediately following saliva collection (see section 3.3.3) an oral examination was carried out. This involved a thorough soft tissue examination, oral hygiene evaluation and the recording of the Gingival Index (GI) on a sextant basis.

In each sextant, the most inflamed site was used to record the GI for that region.

The GI values used for each sextant were according to the criteria of Löe 1967 (164). These were:

- | | | |
|---|---|--|
| 0 | = | Normal gingiva |
| 1 | = | Mild inflammation. Gingival redness or oedema without bleeding on probing |
| 2 | = | Moderate inflammation. Gingivitis which resulted in bleeding on probing |
| 3 | = | Severe inflammation. Marked redness and oedema, ulceration and a tendency towards spontaneous bleeding |

The presence of oral lesions which could contribute to bleeding in the oral cavity was noted. An examination for candida was also conducted although scrapings for yeast were not taken.

3.3.3 Sample Collection

(i) Blood Collection

Blood was collected into two 30 ml heparin containing tubes and a 20 ml EDTA containing tube by axial venepuncture at the same visit as the saliva collection where possible. When this proved logistically impossible, the collection of blood and saliva samples were carried out no more than 7 days apart.

(ii) Saliva Collection

A minimum of 5 ml of unstimulated whole saliva was collected from each patient using a LUKi 20ml disposable aspirating tube (Davis and Geck. USA) attached to an low volume dental aspirator at one end and a vacuum pump (Dynavac I. Selby Anax, Australia) via a sterile 2m suction tube (Inoplas Pty.Ltd. Sydney, Australia) at the other (Figure 3.3). Patients who could not produce sufficient saliva alone were provided with a small piece of paraffin wax on which to chew. The final volume of saliva collected was recorded. Saliva samples were collected prior to oral/periodontal examination to avoid inducing bleeding into the mouth prior to saliva collection.

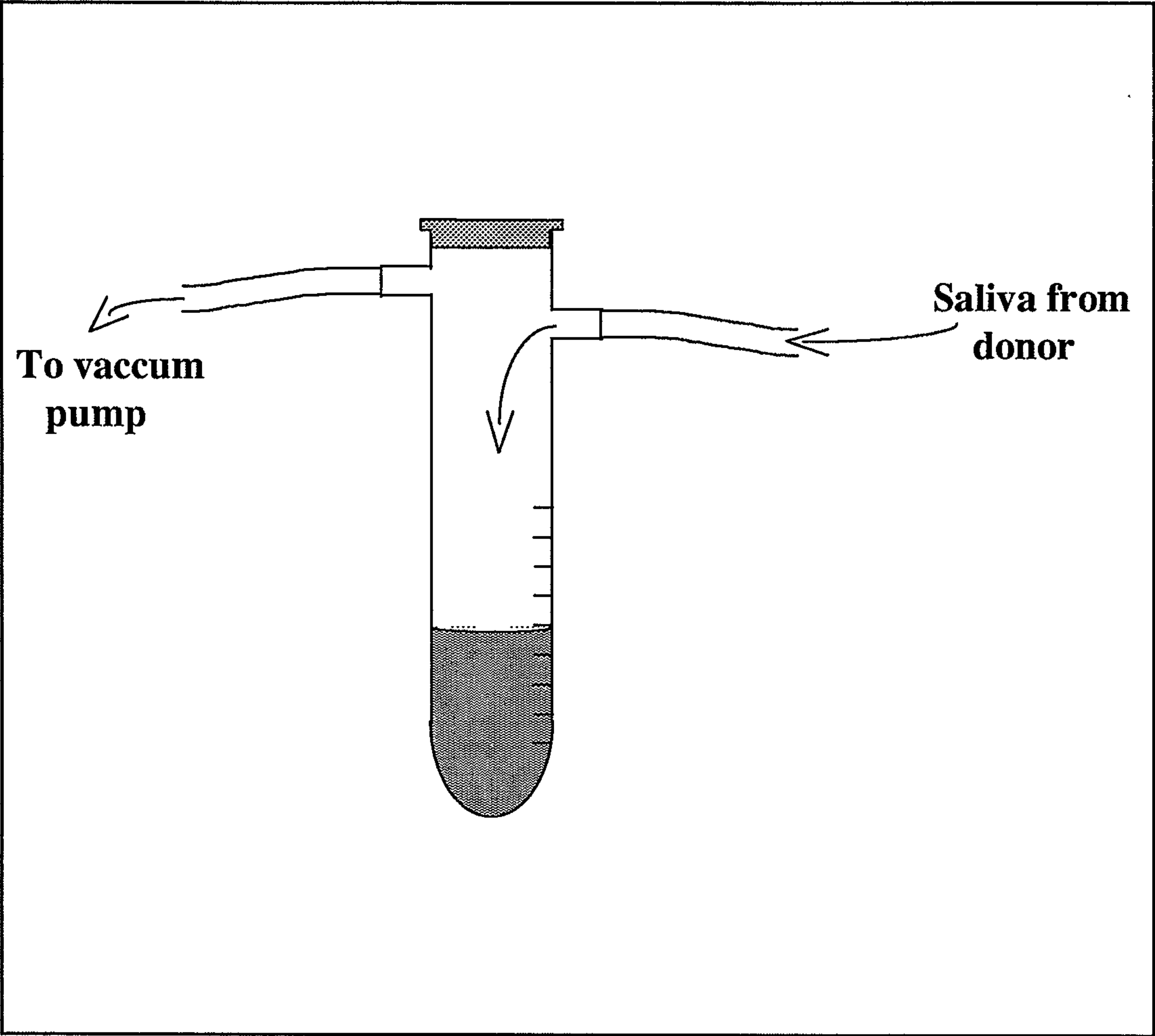


Figure 3.3: LUKi saliva collection tube.

3.4 SAMPLE PROCESSING

3.4.1 Blood Storage

The fresh blood sample was spun at 400 x g for 10 minutes to separate the plasma which was removed, aliquotted and stored in 1ml volumes. The remaining cellular portion was diluted with equal volumes of phosphate buffered saline (PBS) and layered over 15ml of Ficoll-Hypaque (Pharmacia, Switzerland) in 20ml tubes. The tubes were spun at 400 x g for 20 minutes and allowed to decelerate slowly. Peripheral blood mononuclear cells (PBMCs) were harvested with a pipette. The cells were washed twice with PBS, enumerated by cell counting, re-pelleted and stored at -70°C* in 5×10^6 cells/ml aliquots until use.

3.4.2 Saliva Storage

Immediately after collection, the saliva sample was centrifuged at 400 x g for 10 minutes. The supernatant was drawn off and the pellet resuspended in PBS and re-pelleted by centrifugation (400 x g for 10 minutes). A 1ml aliquot of the supernatant was transferred to a cryotube (Nunc. Intermed, USA) and stored. A second aliquot of the supernatant was combined with an equal volume of 5 M guanidium isothiocyanate (GITC) and stored. The remainder of the supernatant was filtered through a 0.8µm filter (Acrodisc. Gelman Sciences, USA) and 1ml stored. The remaining filtered supernatant was added to an equal volume of GITC and stored. The pellet was stored in a similar manner.

* All specimen aliquots were stored at -70°C unless otherwise specified.

3.5 HIV-1 DNA QUANTITATION OF CLINICAL SAMPLES BY PCR ASSAY

3.5.1 Extraction of HIV-1 DNA

One hundred and twenty five microlitres of extraction buffer (see Appendix A) was used to resuspend 5×10^6 PBMCs from each subjects blood specimen and incubated at 56°C for 1 hour. The buffer was inactivated by heating the tubes to 95°C for a further 10 minutes. Extraction of the DNA was then performed according to the protocol described (see Appendix B). A similar protocol was followed for the extraction of DNA from the entire pellet of each saliva sample.

3.5.2 Quantitative PCR

Ten microlitres of DNA solution from the blood or saliva of each subject was combined in a 150µl Eppendorf tube with 40µl of PCR reaction mix as described in section 3.2.2. For each batch of specimens assayed, a concurrent external standard of serially diluted NY-M10 cellular DNA was processed using the same stock reaction mix. Thermocycling was carried out on clinical samples and the standard solutions simultaneously. The products of the PCR assay were labelled according to the method described in section 3.2.5. The β emissions from the labelled product of each tube was plotted against the standard curve derived from the simultaneous amplification of the known standards (as described in section 3.2.7). Hence, an approximation of the HIV-1 DNA copy number per 10µl of cellular DNA could be estimated.

3.6 ASSAY CONTROLS

3.6.1 Overall Saliva Controls

Saliva taken from 4 HIV-1 negative individuals in low risk groups and matched for age and oral hygiene was processed blindly along with those of the test specimens.

3.6.2 DNA Amplification

Positive controls derived from clinical samples which had previously shown to be strongly positive to the PCR assay were included in each PCR run as part of the standard laboratory quality control practice. Negative controls, usually water, were also used for each assay run.

3.6.3 Labelling and Quantitation

Positive controls, in the form of positive post PCR product was used during each batch of labelling. Water and the negative controls used in the PCR step were used as negative controls.

3.7 VIRUS ISOLATION

The procedures used to isolate and identify HIV-1 have been adapted from the U.S. Department of Health and Human Services procedural guide (165) by the virology laboratory at The Institute of Clinical Pathology and Clinical Research at Westmead Hospital.

3.7.1 Cells

Twenty millilitres of whole blood from HIV-1 positive patients was collected into a tube containing 5% w/v EDTA. PBMCs were harvested using Ficoll-Hypaque centrifugation (800 x g for 20 min.) and resuspended in culture medium (see Appendix A) to a final concentration of 2×10^6 PBMC/ml. These were co-cultured with an equal concentration of PHA stimulated healthy (HIV-1 negative) donor PBMCs, at 37°C in a 5% CO₂ atmosphere for 28 days. On the fourth, eleventh and eighteenth days half of the cell culture volume was drawn off and spun at 400 x g. The supernatant was removed and the cells replaced with fresh culture media. On days 7, 14, and 21 half the culture volume was removed and replaced with the same volume of fresh media containing 5×10^5 PHA stimulated donor PBMCs. The cultures were monitored for cytopathic effect (CPE) for up to 21 days. Culture supernatants were assayed for reverse transcriptase (RT) activity (166) and the presence of extracellular HIV p24 antigen using a commercial ELISA assay (Abbott, USA) (111). The criteria for a positive HIV isolation was two consecutive positive RT or HIV-Ag assays, and the converse being positive at least once.

