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WHO-UNAIDS Guidelines for Standard HIV Isolation and Characterization Procedures (Second Edition, 2002)



**WHO-UNAIDS HIV Vaccine Initiative (HVI)*
Initiative for Vaccine Research (IVR)
Health Technology and Pharmaceuticals (HTP)
World Health Organization (WHO)**



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1. Introduction

The human immunodeficiency virus (HIV) is characterized by extremely high variability and evolution rates. This results in the emergence of viral strains with widely divergent nucleotide sequences harbored from diverse geographical locations and different populations. Based on genetic characterization data, HIV strains have been classified into two major types, HIV-1 and HIV-2. The contribution of HIV-2 infections to the epidemic has been limited to a low prevalence in some West African countries and reported cases elsewhere have been rare. On the other hand, to date most of the global acquired immunodeficiency syndrome (AIDS) cases have been caused by HIV-1 strains, which in turn have been classified into 3 groups, including group M (or a major group), group O (“outlier” group) and group N (non-M/non-O group). The HIV-1 group M strains play a specially important role in the pandemic and have been the subject of detailed molecular epidemiology studies, which facilitated the identification of distinct genetic clusters/clades, also defined as genetic subtypes within HIV-1 group M. At least 11 different genetic subtypes have been recognized to date (designated A to K) based on phylogenetic analysis of sequences derived from the envelope gene of HIV-1. However, the recently discovered phenomenon of genetic recombination, has further complicated the classification of HIV-1 strains. The latest molecular epidemiology data provide evidence that certain recombinant HIV strains are emerging as important factors in different sub-regional epidemics and outbreaks. This has justified their recognition as circulating recombinant forms (CRF), e.g. the AE recombinant virus in Thailand designated as CRF01_AE, the A/G (IbNG) recombinant in a number of African countries as CRF02_AG, and the AB recombinant in Eastern Europe as CRF03_AB.

HIV strains can also vary significantly with regard to their biological and immunological properties, an aspect which may have important implications for the natural history of HIV infection, as well as for the development of effective anti-retroviral drugs and HIV vaccines. However, the present state of the art does not provide a clear-cut correlation between nucleotide sequences and viral properties such as infectivity, transmissibility, pathogenicity and antigenicity. Detailed information on the number of distinguishable HIV serotypes and immunotypes circulating in different communities is lacking, and the mechanisms for inducing protective immunity are still unknown. Therefore, it remains to be resolved whether or not effective HIV vaccine preparations would need to contain immunogenic material derived from various viral variants that are either geographically associated with a target populations or temporarily matched with epidemic phases. Thus, it is important to support international HIV surveillance networks, which should be designed to monitor genetic, biological and antigenic variation of HIV on a global basis. The results from such surveillance studies would help determine the impact of antigenic variation on the efficacy of HIV vaccines, and potentially on the diagnosis and treatment of HIV/AIDS.

In 1989, a global Network for HIV Isolation and Characterization was established by the WHO Global Programme on AIDS, which continued to function within UNAIDS. Since the year 2000, the Network is being implemented as part of the joint WHO-UNAIDS HIV Vaccine Initiative (HVI). The Network has a three-tier structure and, comprises research laboratories, that are specialized in different types of activities, but with overall common strategies, goals, and coordinated activities, including the following:

- a) “Primary” collaborating laboratories in different geographical locations representative of various patterns of the HIV/AIDS pandemic, usually from developing countries representing potential HIV vaccine evaluation sites;
- b) “Secondary” specialized expert laboratories for detailed genetic, biological and immunological characterization of globally prevalent and epidemiologically important HIV-1 strains, especially those from developing countries;
- c) Centralized facilities and repositories for the development and wide distribution of HIV strains and other HIV vaccine-related reagents.

The HIV isolates and reagents generated by the WHO-UNAIDS Network are made widely available to scientists from academic and private institutions working in the area of HIV vaccines. This is managed through a centralized facility for HIV isolation at Georg-Speyer-Hause Institute for Biomedical Research in Frankfurt, Germany, and two repositories at the National Institute for Biological Standards and Control (NIBSC) in London, U.K. and the HIV AIDS Research and Reference Reagent Programme at the National Institutes of Health (NIH), U.S.A.

The information and database generated by the WHO-UNAIDS Network is made publicly available by dissemination of results in a wide range of scientific journals and by inclusion in the Database on Human Retroviruses and AIDS at the Los Alamos National Laboratory, U.S.A.

An important priority for the WHO-UNAIDS Network has been the development, evaluation and standardization of various laboratory methods for HIV isolation and characterization. These new laboratory methodologies are further transferred to selected developing countries and practical training is sponsored by the WHO and UNAIDS through organization of specialized training workshops in developed and developing countries.

The first 1994 edition of the Guidelines was widely distributed and has served as a source of detailed practical information to ensure appropriate technology transfer. Moreover, it has also served as a teaching material for scientists starting to work in the area of HIV characterization and HIV vaccines. The second edition (2002) of the Guidelines has been expanded and further improved to include the latest developments in this area with a special emphasis on their applicability for capacity building in developing countries in relation to HIV vaccine development and HIV vaccine trials.

2. Scientific Background

Viral vaccines represent major advances in biomedicine. The eradication of smallpox from the world and the control of poliomyelitis and measles, at least in some areas, are examples of the advances made in this area. The identification of a retrovirus as the etiological agent of AIDS means that the development of a vaccine to prevent this disease is a possibility. Vaccine studies aiming at prevention of HIV infection, or the subsequent immunosuppression caused by the virus, have to consider three factors that are critical for viral pathogenesis:

- the capacity of the virus to persist;
- the progressive destruction of humoral as well as cell-mediated immune functions;
- the antigenic variability of HIV.

To understand these problems, a short description of the retroviral life cycle and the causes and consequences of variability will follow.

2.1 Genome organization and protein products

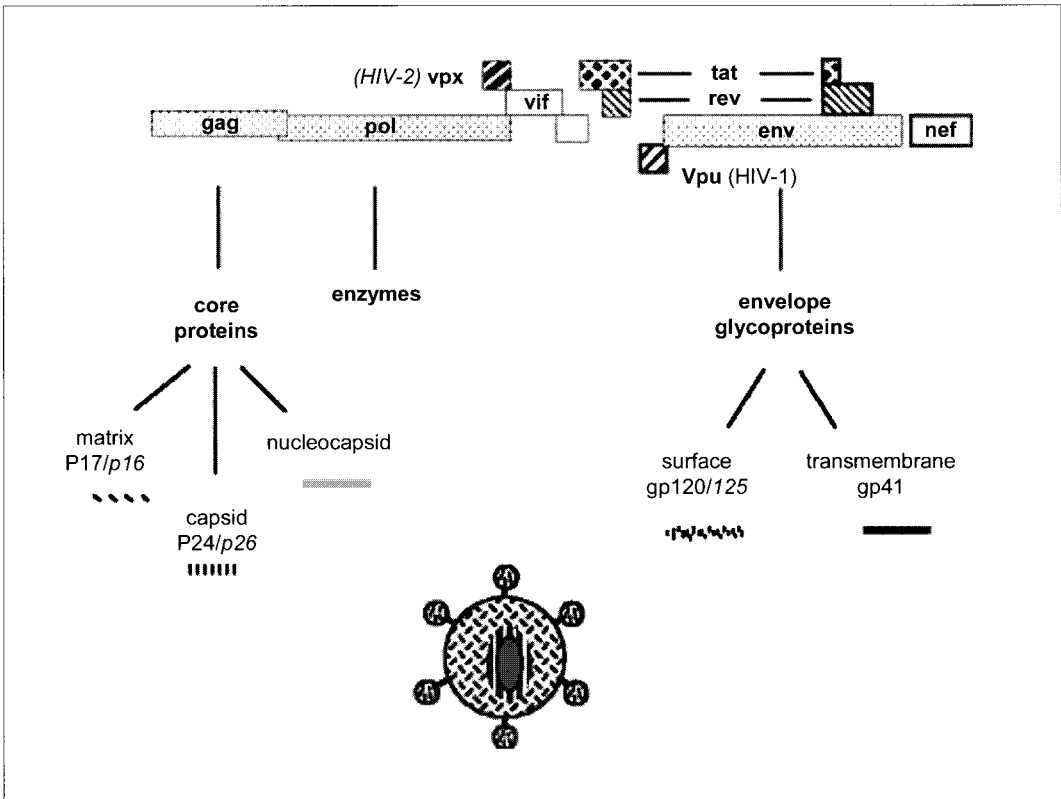
Based on studies of morphology, genomic organization and pathogenic features, HIV has been classified as a member of the lentivirus subfamily of retroviruses. Lentiviruses cause diseases with typically long incubation periods and protracted symptomatic phases. Like all replication-competent retroviruses, HIV has *gag* and *env* genes, encoding structural proteins, and *pol*, encoding enzymatic proteins. Three classes of messenger RNA (mRNA) sizes can be distinguished, namely genome length mRNA, singly-spliced mRNAs and multiply-spliced mRNAs. Translation of genome length mRNA yields a large polyprotein encoding all of the *gag* and *pol* products. This precursor is cleaved in several steps by the viral protease to produce the inner structural components and enzymes of the viral particle. Translation of singly-spliced mRNAs yields the envelope precursor protein (gp160) that is cleaved by host cellular enzymes to give the major external envelope glycoprotein (gp120) and the transmembrane protein (gp41). This process and the localization of final products in the viral particle are depicted in Figure 1. Individuals infected with HIV produce antibodies to viral proteins, and the presence of specific antibodies against viral antigens is the basis of the diagnosis of HIV infection.