3.7.2 Saliva

One millilitre of filtered saliva (0.8µm-Acrodisc) was inoculated into healthy PBMC culture as in section 3.7.1.

3.8 QUANTITATIVE CULTURE

Standard PBMC cell co-culture techniques with serial dilutions of test specimens were used to assay the quantity of HIV-1 virus present in the blood PBMCs and saliva from HIV-1 infected subjects.

3.8.1 Cells

Serial dilutions (1:2, 1:10, 1:50, 1:100, 1:1000) of patient's PBMCs, starting with 1×10^6 PBMC/ml, were made in sterile 10 ml centrifuge tubes containing growth media. Half a millilitre of each dilution was added to consecutive wells of a 24-well microculture plate (Nunc. Intermed, USA) containing 0.5 ml of PHA stimulated normal PBMC (1×10^6 cells/ml). The assays were cultured in a 5% CO₂ incubator at 37°C. On days 7, 14, and 21 one half of the culture volume from each well (0.5ml) was removed and replaced with fresh culture media containing 5×10^5 PHA stimulated normal PBMCs. The collected media was spun at $800 \times g$ for 7 min. and the supernatant assayed for HIV p24 antigen. Quantitation was calculated as the greatest dilution at which HIV p24 was still detected.

3.8.2 Saliva

Two millilitres of filtered (0.8µm-Acrodisc) saliva supernatant was used to suspend 1×10^7 PHA stimulated PBMC's for two hours before addition of medium. Similar dilutions were used for saliva as for blood cells. Fresh media and PBMCs were added at days 7 and 14. Assessment of results was as described above.

3.9 STATISTICAL ANALYSIS

To test the hypothesis that the cpm value derived from PCR amplification of known concentrations of target DNA is in fact a function of the original DNA concentration, a Pearson's correlation coefficient (Pearson's r) can be calculated. Owing to the low number of dilution factors ($n=7$), the test for the null hypothesis (that there is no statistically significant correlation between the two parameters, i.e. $r=0$) required the use of the t test as follows:

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$$

In addition, the coefficient of determination (r^2) will indicate what percentage of change in one value can be predicted by a change in the other.

To test the hypothesis that the degree of gingival inflammation was reflected in the concentration of HIV-1 DNA in saliva, gingival index scores were used to group patients into those not exhibiting any gingival bleeding (GI of 0 or 1; Group A) and those which showed one or more sites with $GI > 1$ but < 3 (Group B) and those with at least one site with $GI = 3$ (Group C). Chi squared tests were conducted to compare the number of subjects in the three groups with the number who exhibited salivary HIV-1 DNA counts above or below the group average. i.e.:

	Group A	Group B	Group C
Above Average			
Below Average			

CHAPTER FOUR

RESULTS

4.1 VALIDATION OF PCR ASSAY

4.1.1 Reference Samples

Using SK 38/SK 39 primers, known quantities of HIV-1 DNA, derived from 8E5 cell culture, were assayed by PCR. Banding on ethidium bromide stained PAGE gels was obtained in the expected region. The procedure was repeated with a P³² labelled probe (SK 19) annealed to the PCR product using the method described in section 3.2.5. Banding observed on the gels in the 110 bp region was recorded by autoradiography, see Figure 4.1. The HIV-1 DNA derived from the 8E5 cells was retained as a reference sample and used for all further PCR assay runs in the development and optimisation of the PCR assay.

4.1.2 Optimisation of Assay Conditions

(i) Magnesium Ion Concentration of PCR Buffer

For the primer pair SK 38/SK 39 a magnesium ion concentration (in the 10 x PCR buffer) of 2.5 mM was found to produce the strongest banding on ethidium bromide stained PAGE gels for a standardised concentration of target HIV-1 DNA. In contrast, ethidium bromide stained banding on PAGE gels using 2 mM and 2.5 mM magnesium ion concentration showed equal strengths when tested the SK 68/SK 69 primer pair. For simplicity and uniformity it was decided that a stock buffer containing a magnesium ion concentration of 2.5 mM would be used for both primer pairs. These findings were recorded and the results applied to all further assays using either primer pairs.

(ii) Annealing Temperatures

The optimal annealing temperatures for the SK 38/SK 39 primers were determined to be in the range of 50 °C to 60 °C. Hence, 55 °C was chosen as the standard for this primer pair. Similarly, the optimal temperature for the SK 68/SK 69 primers was both 45 °C and 50 °C. The annealing temperature subsequently chosen for this primer pair was 50 °C as it was consistent with greater specificity of the assay and represented a considerable time saving over 45 °C for PCR cycling.

(iii) Cycle Number

The results of repeated assaying of the same reference sample using various PCR cycle numbers showed that 25 cycles of the PCR produced a standard curve (see section 4.1.4) at which comparatively large differences in cpm were evident between the various dilutions of HIV-1 DNA.

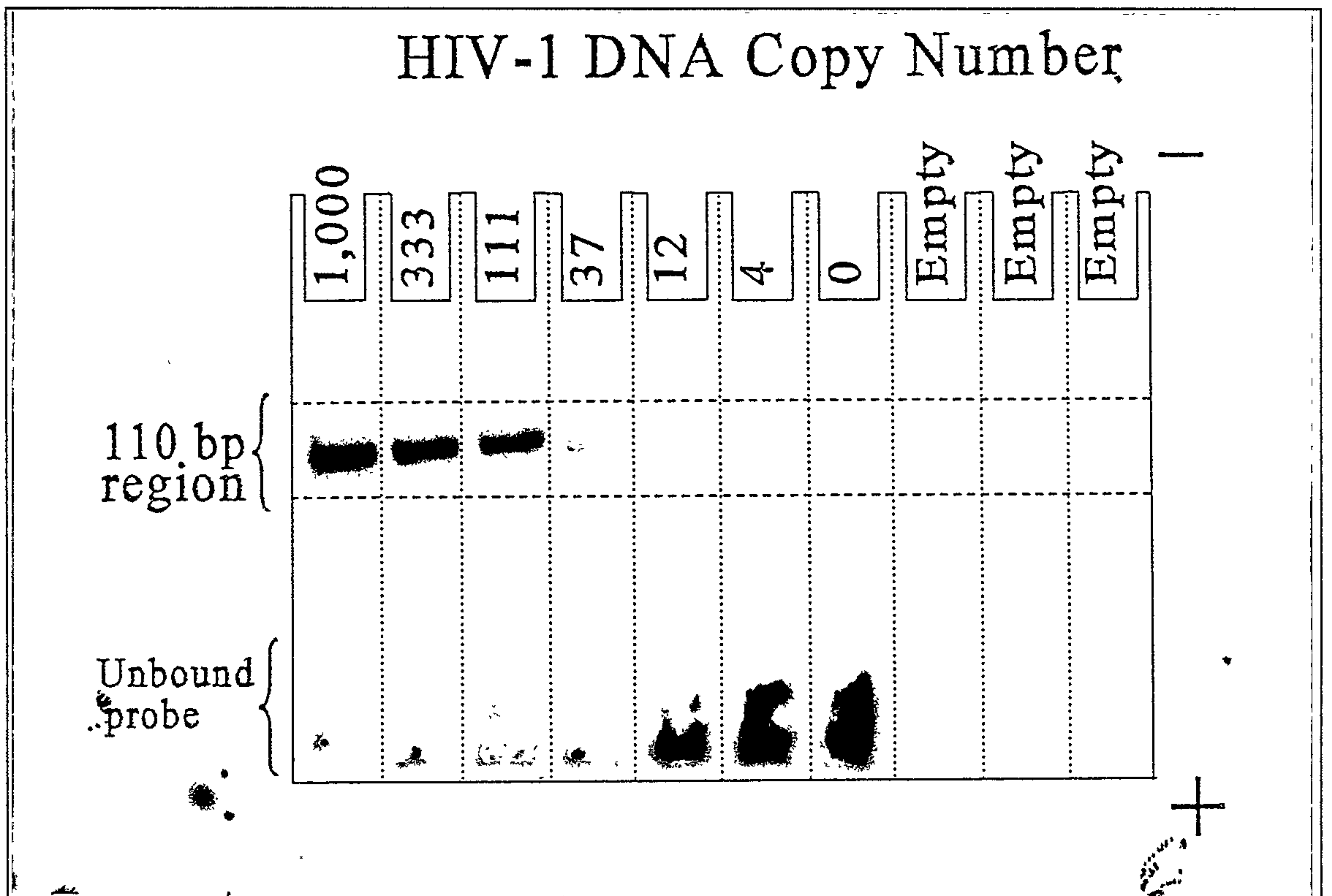


Figure 4.1: Autoradiograph showing banding in the 110 bp region as a result of P^{32} labelling of a post-PCR region of the HIV-1 genome. Band intensity is relative to the concentration of target DNA.

4.1.3 Positive Controls

The assay was applied to a 10µl aliquot of DNA derived from co-culture cells (PBMCs) previously demonstrating CPE. Distinct banding was seen on the PAGE gel using ethidium bromide staining. This DNA batch, derived from a wild type strain of HIV-1, was subsequently retained and used in later PCR assays as a positive control.

4.1.4 Standard Curve

When the HIV-1 DNA copy number was plotted against the cpm values (derived from scintillation counting of the labelled PCR products) over a range of dilutions (Table 4.1), a standard curve was generated. A typical standard curve derived from a serial dilution of 8E5 cellular DNA and amplified using SK 38/SK 39 primers and the SK 19 probe under optimal PCR conditions is shown (Figure 4.2). This standard curve assay run was referred to as Run #1.

Table 4.1: Scintillation Counts derived from step-wise serial dilutions of known concentrations of HIV-1 DNA .