In addition to *gag*, *pol* and *env* genes common to all retroviruses, the HIV genome also encodes regulatory genes, designated *tat* and *rev*, which control viral gene expression at the transcriptional and post-transcriptional level, respectively. The Tat and Rev proteins are produced from overlapping reading frames by multiply-spliced mRNAs. Tat increases the steady state levels of all HIV mRNAs, through a

mechanism called transactivation, through binding to the Tat-responsive-element (TAR) within a sequence termed the long terminal repeat (LTR; LTR sequences are found at the two ends of the viral genome, and components of it are found at both ends of the viral RNA; see below). Rev regulates the export of the viral mRNAs encoding structural proteins from the nucleus to the cytoplasm, through binding to the Rev-responsive-element (RRE) present in all unspliced or singly-spliced HIV mRNAs. Conceivably, the complex regulation of viral replication enables HIV to rapidly change from the latent to the productive phase, the latter being characterized by the output of large amounts of virus within a short time. This complexity also gives hope that interfering with virus replication will be an effective means by which disease progression and infectiousness of infected individuals can be controlled. With the introduction of effective antiviral therapies, such as highly active antiretroviral therapy (HAART), we are just beginning to experience these effects.

As a complex retrovirus, HIV encodes numerous auxiliary genes, *vif*, *vpr*, *vpu* (HIV-1 only), *vpx* (HIV-2 only) and *nef*, the functions of which have not yet been fully elucidated. Based on studies of virus replication in tissue culture systems, the functions of these genes are not strictly required, but rather appear to modulate aspects of the viral life cycle. This suggests that tissue culture systems may not accurately reflect the conditions important to regulate viral replication in the infected host. The fact that both antibodies and cell-mediated immune responses to the accessory proteins have been detected in HIV-infected people, indicates that these genes are expressed *in vivo* and might play important roles in the virus-host relationship.

Figure 1: The HIV genome and encoded protein products



2.2 The viral life cycle

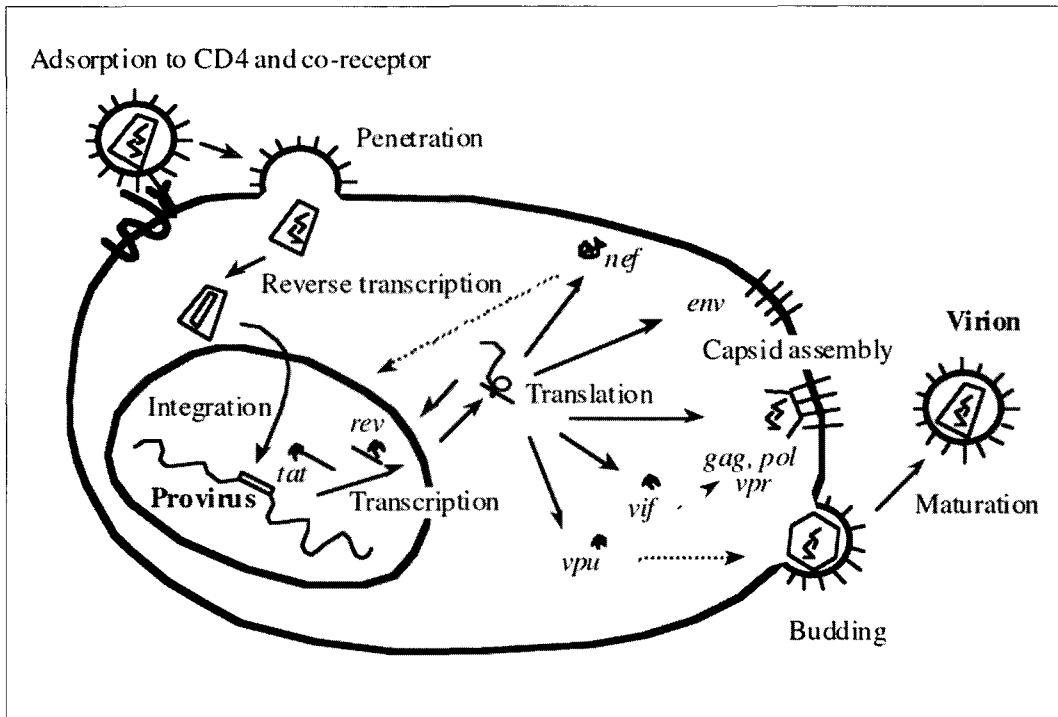
Retrovirus genomes are unique in that they can exist in two forms, both as RNA within virus particles and host cell-associated DNA. As will be discussed below, the integration of the viral DNA allows persistence in the chromosomes of cells of an infected organism.

The first step in viral infection of cells is attachment of virus particles to cellular receptors. In the case of HIV there are several cell surface proteins involved. The CD4 molecule, present mainly on some T-lymphocytes and monocyte/macrophages, has long been recognized as essential for HIV attachment. Recently, it has been shown that a second protein, one of several chemokine receptors (chemoattractant cytokine), is also required, serving as a coreceptor for HIV entry into cells. Since the coreceptor usage pattern of HIV is important for the virus biological phenotype, this will be discussed in more detail in section 2.3. Attachment of HIV to cellular receptors is followed by a complex series of events allowing penetration and reverse transcription to begin. The viral reverse transcriptase (also called RT) synthesizes a DNA copy of the viral RNA, the RNA template is degraded and a second complementary DNA strand is synthesized. The double-stranded DNA copy of the viral sequence is then transported to the nucleus and integrated into the host cell DNA (this integrated DNA is referred to as a provirus). Integration of the provirus is assisted by another virus-specific enzyme that functions as an endonuclease (called integrase or IN). Integration completes phase I of the retrovirus life cycle (Figure 2). The integrated provirus may be transcriptionally inactive and behaves like any other silent cellular gene. When the host cell divides, the proviral gene is transmitted to daughter cells as part of the host cell chromosomes.

When the integrated provirus becomes transcriptionally active, the second phase of the retroviral life cycle is initiated. Host cellular enzymes (RNA polymerases) transcribe the integrated provirus DNA template, and cellular mechanisms complete RNA processing and its transport to ribosomes in the cell cytoplasm. Similarly, host cellular machinery mediates viral protein synthesis, further processing of some viral proteins into glycoproteins, and transport to the cell membrane - where maturation of viral particles occurs through a process called budding. A third virus-specific enzyme, a protease, is involved in cleavage of the large precursor proteins into functional capsid, nucleocapsid, and matrix proteins. The same protease cleaves one form of the polyprotein into functional, enzymatic proteins. These cleavage events occur during the formation of the viral particle and are part of the maturation process.

These phases of the general retrovirus life cycle also apply to HIV. However, unintegrated forms of HIV DNA have been demonstrated in dormant T lymphocytes, both after *in vitro* infection and in people with HIV infection. For an effective virus infection to occur, HIV DNA must be integrated into the host cell genome. Since HIV integration depends on the activation state of the host cell, in this case the T lymphocyte, factors influencing T cell activation (mitogenic agents or opportunistic infections) might promote viral integration and thereby virus production.

Figure 2: Replication cycle of HIV-1.



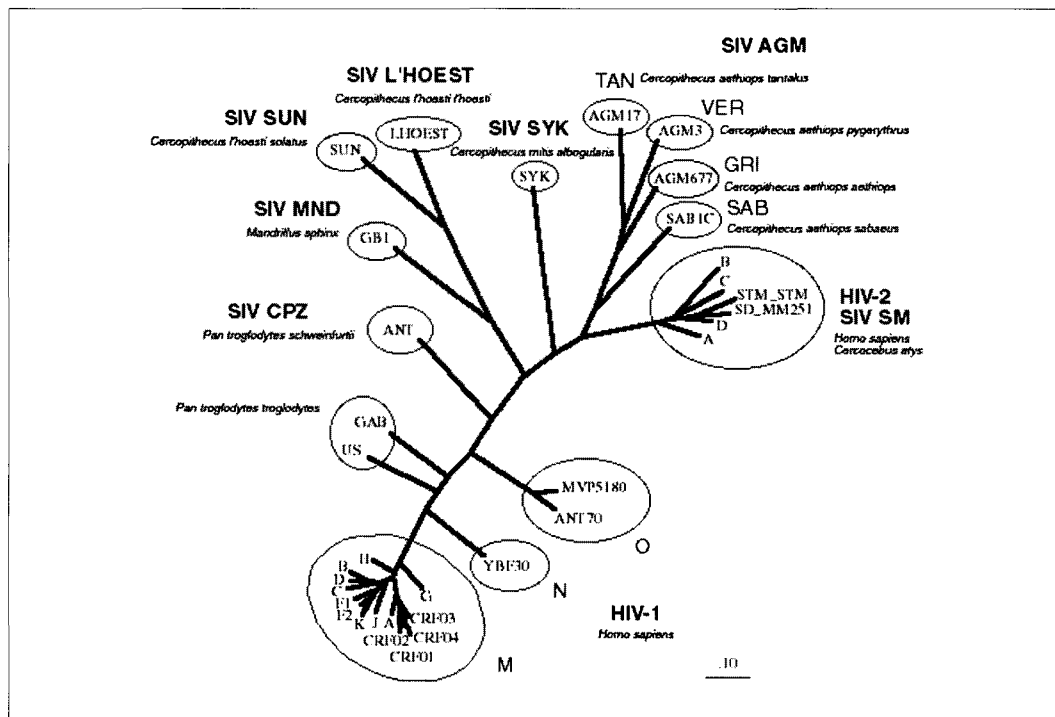
2.3 Genetic, antigenic and biological variability.

a) Genetic variability

High rates of genetic variability, leading to high rates of antigenic variability, are a characteristic feature of the lentivirus subfamily of retroviruses. Reverse transcription of viral RNA to DNA, which occurs each time the virus infects a new cell, has an error rate approximately one hundred thousand to one million-fold higher than the DNA polymerases of animal cells, about one substitution in 1,000 to 10,000 nucleotides synthesized. In addition to substitutions, deletions, insertions and recombination also occur, although at lower frequencies.

The phylogenetic relationships between human and simian immunodeficiency viruses is depicted in Figure 3. The genetic distance between any two viral isolates is found by following the shortest path relating them in the tree. Units are in terms of nucleotide substitutions according to the indicated scale. HIV-1 is divided into three groups: M, N and O. The HIV-1 M group is responsible for the major AIDS epidemic. HIV-1 M is further subdivided into several subtypes (A through K, except I) and circulating recombinant forms (CRF01 through CRF12). Note that the location of the CRF's may differ in trees from different genomic regions depending on their exact recombination pattern. HIV-2 has also been suggested to be divided into subtypes (shown here are subtypes A, B, C, and D), and is closely related to the sooty mangabey simian immunodeficiency virus (SIV SM). HIV-1 is closer related to the chimpanzee virus (SIV CPZ) than to any other currently known virus. The host of each virus is indicated in the tree. Note that this does not indicate the evolutionary relationships among the hosts. SIV CPZ is divided into two different groups, each relating to one (of four) sub-species of chimpanzee. Similarly, African green monkey sub-species are all infected by their own specific SIV variant (SAB, TAN, VER, and GRI).

Figure 3: Phylogenetic relationships between human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) reconstructed from nucleotide sequences of the complete *gag* gene using the maximum likelihood method.



The most variable part of the HIV genome is the *env* gene where the sequence homology between HIV-1 and HIV-2 is less than 50%. Note, however, that the evolutionary distance is even greater, as indicated in figure 3, since it includes multiple substitutions in the same position, i.e., the total number of evolutionary events. The different “clades” or “envelope sequence subtypes” differ by approximately 20-35% at the nucleotide and amino acid sequence level. The clades are phylogenetic groupings that probably represent founder effects, that is, the historical beginnings of HIV-1 epidemics in different groups of people around the world. Thus, classification of sequences into clades is a convenient way of tracking the spread of the virus. *Env* subtype B, for example, was initially identified in viral isolates from North America, Europe and, occasionally, Central Africa. It has now spread to many other parts of the world.

The overall rate of divergence of the *env* gene is close to 1% per year, and slower rates of evolution are found in other parts of the viral genome. This rate of substitution errors is observed both between different infected individuals and within each infected individual. The rate does not seem to be affected by transmissions or other bottleneck effects. For example, the genetic distance between viruses in two individuals infected from the same source partner around the same time would be expected to be close to 10% after 5 years of infection. Similarly, the divergence of *env* sequences from different individuals in a given geographic region or risk group is related to the time the founder virus has been spreading in that region or group. In 1997, the range of divergence was less than 3% in the Kaliningrad region of Russia, (see figure 3) where the virus had been widespread for only approximately 2 years, but was about

10-15% in regions such as Thailand, where the virus has been spreading widely for the previous decade. In some regions, such as Thailand, virus from two different sources (one clade B and one clade E, now recognized as CRF01_AE) were responsible for the initiation of two distinct epidemics.

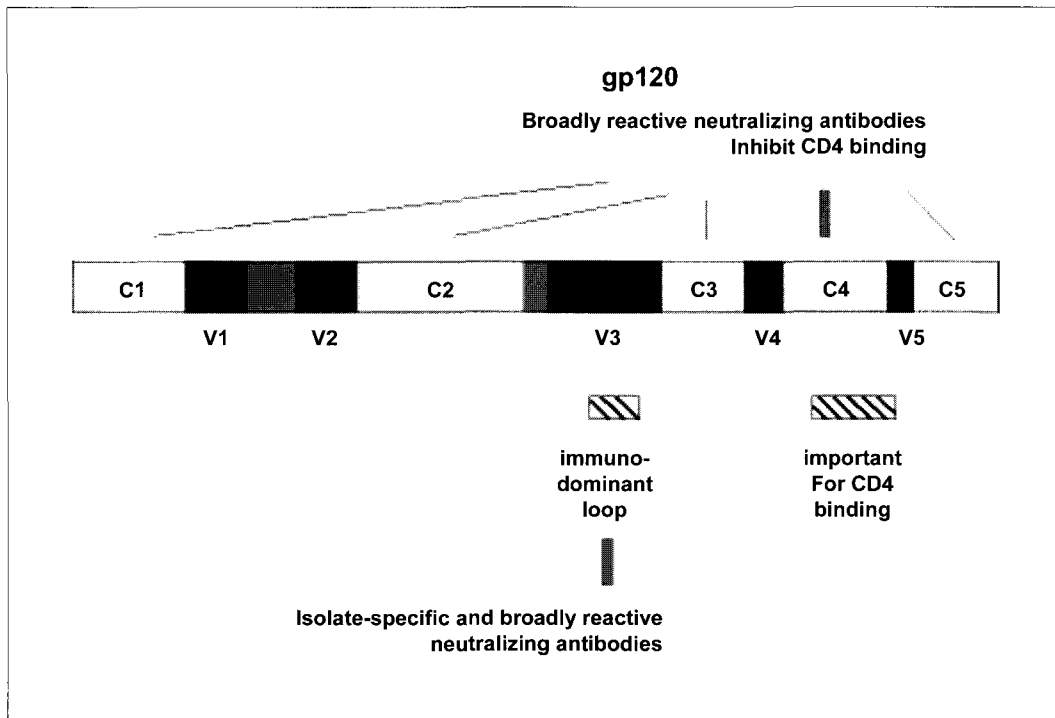
Virus isolates collected in geographically distinct locations are likely to be more divergent than isolates collected from a population group within a defined area. The maximal divergence of clade B strains in the United States is currently about 25-30%, reflecting the widespread introduction of the virus in the early 1980's. The average diversity between strains in different individuals is somewhat lower, reflecting the fact that the most current infections occurred more recently. The maximal divergence between viruses from different M group clades now ranges up to more than 30%, while some clades, such as B and D, have a more recent common ancestor.