Copies of HIV-1 DNA (Dilution Factor)	1000	333	111	37	12	4	0
Scintillation Count (cpm)	785	721	441	168	150	89	65

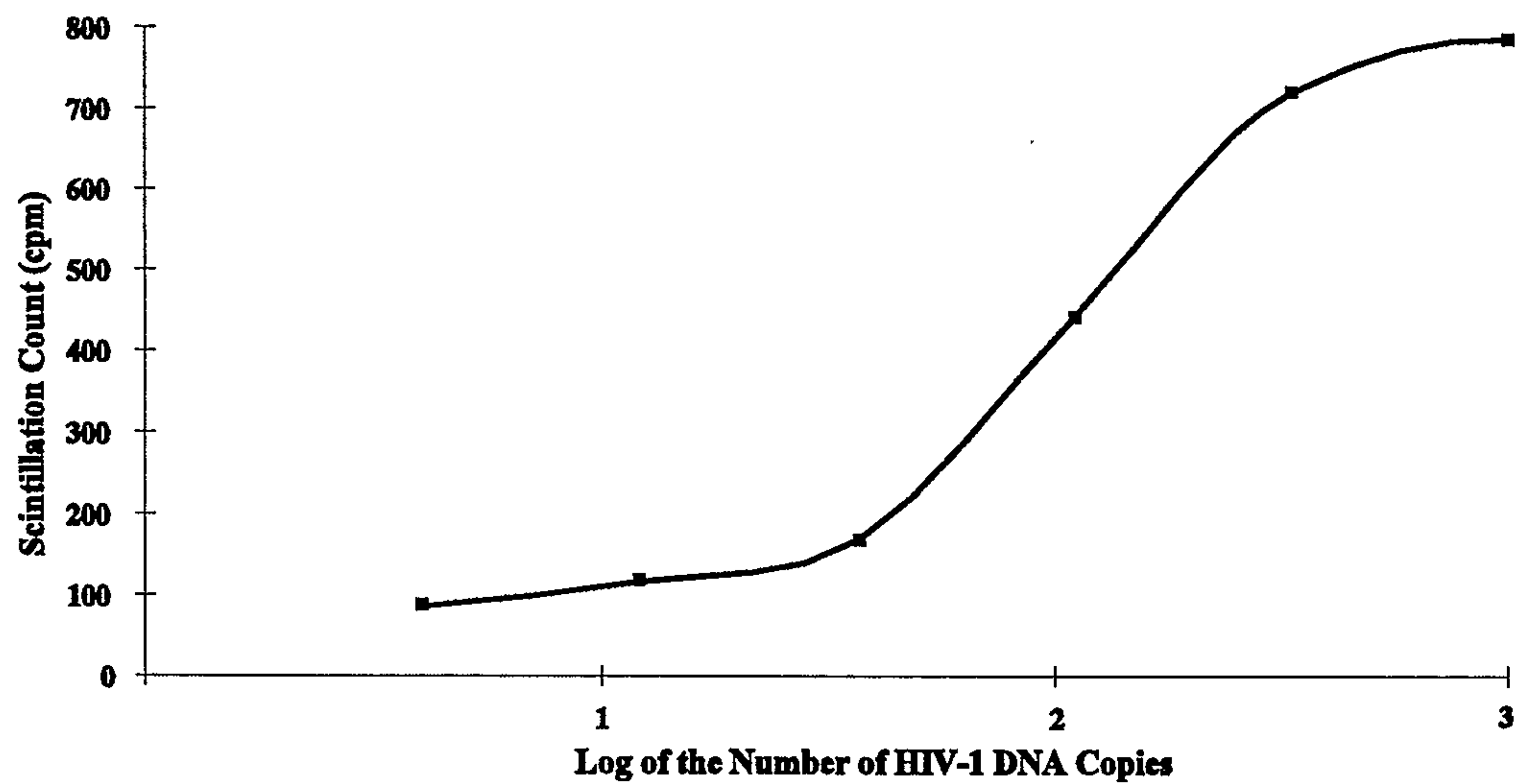


Figure 4.2: Standard curve derived from Run #1. Each data point represents the cpm readings following PCR amplification and P^{32} labelling of a known number of HIV-1 DNA copies. The linear region of the curve is best suited for reading off the number of HIV-1 DNA copies in a given sample.

4.1.5 Reproducibility of Standard Curves

The assay procedure was repeated on two further occasions using the same standard dilutions. The first repetition (Run #2) used the same reagents while Run #3 used the alternative set of primer pairs (SK 68/SK 69) and probe (SK 70). Linear regression was used to compare paired scintillation counts for each dilution across the three test runs (Figure 4.3). Regression statistics were computed for each combination of paired assay results. A high correlation was found between each of the regression lines (Table 4.2). The significance of these correlations was confirmed by regression analysis.

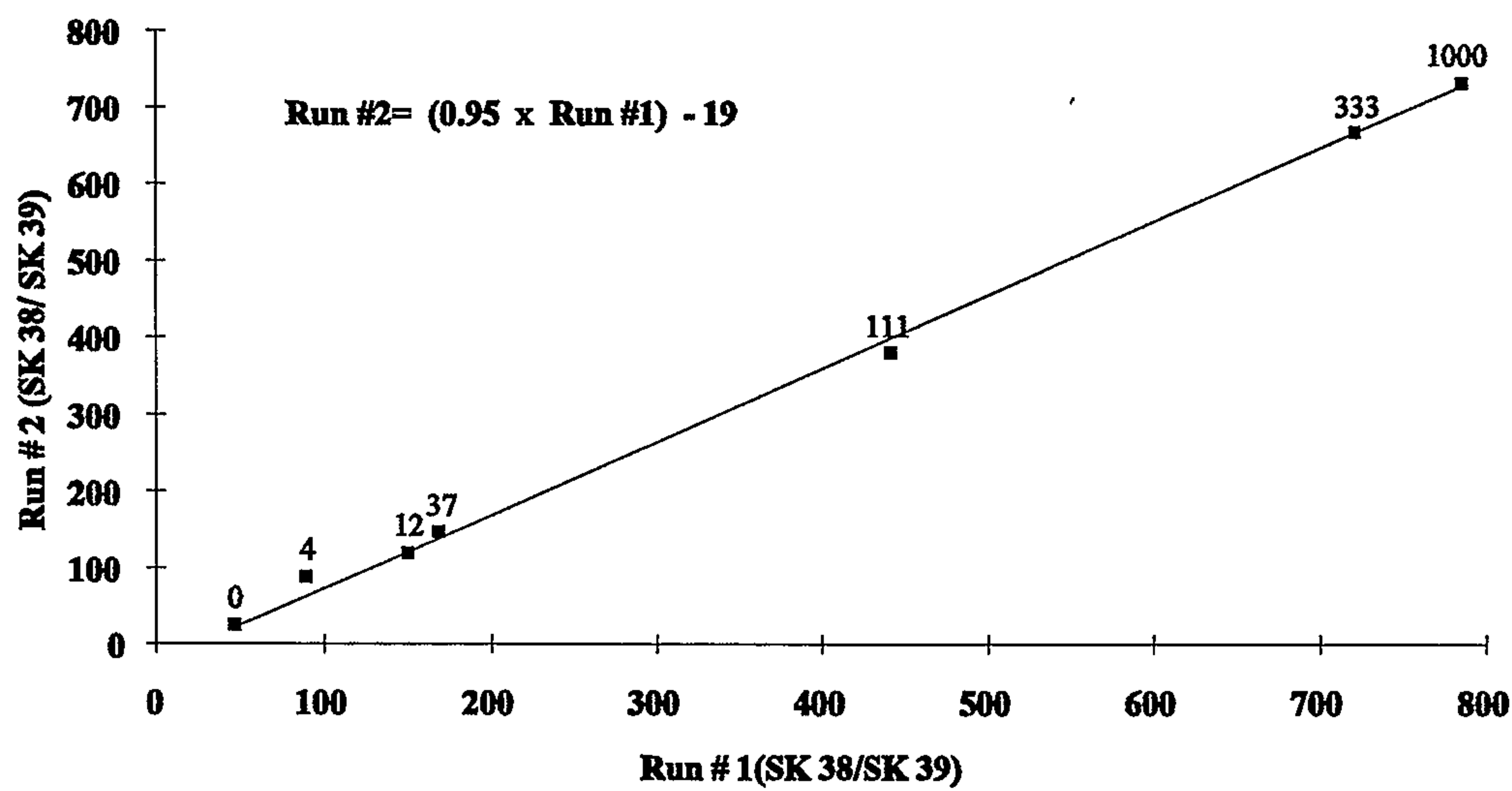


Figure 4.3: An example of the correlation between two runs of the PCR assay (Run #1 and Run #2) on the same dilution series of 8E5 DNA. The numbers above each data point is the number of HIV-1 DNA copies.

Table 4.2: Comparison of Standard Curves between Assay Runs.

Test Runs	#1 and #2	#1 and #3	#2 and #3
Correlation Coefficient	0.999	0.911	0.912
(± S.E.) [§]	(±0.007)	(±0.050)	(±0.700)
r ²	0.99*	0.83**	0.83**

[§] Standard Error of the Correlation
* Statistically significant result at p≤0.001
** Statistically significant result at p≤0.01

4.1.6 Detection Limit

The average scintillation counts for three repeated PCR amplifications of 4 copies of HIV-1 DNA per 400,000 PBMCs were shown to be significantly different to those derived from negative controls (Table 4.3). Standard errors for each was calculated and the differences in mean cpm were tested for using the Student *t* - test. From this analysis the probability of being able to distinguish at least 4 copies of HIV-1 DNA from the background using the present assay was 99.5%.

Table 4.3: Student *t* - Test for Assay Detection Limit.

	4 copies of HIV-1 DNA	0 copies of HIV-1 DNA
Mean cpm*	84.3	58.6
S. D.‡	13.6	18.3
S.E.§	±7.86	±10.6
t - value	9.41	
p - value	0.005	

* Counts Per Minute
‡ Standard Deviation
§ Standard Error

4.2 CLINICAL SPECIMENS

Quantitative HIV-1 DNA PCRs using primer pairs SK 38/SK 39 and SK 68/SK 69 at previously determined optimum conditions were used to assay clinical specimens. The optimisation of the assay conditions is described in section 4.1.

4.2.1 PCR on Blood Specimens

Seventeen blood samples from HIV seropositive subjects were assayed by PCR according to the methodology described in section 3.3 and optimised as in section 4.1.2. The autoradiographs from these samples are shown in Figure 4.4. The standard curves created for each assay run is presented in Appendix C. Each assay was repeated using the alternative set of primers and probe. Table 4.4 shows a comparison of the number of HIV-1 DNA copies (as determined by two PCR assays using different primer pairs) in standard aliquots of 400,000 PBMCs from each subject's blood specimen. For the two different primer pairs used, a highly significant correlation was found between the HIV-1 DNA copy number across the seventeen specimens (Table 4.5 (a)). When the data was re-analysed excluding the three specimens exhibiting cpms in the plateau region of the standard curves (shaded grey in Table 4.4) the correlation between the two primer sets was even higher (Table 4.5 (b)).