Exacerbating the problem of increasing genetic divergence, individuals can sometimes be infected with multiple strains of virus and these viruses may coinfect and replicate in the same cells. In geographic regions and populations in which multiple subtypes are circulating, this has resulted in the infection of some individuals with viruses from more than one subtype, and the formation of virus chimeras, or recombinants, with parts of the resulting viral genomes derived from both clades. The impact of recombination between viruses from different clades on the antigenicity of the virus is unknown, but could conceivably complicate the development of effective vaccines for such populations. The importance of interclade virus chimeras is evidenced by the fact that the Kaliningrad outbreak involves an A/B (*gag* B/*env* A recombinant) chimera (CRF03_AB) and the recent outbreak in central China originated from a B/C chimera. Similar to a new subtype (or clade), three or more viruses with identical recombination pattern from separate patients, who are not directly epidemiologically linked, are required to establish a new CRF.

Intra-patient genetic diversity also occurs, observed as coexisting closely related variants within one individual. The diversity of the virus population within individuals has been found to keep pace with the divergence from the strain with which they were initially infected (the "founder" strain), - about 1% per year in the *env* gene. However, the intra-patient diversity often declines prior to the development of AIDS.

As mentioned previously, different parts of the viral genome change at different rates. While the envelope undergoes rapid change, alterations in the *gag* gene, encoding the viral capsid protein, are more constrained. The slowest evolution, however, is seen in the *pol* gene. Within the *env* gene, variable (V) and constant (C) regions have been identified (Figure 4). A loop structure within variable region 3, called the V3 loop, has attracted particular attention, since it appears to be an important epitope for eliciting neutralizing antibodies (for lab adapted virus strains it serves as the principal neutralization domain or PND), as well as for cell-mediated immune reactions. Changes (variation) in the V3 loop are typically nucleotide substitutions, whereas in other variable regions deletions and insertions are also common. Changes at the nucleotide level often result in changes in the amino acid composition of the envelope proteins whereas deletions and insertions can also alter the folding structure of the envelope molecule.

Figure 4. Organization of the HIV env gene



Alterations in the envelope proteins also appear to be driven by a positive selection advantage over strains with unchanged genotypes. Given the known involvement of the envelope, and the V3 loop in particular, in presenting B cell sites for virus neutralization, selection for changes in V3 suggests that circumventing the immune defence system is a major selective force. As discussed below, changes in the V3 loop can also change coreceptor specificity, and thus change the host cell range of infection of the virus.

b) *Biological variability*

HIV isolates show distinct biological features that correlate with the severity of HIV infection in the host. Viruses from people with asymptomatic HIV infection, or with mild disease, replicate slowly and inefficiently in *in vitro* cultures of peripheral blood mononuclear cells (PBMC). Attempts to passage these isolates in CD4 positive cell lines usually either fail or result in only transient replication. In contrast, viruses from subjects with severe immunodeficiency and symptoms replicate rapidly and efficiently in PBMC and in T-cell lines. Hence, the designation of slow/low and rapid/high isolates, respectively. Two viral phenotypes can also be distinguished by cytopathic effects on PBMC *in vitro*. Rapid/high HIV viruses are characterized by extensive syncytium formation (large cells with multiple nuclei formed as a result of virus-induced cell-cell fusion), whereas syncytia are rarely seen with slow/low viruses. Instead, cultures infected with slow/low viruses show signs of cell death or no cytopathic changes at all. Later, viruses were characterized by their ability to replicate and induce syncytia on the T-cell line, MT-2. In general, the terms syncytium inducing (SI) and non-syncytium inducing (NSI) correspond broadly to the rapid/high and slow/low phenotypes, respectively. In some infected individuals, the shift from the slow/low or NSI to the rapid/high or SI phenotype may be concomitant with, or occurs shortly prior to, disease emergence. Transmission of HIV with rapid/high

or SI phenotype leads to a more rapid deterioration of the immune system than the more common transmission of HIV with slow/low or NSI phenotype. Viral biological phenotype, therefore, appears to be a marker for HIV virulence.

The recent advances in our understanding of the relationship between HIV-1 biological phenotype and co-receptor usage has allowed us to associate phenotypic traits, such as replication rate and cytopathology in culture, with molecular mechanisms. The majority of HIV-1 phenotypic variation can be explained by usage of distinct, transmembrane domain chemokine receptors as co-receptors to CD4 for virus entry into permissive cells. The two most well-defined HIV-1 co-receptors are CXCR4 and CCR5, members of the CXC (a) and CC (b) chemokine receptor subfamilies, respectively. CXCR4 was the first HIV-1 co-receptor to be characterized, and was shown to be required for the fusion of T cell line-adapted viruses with non-human cells expressing human CD4. It is expressed on many cell types including transformed T cells, fibroblasts, primary T cells, and macrophages. Subsequently, CCR5 was shown to be the principal co-receptor for primary HIV-1 isolates with the NSI phenotype, whereas the SI phenotype was associated with the use of CXCR4 alone, or in combination with CCR5. Other members of the CC chemokine receptor family, such as CCR2b and CCR3, may also function as co-receptors for HIV entry, although generally in a less efficient manner than CCR5, as assayed in tissue culture. Individuals carrying a deletion of 32 base pairs in both alleles of the CCR5 gene appear to be completely resistant to infection by slow/low, NSI-type viruses, confirming the importance of CCR5 molecule in HIV-1 transmission *in vivo*. The co-receptor usage patterns of HIV formed the basis of a new classification system (Table 1). Thus, viruses previously termed NSI (slow/low) viruses are defined by their use of members of the CC-chemokine receptor family, principally CCR5, and are termed R5. Viruses previously termed SI (rapid/high) are defined by their use of the CXC-chemokine receptor CXCR4 and are termed X4. Some of the SI (rapid/ high) viruses use both CXCR4 and CCR5 (and/or CCR3) receptors and these are termed R5X4 (R3R5X4 or R3X4) viruses.

Table 1: Classification of HIV-1 biological phenotypes

Chemokine receptor	New classification	Previous terminology based on usage	
		cytopathology in MT-2 cells	replication rate in PBMC
CXCR4	X4	SI	rapid/high
CCR5 /CCR3/CCR2b	R5 R3/R2b	NSI	slow/low
CXCR4 and CCR5 and /or CCR3	R5X4 R3R5X4 or R3X4	SI	rapid/high

Different HIV-1 strains enter different cell types with varying efficiencies, depending on their co-receptor preference. These differences have been observed for more than 10 years, and have been used to classify HIV-1 primary isolates.

As detailed above, classification by growth kinetics and cytopathology is indicative of replication and syncytium induction in PBMCs. Virus isolation involving mixed cultures with donor PBMC, results in HIV-1 replication patterns that are slow or fast, low- or high-titre, respectively. The basis of PBMC suitability for the isolation of a wide variety of HIV-1 isolates is that activated CD4⁺ T lymphocytes contain populations expressing either the CCR5 or the CXCR4 chemokine receptor, and that these cell populations can be infected with viruses using either or both molecules. In addition, the level of expression of the relevant co-receptor in the PBMC cultures appears to determine the rate of replication. A practical assay used currently to distinguish these HIV-1 phenotypes is based on the ability of virus to form syncytia in the MT-2 T cell line. Similar to other immortalized CD4⁺ T cell lines, MT-2 cells express high levels of CXCR4, but not CCR5, and are susceptible to viruses employing CXCR4. Syncytia induction in MT-2 cells is used worldwide and continues to be the most efficient way to identify viruses employing CXCR4.

c) *Antigenic variability*

Within an HIV-1 infected subject, more than 10⁹ virus particles are produced each day. This rapid rate of HIV-1 replication, combined with low fidelity of reverse transcription, may generate mutations in immunogenic regions of viral proteins on a daily basis. Indeed, HIV-1 resistance to neutralization by antibodies, as well as to cytotoxic T lymphocyte - induced death (CTL), has been demonstrated. Furthermore, it has been postulated that accumulation of mutations in epitopes recognized by humoral or cellular immune mechanisms may serve as a dominant mechanism of immune escape by HIV-1.

Escape from neutralizing antibodies. In general, the appearance of neutralizing antibodies in the course of viral infections is considered to be a sign of protective immunity. Moreover, the protective capacity of a vaccine is usually estimated by its ability to induce neutralizing antibodies. Administration of such antibodies will protect from infection, even in cases where the cellular immunity has a crucial role for clearance of an established infection (e.g. *varicella zoster*, measles).

In the course of a primary HIV-1 infection, viremia is followed by antibody production to different viral antigens (seroconversion). As antibody titre increases and CTL activity appears, plasma viral load decreases and becomes undetectable or, more often, stabilizes at a level characteristic for each HIV-1 infected subject. In recent years, it became clear that low amounts of viral RNA in plasma (viral load) are indicative of a better prognosis. The appearance of neutralizing antibodies also varies among HIV-1 infected individuals. Early events in HIV infection, determined by viral virulence as well as the host immune response, appear to determine the subsequent course of HIV infection. A restricted immune response, for example, would allow the emergence of variant viruses resistant to neutralization, thereby leading to increased viral load and rate of progression. In cases where neutralizing antibodies appear at seroconversion, these are isolate-specific and recognize the subject's own virus isolated during the primary HIV-1 infection. These early neutralizing antibodies may not neutralize virus isolated from the same person six or more months after the primary HIV-1 infection. Thus the virus has changed over time and is no longer neutralized by the infected person's own antibodies. The emerging variant viruses are not resistant to neutralization *per se*, since they can be neutralized by sera from other HIV-1 infected people.

Escape from CTL. Cellular immune responses to HIV-1 have been suggested to mediate potent suppression of viral replication *in vivo*. Activated HIV-1-specific CTL occur at high frequency in many HIV-1 infected individuals and have been shown in *in vitro* experiments to kill HIV-1 infected cells and inhibit viral replication. Despite the presence of a vigorous CTL response, however, virus replication may continue *in vivo*. Recent evidence suggests that in the course of HIV-1 infection, both early and late, mutant viruses emerge with decreased or abrogated sensitivity to CTL recognition. In primary HIV-1 infection, the initial CTL response may be directed against only one or two CTL epitopes, thus concentrating selective pressure of the CTL response on a single epitope. Indeed, unrecognized variant sequences have been detected in several individuals within a few weeks and these unrecognized sequences became dominant in plasma viral RNA 4-5 months later. These studies support the hypothesis that sequence variation may serve as one of the mechanisms of escape from CTL responses.

Broadly cross-reactive immune responses. In spite of the antigenic variation and immune escape that exists in individuals with increasing severity of HIV-1 infection, some HIV-1 antibody-positive sera are able to neutralize virus from many HIV-1 infected individuals, i.e. contain broadly reactive neutralizing antibodies. In particular, sera from HIV-1 infected individuals who fail to develop symptoms for many years, i.e. long-term non-progressors, often contain antibodies capable of neutralizing many - even genetically diverse - primary HIV-1 isolates at relatively high titre. This suggests an association between effective neutralizing antibody responses and delayed disease progression. Moreover, the results from several laboratories have shown that classification of HIV-1 by genetic subtypes does not correspond to neutralization serotypes. Within the framework of the WHO Network for HIV Isolation and Characterization, neutralization assays have been carried out with primary isolates from four geographical areas belonging to five different genetic subtypes (A-E). Half of the sera did not neutralize any of the isolates tested (2-6 isolates tested with each serum), whereas the other half neutralized viruses regardless of genetic subtype. These data clearly show that genetic subtypes do not identify neutralization serotypes. The broad cross-neutralizing activity of some sera (25% of samples tested) suggests that genetic variation may not present as serious an obstacle for vaccine design as previously thought. Similarly, CTL responses appear to be broadly cross-reactive between HIV-1 subtypes. Taken together, these results suggest that more attention should be focused on such cross-reactive immune responses and less on virus variability. There is a need for a strategy to elicit broadly cross-reactive immune responses in vaccinees. Since the form of immunogen affects the type of immune response, it has been argued that vaccines must provide antigens in three forms so as to stimulate humoral as well as both cellular arms of CD4+ and CD8+ cell-dependent immunity. For stimulation of humoral immunity, antigens in native form must be available on the cell membrane to the surface immunoglobulin receptor of B cells. Evaluation of vaccine efficacy will be necessary in large seroepidemiological studies. For this purpose it is feasible to use neutralization assays, since measurement of neutralizing antibody responses is a lot less difficult than measurement of CTL, where epitope recognition is further complicated by human leukocyte antigen (HLA) type. Assuming that neutralization is an indicator for protection, neutralizing antibodies may provide important clues for vaccine efficacy.

How to elicit a broadly cross-reactive neutralizing antibody response?

There are a number of ways for generation of neutralizing antibody responses: immunization with virus particles, infected cells, oligomeric or monomeric viral proteins or all of these antigens complexed with immunoglobulins. The questions are: which form of the immunogen will give a broadly cross-reactive response and which epitopes on the viral envelope are involved in binding neutralizing antibodies with broad specificity? Monoclonal antibodies are important instruments to dissect this problem and have already been used to delineate epitopes that fulfil the requirement of neutralizing of several genetic subtypes of HIV-1. Three HIV-1 - specific human monoclonal antibodies with such capacity have been described to date. In tissue culture systems, the antibodies see distinct epitopes on the viral envelope: IgG1b12 detects an oligomer-dependent epitope that overlaps the CD4-binding site on gp120, 2G12 detects a glycosylation dependent epitope on gp120, and 2F5 recognizes a continuous but conformationally sensitive peptide sequence on gp41. One of the problems may be that most HIV-1 infected subjects appear to lack antibodies that recognize the epitopes specified by these three monoclonal antibodies. If so, then the main task would be to find an immunogen that would elicit potent neutralizing antibody responses in vaccinees.

The fact that CCR5 usage is a general property of many primate lentiviruses suggests that highly conserved regions must be present in the otherwise highly variable surface envelope protein. Analysis of the crystal structure of a core gp120 molecule binding CD4 and a neutralizing antibody (monoclonal 17b known to bind to an epitope, CD4i, induced on gp120 by CD4 binding), revealed that the binding sites for neutralizing antibodies cluster on one gp120 surface. The conserved receptor binding site is, however, effectively shielded by variable regions, such as V1/V2 and V3, and by heavy glycosylation of the surrounding outer parts of the molecule. Binding of gp120 to CD4 results in a conformational change that exposes this conserved epitope (the CCR5 binding site). This causes further dislocation of the molecule and induces gp41 to execute fusion of the viral and cellular membranes. This scenario predicts that the conserved epitope is transiently exposed in the process of virus-receptor binding and, even then, may be relatively inaccessible to antibodies. “Freezing” the structures formed during the fusion process between the HIV-1 envelope and cellular receptors and using these as immunogens could elicit a broad neutralizing antibody response. Even if these experiments involved immunization of mice with mixtures of human cells undergoing fusion, this model may be used to explore the exact nature of the immunogen. The knowledge obtained may then be applied to the development of an effective HIV vaccine.