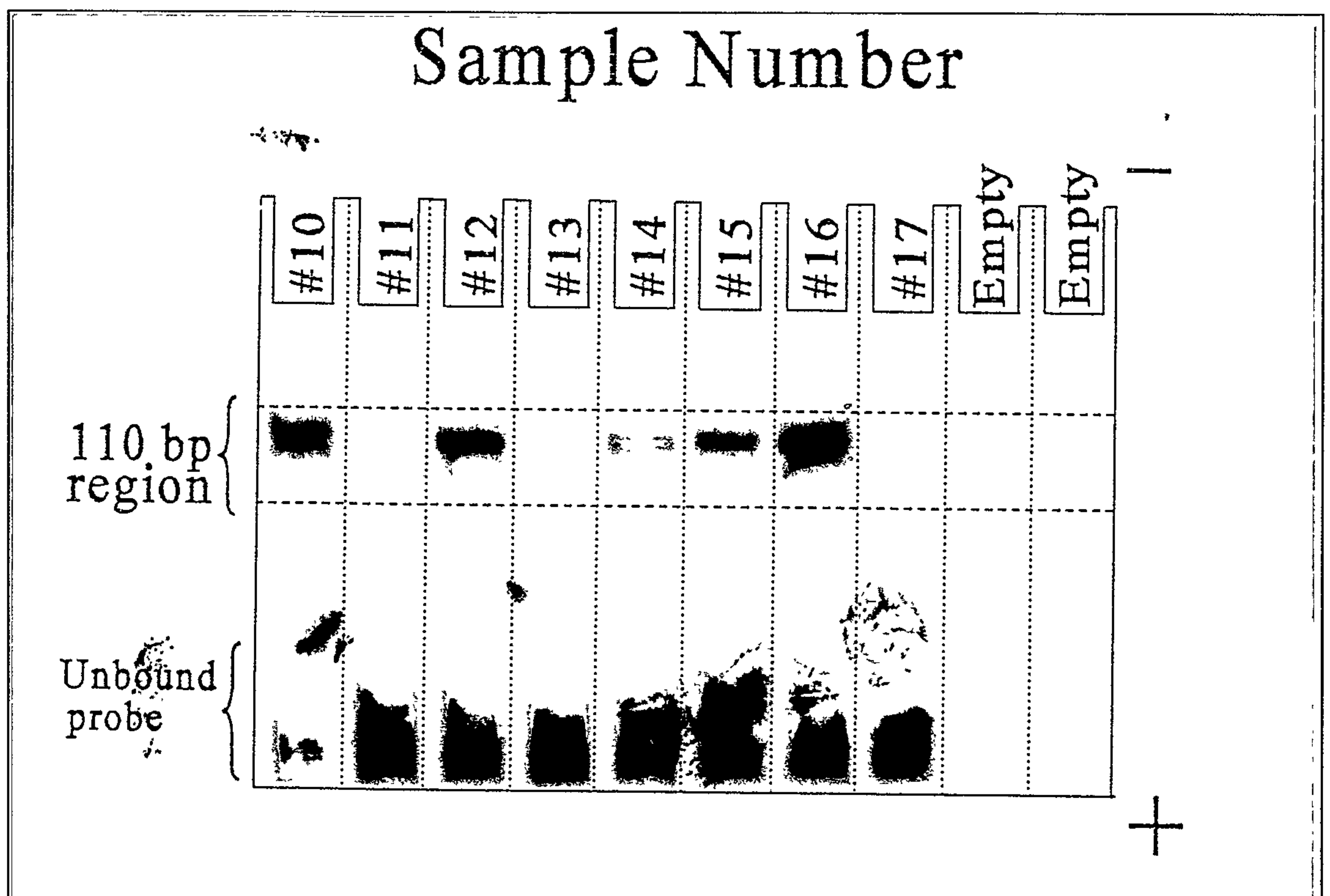
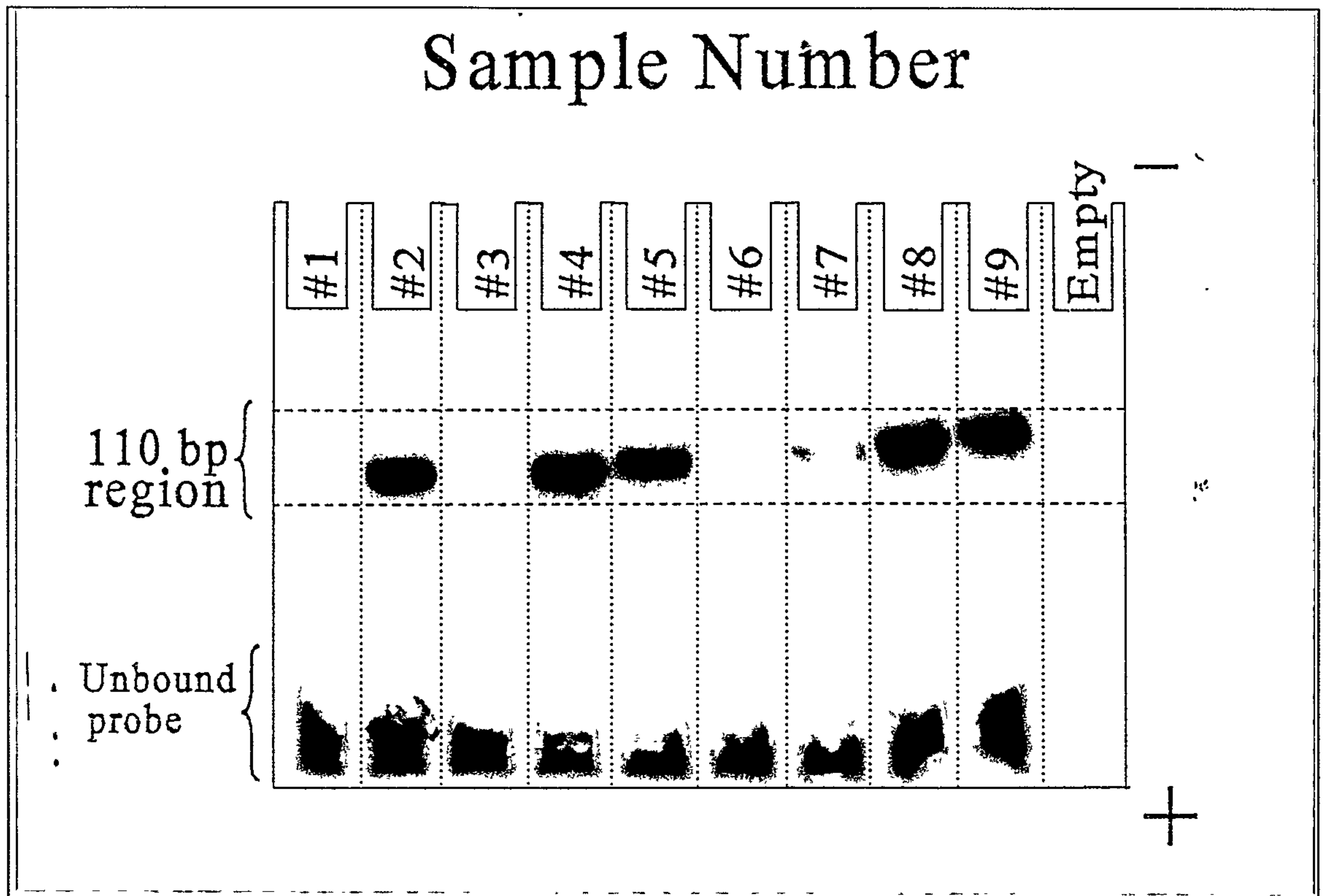


Figure 4.4 (a) & (b): Autoradiographs from 17 clinical blood specimens. Each specimen was assayed by HIV PCR and labelled with the P^{32} probe.

Table 4.4: HIV-1 DNA Quantitation of 17 Blood Specimens Using Two Primer and Probe Sets. Shaded cells represent three samples with very high DNA copy number.

Subject Identification (Initials/Sex/Age)	Copies of HIV-1 DNA in a 400,000 cell sample using SK 38/SK 39	Copies of HIV-1 DNA in a 400,000 cell sample using SK 68/SK 69
TA/M/49	1.1	1.1
BV/M/29	25	1.01
RM/M/42	1	0.9
CD/M/42	28	12
BV/M/29	40	32
LB/F/28	1.4	2.2
GE/M/31	4	0.5
CS/F/38	2300	1080
DB/M/45	3000	1500
GE/M/31	1	2
MM/F/80	3000	800
IL/M/35	105	90
PM/M/34	60	27
NN/M/30	12	21
BL/M/55	570	402
CD/M/42	5.8	3
WP/M/42	320	208

Table 4.5 (a): Correlation for HIV-1 DNA Copy Number Between Two Primer Pairs (17 specimens).

Statistic	Value
Correlation Coefficient (r)	0.95
r^2	0.90
S.E. [§]	± 0.19
t	11.99
p	<<0.001

§ Standard Error of the Correlation

Table 4.5 (b): Correlation for HIV-1 DNA Copy Number Between Two Primer Pairs (excluding 3 specimens with very high copy numbers).

Statistic	Value
Correlation Coefficient (r)	0.99
r^2	0.99
S.E. [§]	± 0.03
t	40.25
p	<<0.001

§ Standard Error of the Correlation.

4.2.2 Comparison with Co-Culture Technique

Ten blood samples from HIV-1 seropositive donors were used to compare the sensitivities of the PCR assay and conventional virus isolation on clinical samples. The presence and number of viral DNA copies was detected in nine out of the ten samples using the PCR assay. By comparison cell co-culture detected the presence of the HIV in seven specimens. When the HIV-1 DNA copy numbers, as determined by the PCR, were plotted for each sample it was noted that the co-culture technique failed to detect the virus in the samples exhibiting the lowest copy number by PCR (Figure 4.5).

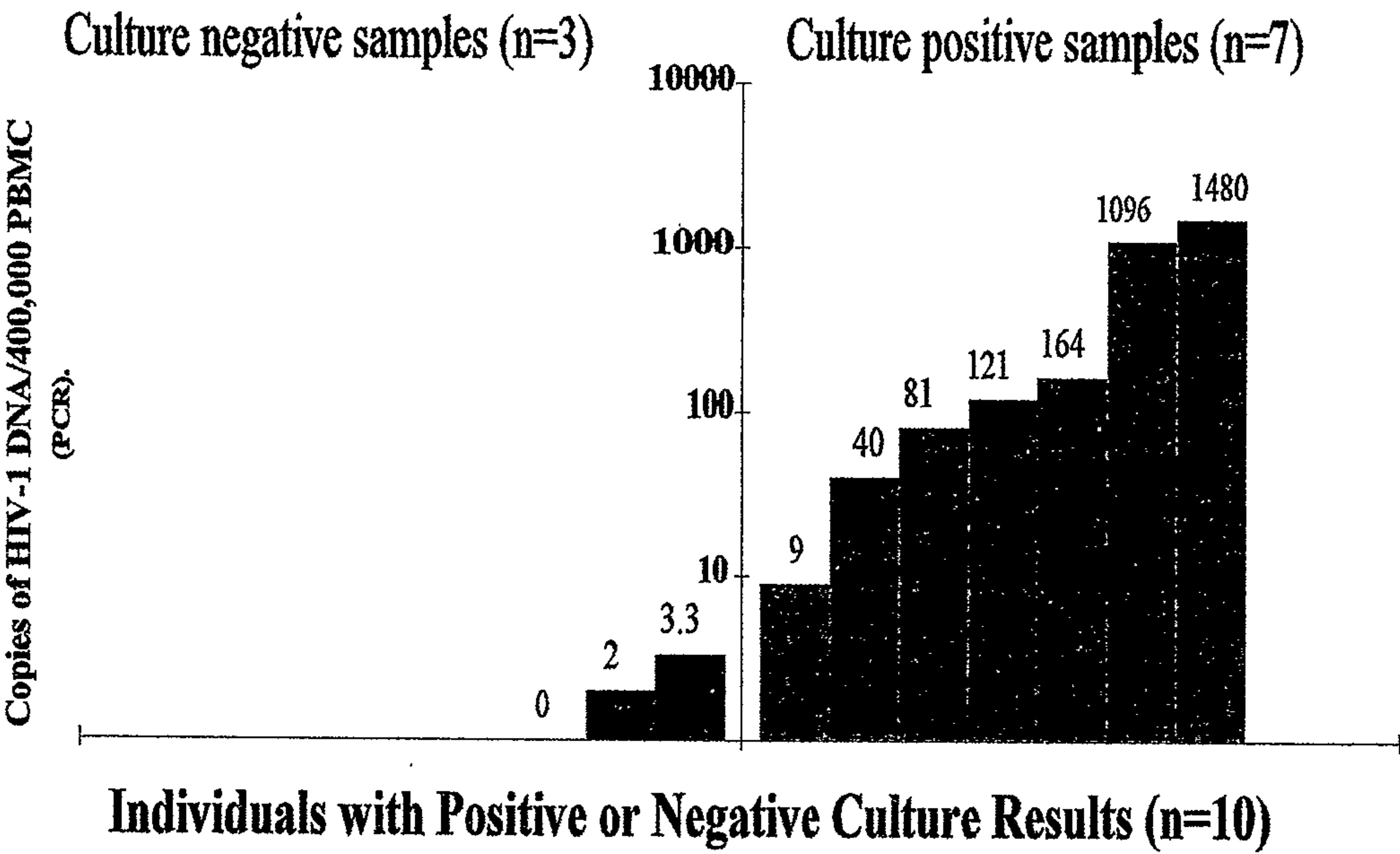


Figure 4.5: Comparative sensitivity of cell co-culture and PCR. Ten specimens were cultured and presence of HIV, as determined by reverse transcriptase assay, was detected in 7 cases. The blue and red bars represent blood specimens returning a negative and positive result for HIV isolation respectively. The numbers at the top of each bar represent the HIV-1 DNA copy number estimated by the PCR assay.

The average number of HIV-1 DNA copies per 400,000 PBMCs was determined for samples testing positive and negative by cell co-culture. A statistically significant difference was noted at the 95% level of confidence. This relationship is shown (Figure 4.6).

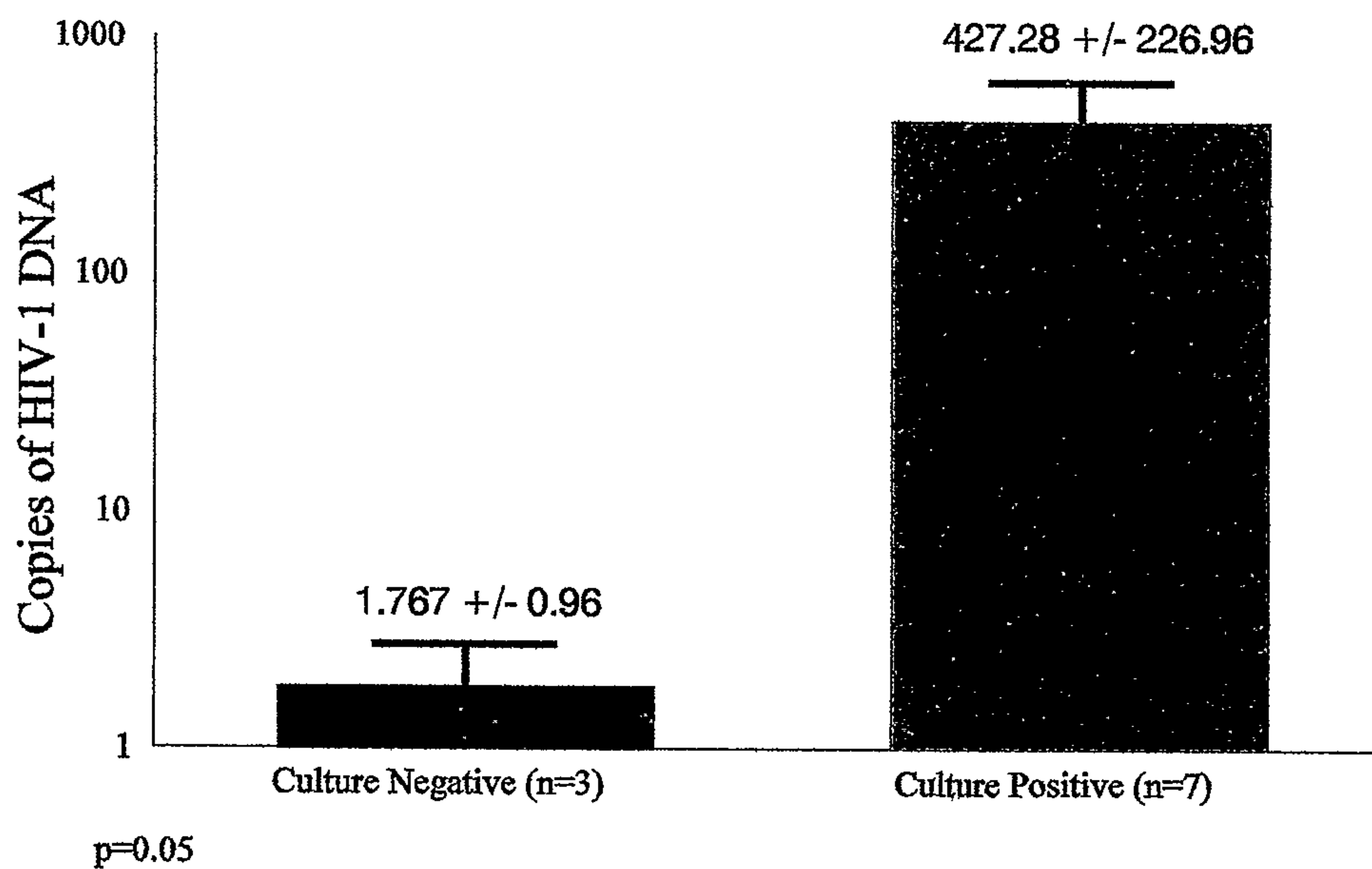


Figure 4.6: Average number of HIV-1 DNA copies per 400,000 PBMCs for co-culture negative vs co-culture positive blood samples taken from HIV seropositive subjects.

Four clinical samples, chosen at random, were used to compare the result of quantitative co-culturing (See section 3.8) and quantitative PCR (Figure 4.7). Linear regression was used to determine the correlation between the two methods of quantitation. Despite the low number of samples assessed, a strong linear relationship was found (Table 4.6). The null hypothesis, that there is no relationship between the two methods, was tested. Computation of the t distribution returned a t -value of 49.08 which allowed the rejection of the null hypothesis with a p -value of <0.001 .