The crystal structure of the core gp120 molecule also provides clues for the “occupancy model”, which assumes that antibodies to all conserved and well-exposed epitopes on the mature envelope may be equally effective in virus neutralization. Thus, the important factor in neutralization is the fraction of virion binding sites occupied rather than epitope specificity. Indeed, clustering of the neutralization epitopes delineates a “neutralization face” on the gp120 molecule. The conserved epitopes buried in this region might be those immunogens for which we are looking. Consequently, efforts should be focused on increasing the immunogenicity of the native oligomeric envelope for presentation to the immune system. Virus neutralization will then be an important tool to assay efficacy of immunization.

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2.4. Approaches to vaccination against HIV/AIDS

At present, the HIV pandemic continues to spread at an accelerated rate with 15,000 new infections occurring every day, generating a total of more than 5.4 million newly infected individuals every year. According to the WHO and UNAIDS estimates, since the start of the epidemic there have been more than 60 million people worldwide who have been infected with HIV. An overwhelming majority of these infections – some 95% of the global total – have occurred in developing countries. Over the past years, the AIDS pandemic has claimed the lives of more than 20 million people, placing HIV/AIDS among three major killer infectious diseases of mankind, together with malaria and tuberculosis.

Facing this situation, there is a growing consensus among scientific, public health and grass-root communities, that availability of a safe, effective and widely affordable HIV vaccine would offer the best hope and an effective complementary tool to control this epidemic, especially in less developed countries.

2.4.1. *Potential uses of HIV/AIDS vaccines*

Traditionally, there are 3 different types of vaccines:

- ***Preventive vaccines***, which are given to non-infected individuals with a goal of preventing an infection and/or disease. Preventive vaccines are known to be the most cost effective tools for the control and potential eradication of some epidemics, e.g., smallpox, polio, measles;
- ***Therapeutic vaccines*** are given to infected individuals aiming at modulation of the host immune responses that would allow for better control of infection and would prevent the development of the disease. In some cases, therapeutic vaccines could produce secondary preventive effects by decreasing the viral load levels in infected individuals and thus rendering them less infectious;
- ***Perinatal vaccines*** are given to infected women of childbearing age to prevent vertical transmission from mother to child. At the same time, perinatal vaccines could act as a therapeutic vaccine in an infected pregnant woman.

However, the ultimate goal of HIV vaccine development, and high priority for WHO and UNAIDS, is to develop safe and effective preventive vaccines. Such vaccines would have the highest public health impact, not only by protecting individuals from infection, but also by interrupting different chains of transmission. However, it should be anticipated that future HIV vaccine may not be able to completely replace other HIV preventive interventions, especially if the first generation of vaccines would offer only modest levels of protection. These vaccines will need to be delivered as part of comprehensive HIV prevention packages, including other health promotion and behavioural interventions.

The development of therapeutic and perinatal vaccines is important, not only because of their own merits, but also because of their potential value as a source of critical information, which could help identify correlates of immune protection and more effectively guide the development of preventive vaccines. The introduction of HAART provides additional possibilities to improve therapeutic approaches by combining HAART and therapeutic vaccination. The potential benefits of this approach could include an increased control of HIV infection, engendering virus free state, reducing viral loads and minimizing chances for the emergence of drug-resistant strains.

2.4.2. *Approaches to HIV vaccine production*

A major conceptual problem in HIV vaccine development is the lack of information on correlates of immune protection against HIV infection or disease. Natural history studies have demonstrated that in an infected person, HIV can persist in a chronic form, and in most cases AIDS ultimately develops, even in the presence of specific immune responses. Thus, it is not completely clear what type of immune responses should be induced by vaccination in order to protect against HIV/AIDS. In addition, it should be noted that the immune responses induced in an infected individual are driven by the virus, in particular by immunodominant regions of the viral envelope, which may not be relevant for vaccine-induced protection. The type and the quantity of immune responses in the form of pre-existing (vaccine-induced) immunity required for protection against HIV infection, may be unrelated to those observed in an infected individual. Furthermore, protection mechanisms against sexual transmission at

mucosal level also may be different, as compared to the intravenous route of transmission. These scientific uncertainties justify a need for parallel development of different vaccine approaches targeting the stimulation of different arms of the immune system. The following HIV vaccine approaches have been exploited to date:

- Sub-unit vaccines: peptides and recombinant proteins
- Live-vectored vaccines
- DNA vaccines
- Whole-inactivated vaccines
- Live-attenuated vaccines

Although most of the viral vaccines used today in humans are based on the use of live attenuated viruses (e.g., polio, measles), this approach has not been promoted in the case of HIV vaccines, due to safety concerns. Similarly, inactivated viral vaccines are perceived as inherently unsafe for administration to people who have not been exposed to HIV, although these are being studied as potential therapeutic vaccines. However, both whole-inactivated and live-attenuated vaccines produced encouraging results in experimental animal models and are generating important information, which is being used in the development of safer vaccination approaches.

A large majority of experimental (candidate) vaccines for HIV/AIDS being developed today are based on the use of HIV-1 envelope proteins (gp120 or gp160), produced by genetic engineering. Related to this approach is the use of chemically-produced synthetic peptides derived from functional epitopes of the virus. The recombinant or peptide-based vaccines mostly are targeting the induction of humoral (antibody) responses. In particular, these vaccines aim to stimulate the production of anti-HIV neutralizing antibodies, which is mediated by the major histocompatibility complex (MHC) class 2 (Th2) arm of the immune system. The latest basic research on the crystal structure and function of the HIV-1 envelope has provided further guidance for the development of new generation candidate vaccines, which are based on recombinant proteins with properties approximated to those of naturally circulating viruses, i.e. epidemiologically relevant prototype strains (subtypes C, A and E) of an appropriate biological phenotype (NSI/R5), and a natural conformation structure (oligomeric, fusion-competent proteins).

In parallel, other candidate vaccines are under development aiming at stimulation of cell-mediated immunity (CTLs and lymphoproliferative responses), which is driven by the MHC class 1 response. Various viral and bacterial vectors are being evaluated as part of this strategy, including vaccinia virus, modified vaccinia Ankara strains (MVA), Canarypox virus, adeno-associated virus (AAV), Venezuelan Equine Encephalitis replicons (VEE), BCG, *Salmonella* and others. If safety concerns are addressed, live vectored vaccines, in general, are of considerable interest, since they could be easier and less expensive to manufacture, and if properly designed, could also be easier to administer. Oral administration, for example, may offer further potential benefits such as inducing mucosal immunity required for protection against sexual transmission.

More recently, direct immunization with DNA encoding HIV-specific antigens was proposed. The first results have demonstrated that this type of vaccine, when administered into skeletal muscle or dermal tissue, results in protein expression and subsequent induction of antibodies and CTLs against the encoded protein. However, the immunogenicity results, which were achieved in animal models, are not reproducible in initial human trials. Thus, further efforts are required to improve the DNA constructs and their delivery, in order to optimize immune responses with these constructs in humans.

Finally, in an effort to induce both CTL and antibody responses, a strategy has been proposed, based on the combination of two different types of vaccines, which is referred to as “prime-boost” approach. The most common combination being evaluated as part of this strategy includes priming with a vectored vaccine (e.g. Canarypox or MVA) and boosting with a recombinant protein (gp120) or DNA.

2.4.3. Phases of HIV vaccine development

Vaccine development proceeds through a series of well-defined pre-clinical and clinical phases. In the pre-clinical phase, experimental vaccines are tested in animal models to assess safety, the ability to induce specific immune responses (immunogenicity) and, in some cases, the ability to protect against experimental challenge with the virus (protective efficacy). To date, most of the known vaccine concepts have been evaluated in animals.

Some vaccine candidates have undergone phase I trials in humans, which are designed to evaluate safety and immunogenicity in a limited number of volunteers, normally between 30-50 per trial. Phase II trials are designed to obtain additional information on safety and immunogenicity, usually including larger numbers of volunteers. In addition, phase II trials are conducted in order to optimize immune responses by adjusting doses, routes of administration and use of adjuvants. In some cases, phase I and II trials are conducted as a single phase to assess or to confirm the preliminary results on safety and immunogenicity in a broader sense. Finally, phase III trials are conducted on a large-scale basis involving several thousand volunteers. These are designed as population-based field trials to assess efficacy of candidate vaccines in protecting against HIV infection or disease, but also to evaluate safety with regard to rare side effects after vaccination.