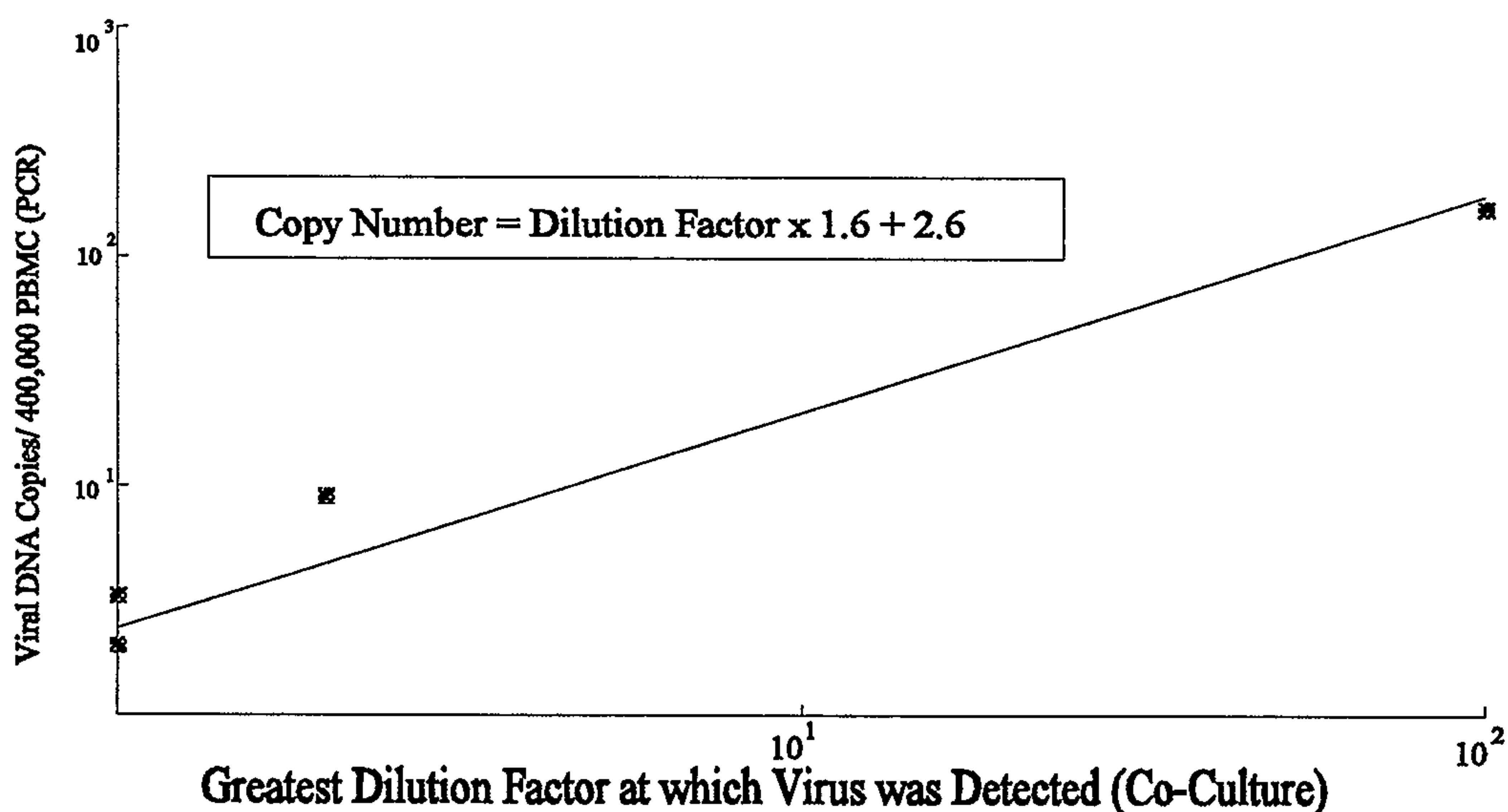


Figure 4.7: Relationship between quantitative DNA PCR and quantitative viral co-culture. The values along the abscissa are derived from the greatest dilution of the subject's PBMCs in donor cells at which the presence of HIV was detected. The solid line represents the regression line, its formula given in the box.

Table 4.6: Regression Analysis for the Correlation between Two Methods of Assessing Viral Load (Co-culture and PCR).

Statistic	Value
Correlation Coefficient (r)	0.999
r ²	0.999
S.E. [§]	± 0.032
t - value	49.08
p - value	<0.001

§ Standard Error of the Correlation.

4.2.3 PCR on Saliva Specimens

Quantitative PCR, using the SK 38/SK 39 primer pair, was carried out on 31 saliva specimens from 28 HIV-1 seropositive donors at all stages of HIV disease as well as 4 specimens from HIV-1 seronegative volunteers with no known risk factors for HIV. None of the specimens assayed returned values significantly greater than that of the negative controls. Tables 4.7 (a), (b), and (c) depict the distribution of the sampled subjects according to their greatest GI score.

Of the 31 saliva samples derived from seropositive donors, 13 specimens were also assessed for the presence of virus by cell co-culture. All cultures consistently tested negative by reverse transcriptase assay over the 21 day period.

Table 4.7 (a): [Group A] Data for laboratory results on 10 saliva specimens from HIV-1 seropositive subjects with no bleeding on probing (GI = 0 or 1 only).

No.	GI ^β	QUANTITATIVE PCR		CULTURE ^α		AR ^ε	HIV STATUS
		Saliva ^γ	Blood ^δ	Saliva	Blood		
1	100110	0		Neg	Pos	N	Stage II
2	101101	0	1			N	Stage II
3	111101	0	2300		Pos	N	Stage II
4	000010	0			Neg	N	Stage II
5	000000	0	3000			Y (AZT)	Stage IV
6	000010	0	105		Pos	N	Stage IV
7	001000	0	4			N	Stage IV
8	001000	0				N	Stage IV
9	001001	0		Neg	Pos	N	Stage IV
10	111111	0	40			N	Stage IV

^α Presence or absence of HIV-1 in blood or saliva using co-culture technique.

^β Gingival index. Each digit represents the highest value for that sextant.

^γ Number of HIV-1 DNA copies found in a 10µl aliquot of extracted DNA from the cell pellet of a 5ml saliva specimen using PCR with SK 38/SK 39 primer pairs.

^δ Number of HIV-1 DNA copies found in 400,000 PBMCs taken from sampled subject using PCR with SK 38/ SK 39 primer pairs.

^ε Antiretroviral therapy. When antiretrovirals were being taken at the time of sampling ('Y') the type of medication is included in brackets.

Table 4.7 (b): [Group B] Data for laboratory results on 16 saliva specimens from HIV-1 seropositive subjects with slight bleeding on probing at one or more sites (GI = 2 but not GI = 3).

No.	GI ^β	QUANTITATIVE PCR		CULTURE ^α		AR ^ε	HIV STATUS
		Saliva ^γ	Blood ^δ	Saliva	Blood		
1	111121	0	1.4	Neg	Neg	N	Stage I
2	111122	0	25	Neg		N	Stage I
3	111120	0		Neg	Pos	N	Stage II
4	121122	0	5.8			N	Stage II
5	211222	0	570		Pos	N	Stage II
6	222122	0		Neg	Pos	Y (AZT)	Stage II
7	222222	0	3000		Pos	N	Stage II
8	000210	0		Neg	Pos	N	Stage II
9	112121	0	1.1			Y (AZT)	Stage III
10	011012	0	12	Neg	Pos	Y (DDC)	Stage IV
11	111112	0	4	Neg	Pos	N	Stage IV
12	111121	0			Pos	Y (DDI)	Stage IV
13	112121	0			Pos	N	Stage IV
14	121122	0	60		Pos	N	Stage IV
15	122221	0			Pos	N	Stage IV
16	221221	0			Pos	N	Stage IV

^α Presence or absence of HIV-1 in blood or saliva using co-culture technique.

^β Gingival index. Each digit represents the highest value for that sextant.

^γ Number of HIV-1 DNA copies found in a 10µl aliquot of extracted DNA from the cell pellet of a 5ml saliva specimen using PCR with SK 38/SK 39 primer pairs.

^δ Number of HIV-1 DNA copies found in 400,000 PBMCs taken from sampled subject using PCR with SK 38/ SK 39 primer pairs.

^ε Antiretroviral therapy. When antiretrovirals were being taken at the time of sampling ('Y') the type of medication is included in brackets.

Table 4.7 (c): [Group C] Data for laboratory results on 5 samples from HIV-1 seropositive subjects with marked or spontaneous bleeding on probing in at least one region (GI = 3).

No.	GI ^β	QUANTITATIVE PCR		CULTURE ^α		AR ^ε	HIV STATUS
		Saliva ^γ	Blood ^δ	Saliva	Blood		
1	333333	0	1	Neg	Neg	N	Stage I
2	112321	0		Neg	Pos	N	Stage III
3	312212	0		Neg	Pos	N	Stage III
4	213212	0		Neg	Neg	N	Stage III
5	202232	0*	320		Pos	N	Stage IV

^α Presence or absence of HIV-1 in blood or saliva using co-culture technique.

^β Gingival index. Each digit represents the highest value for that sextant.

^γ Number of HIV-1 DNA copies found in a 10µl aliquot of extracted DNA from the cell pellet of a 5ml saliva specimen using PCR with SK 38/SK 39 primer pairs.

^δ Number of HIV-1 DNA copies found in 400,000 PBMCs taken from sampled subject using PCR with SK 38/ SK 39 primer pairs.

^ε Antiretroviral therapy. When antiretrovirals were being taken at the time of sampling ('Y') the type of medication is included in brackets.

* Sample containing visible blood in the saliva specimen at the time of sampling.

CHAPTER FIVE

DISCUSSION

A PCR assay was established for the detection of two regions of the HIV-1 genome from reference samples in accordance with previously published data (141, 144, 147). Due to the high technique sensitivity of the PCR method, optimisation of the assay conditions prior to its application to clinical samples has been suggested (138, 144). In the present study the use of an optimal magnesium ion concentration of 2.5 mM is in agreement with Abbott *et al* (1988) (167) and Ferre *et al* (1992) (168) but far lower than others using the same primers (141, 162). Similarly, the use of 25 amplification cycles in this study was fewer than that reported in previous papers (145, 161, 162, 167, 168). No data is provided by these studies to indicate the criteria used to determine the number of cycles to be performed. The optimal annealing temperature found in this study was within 5°C of previously reported studies using these primer pairs (162, 167, 170, 171).

The reproducibility of the standard curves used for clinical samples has been previously reported (168). Although not evaluated statistically, the authors present

their results graphically and report "... when using the same preparation of standard DNA, the slopes of the linear standard curves of four separate and consecutive experiments, run on different days, were equivalent." In the present study, this relationship was expressed numerically through the determination of the coefficient of correlation between each of three assay runs (see Table 4.2). The high correlation found between each run supports the observations reported previously.

The detection limit of the HIV-1 DNA PCR assay using SK 38/SK 39 or SK 68/SK 69 primer pairs was examined in two previous studies (161,168). Oka *et al* (1991) reported a significantly greater mean cpm from samples containing 3 or more HIV-1 DNA copies per 100,000 copies of genomic DNA compared to that of negative controls (161). Similarly, Ferre *et al* (1992) determined that 10 copies of HIV-1 DNA marked the lower limit for quantitation per 400,000 copies of genomic DNA (168). These findings are in agreement with the present study which found that four or more HIV-1 DNA copies produced an average cpm significantly greater than the background ($p=0.005$). Further dilution of the reference sample beyond 4 copies per 400,000 was not performed due to the potential errors inherent in the stochastic partition of low copy numbers.

The present assay detected HIV-1 DNA in blood but not saliva samples from seropositive subjects across concentrations ranging between 1 and 3000 copies per

400,000 PBMCs. Similar results for blood specimens have been previously published using different PCR assay conditions (141,145,147). Specimens containing high copy numbers showed greater variation between assay runs. This was presumably due to the plateauing of the standard curves at high concentrations and hence a loss of accuracy when reading the copy numbers off the standard curve. Greater accuracy across a wide range of concentrations can be achieved by serially diluting each clinical sample and determining the HIV-1 DNA copy number from the dilution which gives a cpm in the linear part of the standard curve. Using the SK 38/SK 39 primers we found 76% of blood samples from seropositive subjects showed HIV-1 DNA copy numbers significantly above the background. In contrast, only 59% of these samples had significant results using the SK 68/SK 69 primers. Ferre *et al* (1992) have suggested that such variation may be due to the SK 38/SK 39 primers coding for a highly conserved region of the HIV-1 genome and are therefore less likely to containing low affinity sites for the primers or probe (146, 147, 168). Whether this reflects genetic variation among isolates or merely a technical difference remains unclear. However, this finding underlies the need to use a second primer pair to minimise false negative results.

The correlation between virus isolation using co-culture and high HIV-1 DNA copy numbers, determined by the PCR assay, is in agreement with previous studies (141, 168,

169). Using the same primer pairs as those of the present study, Ou *et al* (1988) were able to detect HIV-1 DNA in 11/11 (100%) culture positive samples and 6/11 (55%) culture negative samples (141). This correlates closely with the 100% and 66% respectively for the present study. The present study also confirmed the value of the PCR at detecting the presence of HIV-1 at concentrations below the detection limit of co-culture.

Comparison of quantitative PCR and co-culture methods was recently reported by Escaich *et al* (1992) and Bieniasz *et al* (1993) (169, 170). Both groups found a highly significant correlation ($p < 0.01$ and $p = 0.0006$ respectively) between the quantitative methods when applied to blood specimens from HIV-1 seropositive subjects. A significant correlation was likewise found from the four samples assayed by quantitative co-culture and quantitative PCR in the present study. Thus, the PCR assay may be considered a valuable and reliable alternative for the quantitation of HIV-1 DNA in human biologic specimens. Its advantages of speed and sensitivity make it an ideal screening tool for the assessment of new antiretroviral therapies on viral titres or when HIV antibodies are not considered reliable (such as in neonates).

In the present study, HIV-1 DNA failed to be detected in the saliva of 31 seropositive subjects at a concentration above the detection limit of the assay previously developed

and optimised on reference samples, positive controls and clinical blood specimens. This finding partially supports two recent studies using HIV-1 DNA PCR on saliva specimens (171, 172). Goto *et al* (1990) found HIV-1 DNA in 20% of saliva samples taken from seropositive subjects using the SK 38/SK 39 and SK 68/SK 69 primers (171). In addition, 50% of samples were found to be positive using a primer pair in the LTR region of the genome. Although the methodology used by Goto *et al* (1991) was similar to that of the present study, the PCR was cycled 45 times in contrast to the 25 cycles used here. The use of high cycle numbers (greater than 30 cycles) was criticised previously (140) due to the risks of obtaining false positive results from the amplification of minor contaminants or non-specific regions. In another study Lucht *et al* (1993) failed to detect the presence of HIV-1 DNA in 63 saliva samples from HIV-1 seropositive subjects despite using the more sensitive technique of nested PCR (172). Taken together, the findings of these studies indicate that HIV-1 genetic material derived from host cells cannot be detected reliably in the saliva of subjects at any stage of HIV-1 disease.

The failure to detect HIV-1 DNA in the saliva of seropositive subjects supports the findings of epidemiologic studies (10, 173), which have found no evidence of salivary transmission of HIV-1, as well as co-culture based studies, which isolated HIV-1 in very few specimens (14, 41, 123, 125, 163). Several reasons are proposed to explain the failure of the present assay to detect HIV-1 in saliva samples.

Unlike blood, saliva contains relatively few nucleated cells of which even fewer are lymphocytes. Schiött and Löe (1970) found the number of lymphocytes in saliva averaged 3,240/ml using mouth washings (15). Furthermore, only approximately 1% of CD₄ cells are infected with the virus at any one time (174). These factors in combination with the reduced CD₄ percentage and overall T cell count of HIV-1 affected subjects (175) could have contributed to the non-detection of HIV-1 DNA in the saliva of the subjects in the present study. In addition, the processing of the salivary pellet, by cell lysis and phenol chloroform extraction, causes a further loss and dilution of the genetic material from the sample. This error is most pronounced when small volumes are used. Finally, from the entire DNA extraction, a 10µl aliquot (representing one half of the total DNA extracted from the sample) was used on each occasion of the assay run. Therefore, it is likely that the nature of the saliva sample as well as it's processing resulted in a dilution beyond the limit of the present assay. To overcome this inherent source of error several alternative techniques were investigated. Simple cell lysis, without phenol chloroform extraction, would have been sufficient to release the DNA for the PCR assay. However, the volume of lysis buffer required (approximately 250µl) would have resulted in greater dilution of the DNA. This too could be overcome by pelleting the DNA using ultracentrifugation, removal of the supernatant and resuspending in a smaller volume. Unfortunately, no ultracentrifuge for volumes below 10ml was available for use.

It is possible that in the saliva of the sampled subjects insufficient HIV-1 containing cells were present. This would seem unlikely since in one instance visible blood contamination of the saliva was evident and 21 subjects had gingivitis causing bleeding on probing. Schiött and Loe (1970) reported an increased presence of lymphocytes in human saliva with the onset of experimental gingivitis (15). Decomposition of the genetic material and hence a lack of contiguous lengths of HIV-1 DNA available for amplification may be an alternative explanation for the observed results. Freeze-thawing of clinical samples was attributed to the lack of success in a previous study (172), however no such effect was seen in the present study on blood cells treated in a similar manner. Salivary enzymes such as proteinases, amylases and trypsins may have caused damage to the genetic material (131, 132). The prompt washing of pelleted material in PBS prior to storage was performed in an effort to reduce the risk of damage to the sampled material. The extent of damage to the genetic material can be investigated by performing a human genomic DNA PCR on the sample. This experiment will be carried out on the current samples in a separate series of experiments.

The failure to detect HIV-1 DNA in the saliva of seropositive subjects in the present study does not imply that saliva is not potentially infectious. The present assay sought only to detect and quantify cell associated viral genetic material whose source was the healthy or inflamed gingival crevice. The presence of free virus can not be detected by the present assay which seeks only DNA. Quantitation of free viral particles in human saliva using PCR technology presents several additional obstacles. For

instance, the PCR technique can only be applied to DNA. Hence, the viral genomic RNA must first be converted to DNA by reverse transcription. The efficiency of RNA transcription on clinical samples is unpredictable and thus complicates attempts at quantitation. The construction of a standard curve derived from HIV-1 RNA is likewise affected by reverse transcription. RNA is less biologically stable and is highly susceptible to decomposition by RNAases. This adds to the difficulties in obtaining known quantities of intact HIV-1 RNA copies. For the purposes of future research into the quantitation of HIV-1 RNA in saliva, aliquots of each clinical sample were stored in equal volumes of 5M guanidium isothiocyanate to improve the preservation of any RNA present.

CONCLUSION

The presence of HIV-1 associated DNA was not be detected in 31 saliva samples derived from HIV-1 infected subjects using a PCR assay previously shown to have a high degree of sensitivity and reproducibility. This finding was irrespective of the degree of gingival inflammation. The reasons for the observed lack of success may include the low number of virally infected lymphocytes in a 5 ml saliva sample or that the DNA was decomposed in the salivary environment. Future investigations may be directed towards the detection of HIV-1 RNA in the saliva. Although this study may encounter more technically difficulty it may provide data which are biologically and clinically more relevant.

APPENDIX A

BUFFERS AND REAGENTS

1. DNA Extraction Buffer:

50mM KCl,

10 mM Tris (pH = 8.3),

10 mM MgCl₂,

0.45% NP-40,

0.5µg/µl Proteinase K

2. 10 x PCR Buffer:

500 mM KCL,

100 mM Tris (pH = 8.5),

25 mM MgCl₂,

0.1% Gelatine,

1% Triton.

Appendix A.ii

3. PMA Diluent:

40 mM Tris (pH = 7.6),

20 mM MgCl₂,

50 mM NaCl

4. Culture Medium:

RPMI 1640 with glutamine (GIBCO, USA),

10% Fetal calf serum (CSL, Australia) - heat inactivated at 56°C for 30 minutes,

10% IL-2 (Boehringer Mannheim, Germany),

DEAE-dextran at 3µg/mL,

Pennicillin (100 units/mL)/streptomycin (100 units/mL) or gentamicin (50 µg/mL).

APPENDIX B

PHENOL-CHLOROFORM DNA EXTRACTION METHOD

Resuspend lysed pellet in 250µl STE and vortex for 2 min.

Add 40µl of 10% SDS, vortex for 2 min and spin at 1500 x g for 5 min.

Add 300µl of Phenol-Chloroform-Isoamyl alcohol (49:49:1) and vortex for 5 min.

Spin at 1500 x g for 10 min.

Carefully decant aqueous phase off the top and repeat steps 3 and 4 to the decanted phase.

Add 300µl of Chloroform and vortex for 5 min. Spin at 1500 x g for 10 min.

Decant off aqueous phase and place into fresh tube.

Add 20µg glycogen, 2 volumes of cold absolute ethanol, and 1/10th volume 3M sodium acetate.