2.4.4. Experiments with animal models

Current optimism regarding the possibility of developing an effective HIV vaccine arises from the demonstration of protection in immunized animals. Three animal model systems are most widely used for HIV vaccine development purposes:

- Simian immunodeficiency virus (SIV) infection of macaque monkeys
- HIV-1 infection of chimpanzees
- Chimeric SIV/HIV virus (SHIV) infection of macaques.

Some protection experiments have also used HIV-2 infection in macaques.

In the SIV/macaque model, protective immunity was first demonstrated by using whole-inactivated virus. Protection of macaques was also achieved with live attenuated viruses. However, the overall results with other vaccine approaches have demonstrated them to be less successful and less consistent in inducing sterilizing immunity. Interestingly, using this model, it was found that immunization was more effective in slowing the disease progression, a concept that cannot be tested in the HIV-1/chimpanzee model, since chimpanzees do not appear to develop an AIDS-like syndrome. These observations have led to a shift of a paradigm concerning the expected outcomes of HIV vaccines. Thus, the prevention of disease progression is deemed an acceptable outcome if sterilizing immunity could not have been achieved.

A major breakthrough has been the development of chimeric viruses, containing SIV core and an envelope derived from HIV-1, (SHIV). The use of SHIV constructs has made it possible to evaluate vaccine concepts based on HIV-1 envelope proteins and corresponding peptides in macaques, although the role of the core component and related CTL epitopes cannot be evaluated in this model. A further improvement was the development of SHIV chimeras with pathogenic properties in macaques, providing a valuable model for testing vaccine efficacy in preventing disease progression. Attempts are being made to develop SHIV chimeras derived from field isolates belonging to non-B subtypes.

The experimental animal models have been important for studying the significance of virus variability for vaccine efficacy (heterotypic protection). Another important contribution has been the possibility of testing efficacy of candidate vaccines in inducing mucosal immunity and preventing sexual transmission of the virus.

However, each of the animal models that have been developed to date has its own advantages and disadvantages. The major concern is that there is little understanding of how the results obtained with these animal models are predictive of results in humans. This uncertainty is strengthened by the dichotomy of results obtained with different vaccine approaches in different animal models, whereby one vaccine concept tested in the HIV/chimpanzee model would demonstrate sterilizing immunity and high level of protection, while the same vaccine concept in the SIV/macaque model would exhibit extremely poor results.

In summary, animal models have been of critical importance for the development and evaluation of different vaccine concepts prior to human trials. However, the use of animal results as a criteria for the selection of candidate vaccines for human trials is limited due to the lack of knowledge about correlates of protection in animal models, and their relationship to the human situation. It is, therefore, important that parallel research is continued, whereby the results from first human efficacy trials of vaccine candidates based on safer vaccine approaches are used to validate the existing animal models. Such validation of animal models would greatly facilitate testing of the new generation of HIV vaccines, in particular those which are associated with significant safety concerns.

2.4.5. Preparation for phase III efficacy trials

To date, the progress in the development of HIV vaccines has reached an important milestone, dictating the need to initiate phase III efficacy trials of different HIV vaccine concepts. Vaccine efficacy trials under field conditions in different populations have very specific objectives, which cannot be achieved by other type of research. The questions that should be addressed are related to vaccine efficacy against infection, or against the disease, and measurement of these protection end-points. However, answering these questions require large and very complex efficacy trials.

The complexity of efficacy trials is determined by numerous variables, which need to be evaluated, including:

- a) different vaccine concepts and vaccine candidates;
- b) efficacy in achieving different vaccine end-points (prevention of infection, reduction of disease progression or reduced transmissibility);
- c) efficacy against different genetic subtypes of HIV-1 or their recombinant forms;
- d) efficacy against different routes of transmission;
- e) efficacy in different populations with different genetic, nutritional, social and economic backgrounds.

Obviously it would be impossible to address all these and many other questions in a small number of phase III trials. Therefore, multiple phase III trials will need to be conducted in parallel or sequentially, which will require extensive international collaboration. The developing countries must play an important role in this process. This is especially appropriate, since some of the highest HIV incidence rates are observed in developing countries, and greater feasibility of phase III trials in these populations is obvious. Moreover, because more than 90% of all HIV infections are occurring in developing countries, they eventually would benefit the most from an effective HIV vaccine.

Active and full participation of developing countries as equal partners in the global process of HIV vaccine development requires advanced planning and preparation for large-scale efficacy trials. These preparations should include the following: (i) establishment of infrastructures and national capacity allowing for different types of trials; (ii) conduct of preliminary epidemiological, virological, socio-behavioural and clinical research; (iii) the development of a national consensus and a framework that would ensure high scientific and ethical standards in the conduct of trials in these countries with active participation of communities.

2.4.6. The role of the World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS (UNAIDS) in the area of HIV vaccines

Since 1989, two UN Agencies, first WHO and later UNAIDS, have been actively involved in international aspects related to the promotion and coordination of activities in the area of HIV vaccines. The urgent need to accelerate the development of HIV/AIDS preventive vaccines, prompted WHO and UNAIDS to join forces in establishing a new joint HIV Vaccine Initiative (WHO-UNAIDS HVI), which was launched on January 1st 2000.

The mission of the newly created HIV Vaccine Initiative (HVI) is to promote the development, facilitate evaluation, and address future availability of preventive HIV vaccines, with a focus on the needs of developing countries.

The WHO-UNAIDS HVI maintains the continuity of the global long-term strategy, taking advantage of complementary expertise of both WHO and UNAIDS, playing a role of an “honest and independent broker”. In implementing its strategy, HVI collaborates with multiple partners, both in public and private sectors, in industrialized and developing countries, in order to effect activities in 5 priority areas, including:

- Advocacy, information, education and policy dialogue;
- Guidance and coordination of international efforts, establishing international norms and standards;
- Promoting the development of vaccines appropriate for global use, especially in developing countries;
- Facilitating the conduct of HIV vaccine trials in developing countries, through training and capacity building;
- Addressing issues of availability, access and effective use of future HIV vaccines.

The WHO-UNAIDS HVI activities are guided by a Vaccine Advisory Committee (VAC) comprising representatives of major leading national and international institutions from developed and developing countries, that are involved in HIV vaccine development and evaluation.

Recognizing the gravity and adverse impact of the HIV/AIDS pandemic and its role as a leading cause of illness and death in African countries, WHO-UNAIDS HVI has recently made a major commitment to support African countries in calling for an accelerated development of HIV vaccines suitable for use in Africa, as was recently launched by representatives of African communities on 14 June 2000 (The Nairobi Declaration).

2.5 Results obtained within the framework of the WHO Network

In 1992, the Global Programme on AIDS of the World Health Organization (GPA/WHO) established the “WHO Network for HIV Isolation and Characterization” to monitor HIV variability on a global basis. The initial results were published in the 1994 November issue of *AIDS Research and Human Retroviruses*. These results, along with further developments to date, will be summarized here. HIV-1 strains from different parts of the world were systematically isolated and characterized by the laboratory network established by WHO. Sixty-three HIV-1 isolates obtained from 224 specimens collected during 1992-1993 in Brazil, Rwanda, Thailand and Uganda were characterized [1]¹. Virus strains were first genetically subtyped using three different screening methodologies: polymerase chain reaction (PCR) gag fingerprinting, RNase A mismatch, and heteroduplex mobility assay (HMA). In addition, selected viruses were sequenced over various portions of their envelope genes. Viruses belonging to five different sequence subtypes were identified in the four countries: 16 subtype B and 1 subtype C in Brazil, 13 subtype A strains in Rwanda, 15 subtype E and 2 subtype B strains in Thailand and 3 subtype A and 13 subtype D strains in Uganda. Recent analyses showed that at least 23 of these were intersubtype recombinants formed by viral chimerization of two locally circulating subtypes [13]. Comparison of sequence data with results of the genetic screening efforts identified the HMA as a rapid and reliable method for sequence subtype determinations [2, 3].