Store over night at -70°C to allow DNA precipitation

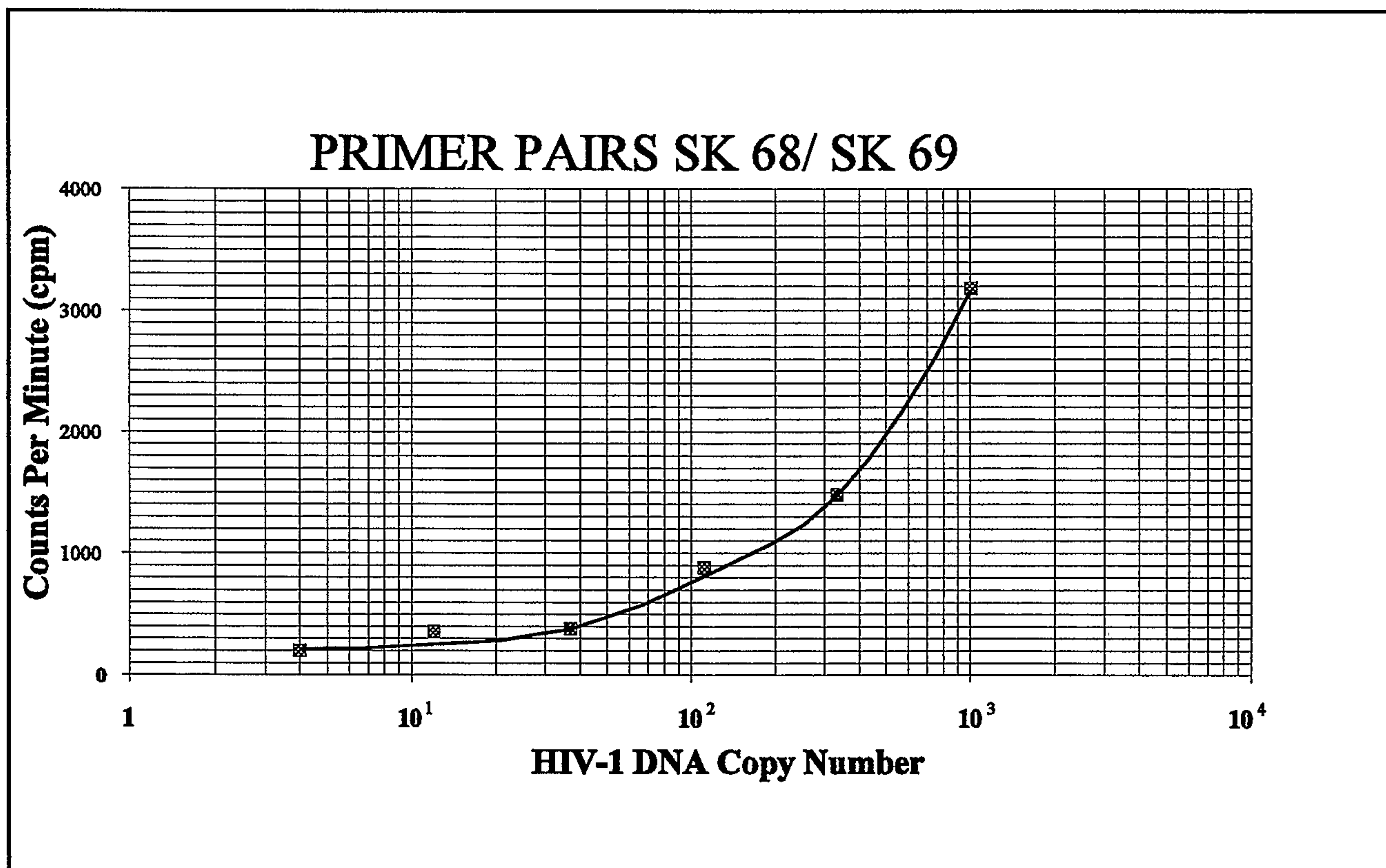
Thaw and centrifuge at 1500 x g for 20 min. Draw off supernatant and resuspend in 20µl water.

Store at -70°C until required.

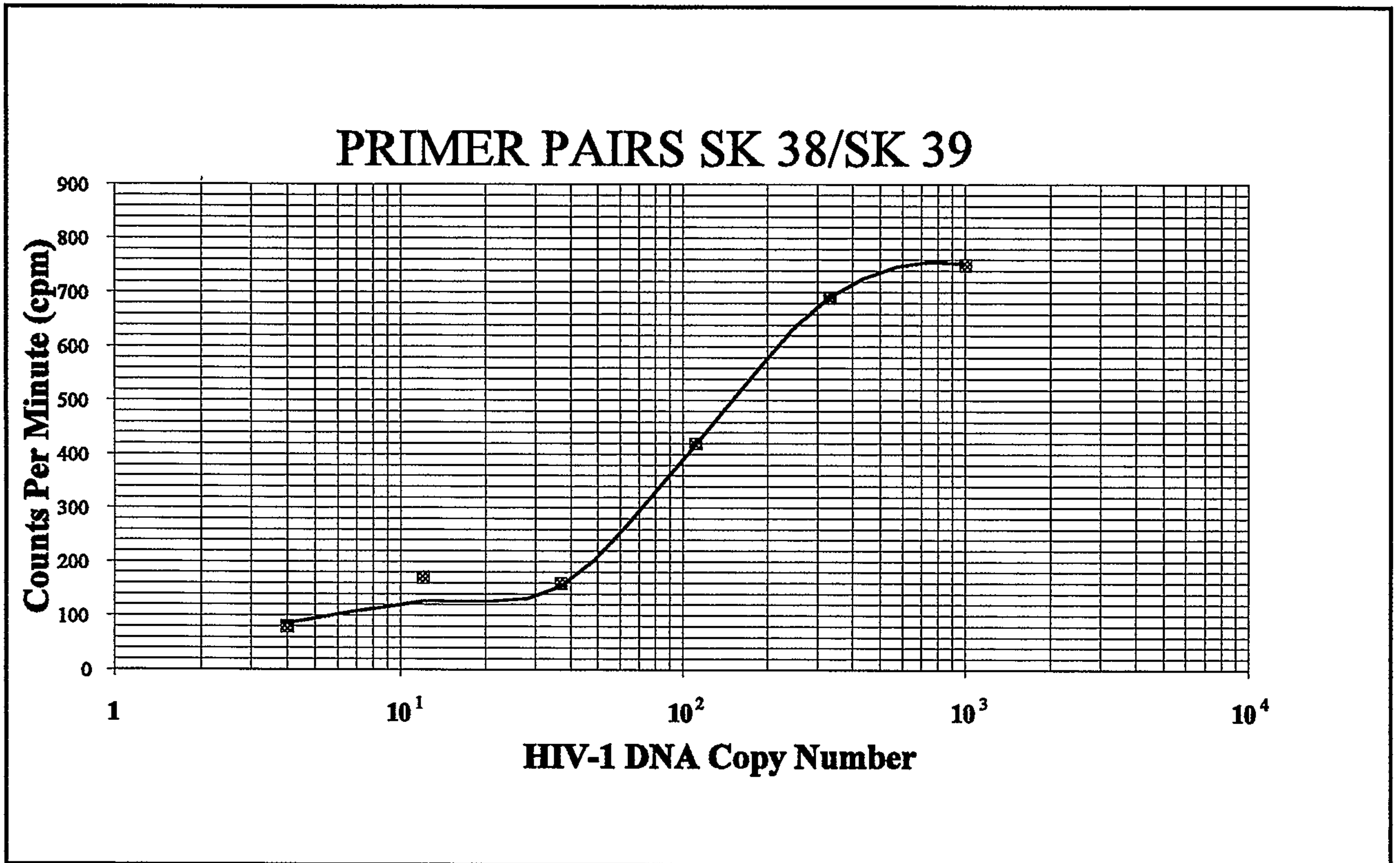
APPENDIX C

STANDARD CURVES

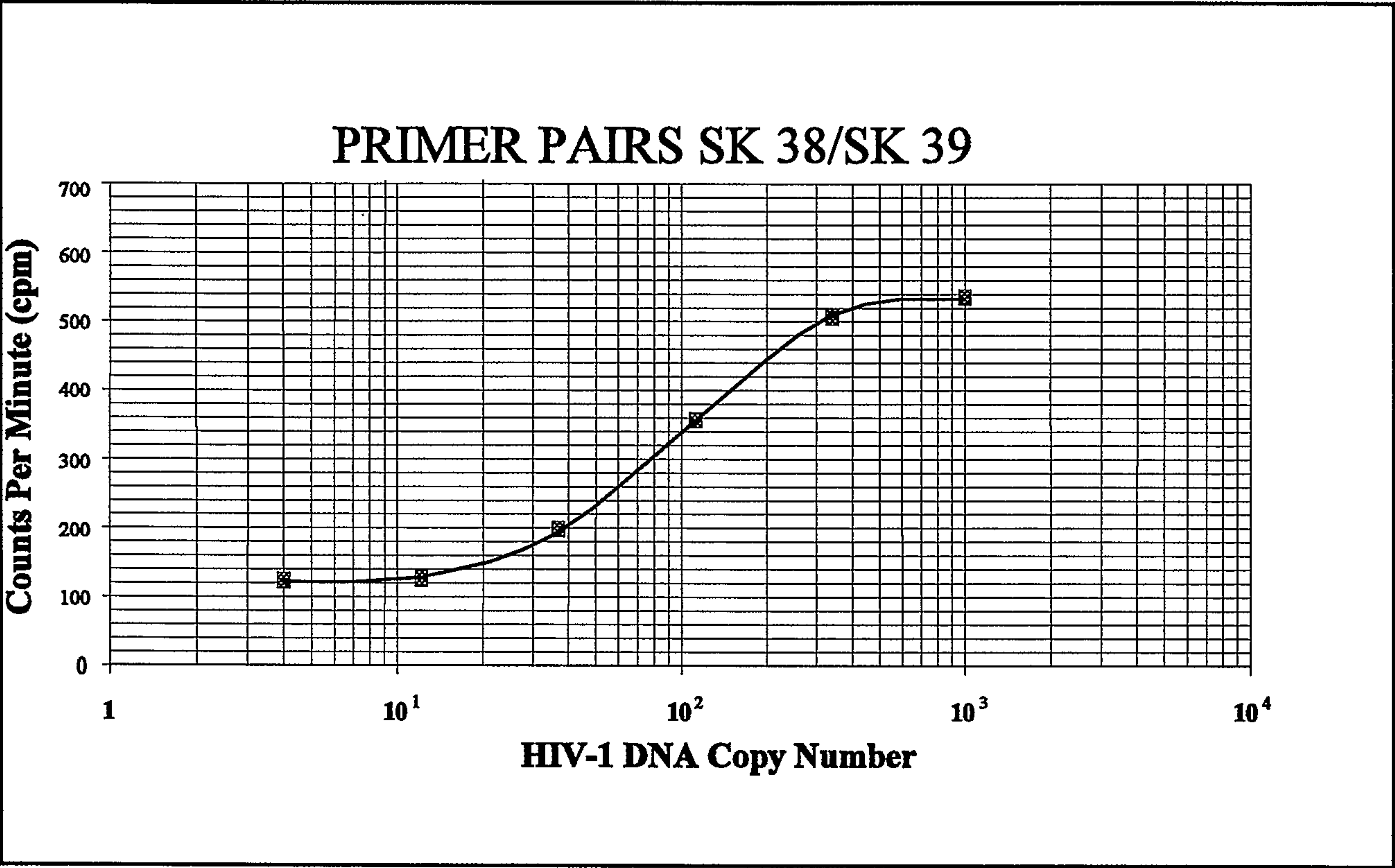
1. Blood Specimens



Appendix C.ii



2. Saliva Specimens



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