The biological properties of patient-derived virus isolates were compared using a standardized protocol based on coculturing of patient's PBMC with phytohaemagglutinin (PHA)-stimulated normal donor PBMCs [16]. As expected, in cases of confirmed early seroconversion most isolates were found to have slow replication and low cytopathic characteristics in PBMC cultures and could be classified non-syncytium-inducing in the MT-2 cell line (slow/low-NSI phenotype) [8]. The biological phenotype correlated with the corresponding genotype and charge of the V3 loop amino acid sequences, thus confirming and extending previous observations for HIV-1 strains belonging to subtype B [7].

Using HMA, the genetic subtype as well as phylogenetic relationships within each subtype were assigned to the 55 viruses initially studied. A relatively homogenous subtype E virus population predominated over subtype B viruses in the sample set from Thailand [2]. Subtype E viruses clustered tightly, indicating little divergence and recent spread from a single point of introduction. Viruses from the other countries were also limited to one or two subtypes, but were more divergent within each subtype. The evolution of HIV-1 epidemic in Thailand was further studied [9, 10]. The results of a peptide-binding immunoassay, HMA and sequencing of the envelope V3 region were concordant and showed that the proportion of new infections due to subtype E viruses among Bangkok injection drug users are increasing significantly over time. The data highlighted the importance of monitoring the molecular epidemiology of HIV-1 in populations being considered for HIV-1 vaccine trials.

¹ For references see Annex 6.

The rapid increase in sequence data resulting from these studies called for standardized HIV sequence nomenclature. The first attempts were made to link elements critical for the specific identification of a particular sequence with biological importance [4]. By adopting standardized sequence names that included informative characters, HIV researchers could improve their interpretation of data in the broader context of previously published work. Computer-assisted analyses of the relationships between sequences and associated geographic, clinical, or viral phenotypic information thus became possible.

The use of peptide serology, with peptides corresponding to the loop structure in variable region 3 (V3) of the viral envelopes belonging to different subtypes, was also explored [5, 6]. V3 peptide serology could predict HIV-1 genetic subtypes in up to 90% of cases for subtypes A, B, C, and E; Ugandan sera from individuals carrying subtype D virus were more broadly reactive. However, there was considerable serological cross-reactivity between some HIV-1 subtypes, in particular between A and C, and, to a lesser extent, between the B and D subtypes. Subtype E sera showed the best concordance with genetic subtype. A more recent large study [12], carried out with 3 different V3 peptide-binding assays in three different laboratories, concluded that the discriminatory power of HIV-1 V3 serotyping was not sufficient to distinguish most HIV-1 genetic subtypes. In fact, with the discovery of the frequent occurrence of intersubtype recombinant viruses [17] it has been suggested that sequencing of many more HIV-1 strains would be necessary than previously anticipated in order to adequately track subtype spread. Phylogenetic analysis of the *gag* gene sequences from plasma samples of 53 individuals infected with viruses previously subtyped on the basis of envelope V3 sequence (WHO collection), showed intersubtype recombinant genomes in 23 cases. Thus, recombination is very frequent among co-circulating subtypes and their occurrence suggests that recombinants may be selected based on particularly beneficial combinations of viral gene products.

Perhaps the most pertinent question from the point of view of vaccine studies is whether there are immunological correlates of the sequence subtypes. Neutralization assays were performed with polyclonal human plasma and HIV-1 primary isolates from the four geographical locations, representing genetic subtypes A, B, C, D, and E. In parallel, independent experiments conducted in three laboratories using diverse methodologies and common reagents, no pattern of genetic subtype-specific neutralization was observed [11]. Those plasma that had neutralizing activity were broadly cross-neutralizing across two or more genetic subtypes. Hence, it was concluded that neutralization serotypes of HIV-1 field isolates are not predicted by genetic subtype. The implications of these findings for vaccine research are encouraging, since even at the level of field isolates of HIV-1, cross-neutralizing epitopes dominate in HIV positive human sera and plasma. If an immune response to these epitopes is important in conferring protection against HIV, and can be elicited by immunization, the considerable genetic variation of HIV-1 may not preclude a successful HIV vaccine.

Beginning in 1996, work done within the Joint United Nations Programme on HIV/AIDS (UNAIDS) Network focused on the implications of HIV variability on transmission and pathogenesis. The implications of genetic differences between HIV-1 subtypes, in terms of ease of transmission in different populations at risk of infection and the differential pathology they may induce, are of considerable public health and scientific interest. Examining these issues, an UNAIDS Expert Group, meeting in Berlin in March of 1996, concluded that laboratory and epidemiological data remain inconclusive and are insufficient to reject the null hypothesis that key ecological and biological properties do not differ significantly between HIV subtypes. The major reason for this uncertainty was related to the lack of studies integrating basic virology, epidemiology and social behavioural approaches. Recommendations have been made for further research to integrate virology and basic science with epidemiology [14]. The UNAIDS HIV Characterization and Molecular Epidemiology Network has become an important part of such an international collaboration effort.

A year later, a workshop jointly organized by the European Commission and UNAIDS in Dar es Salaam, further examined the possible biological differences between HIV-1 subtypes. Subtypes D and C were of particular interest. When compared to other HIV-1 subtypes, the frequency of syncytium inducing (SI) variants appeared to be higher among subtype D viruses [7, 8]. Translated to coreceptor usage, subtype D viruses with SI phenotype were often monotropic for CXCR4 usage (X4) [18, 19], whereas viruses with SI phenotype belonging to subtypes A, B and E were frequently associated with R5X4 dualtropism. Whether X4 viruses are more virulent than R5X4 viruses is an open question [8, 20, 15, 21]. Subtype C viruses were shown to differ from other subtypes in the configuration of important regulatory regions in the LTR, in that they have three or more NFkB binding sites, whereas other subtypes have one (E) or two (all others) such sites [22, 23]. A further difference became evident following this meeting, some but not all studies showed an underrepresentation of CXCR4 usage among subtype C viruses [24, 25, 26]. The workshop concluded that a framework relating genetic variation to pathogenesis and transmission of HIV is necessary to guide global research efforts [15]. Three working groups were established, epidemiology, virology and preventive technology, each emphasizing multidisciplinary and coordination of international efforts as an important requirement to reach understanding of HIV transmission and pathogenesis.

In conclusion, the WHO Network, first set up for HIV Isolation and Characterization, developed reagents, such as primary HIV-1 isolates and their clonal derivatives, and collected sera/plasma from four carefully selected sites. For the first time, a systematic genetic, biological and serological characterization was carried out of the samples collected. These reagents are available to all researchers worldwide and can be accessed through repositories in the USA [18] and in the UK [27]. In the course of the characterization of the reagents, assays were standardized and these will now be described in this Guidelines document.

3. Biosafety in Laboratories Working with HIV

This chapter summarizes the biosafety requirements to be followed throughout the entire procedure of sample collection, virus isolation and characterization, in reference to WHO Biosafety guidelines for diagnostic and research laboratories working with HIV-1.

3.1 General biosafety guidelines

The major hazard to laboratory workers working with HIV-infected blood and body fluids is contamination of hands and mucous membranes of the eyes, nose and mouth. Since there is no evidence that HIV can be transmitted through the airborne route, contamination, if it occurs, is either the result of penetrating injuries caused by sharp objects or arises from the spilling and splashing of specimen materials. The most important elements of biosafety guidelines are:

- laboratory working routines to avoid penetrating injuries and to prevent direct contact of skin or mucous membranes with HIV-infected blood
- simple protective measures designed to prevent contamination of a person or clothing, and good basic hygiene practices, including regular handwashing
- control of surface contamination by containment and disinfection
- safe disposal of contaminated waste.

The role of training in laboratory safety is vital. Poor laboratory practice and human error can negate all safety standards and render equipment hazardous. Continual on-the-job training in safety measures is essential for all laboratory and support staff.

a) Blood sample collection

- When performing venepuncture, wear a protective laboratory gown used for this purpose only.
- Wear disposable gloves and discard them as soon as they become contaminated with blood or other body fluids. Wash hands with soap and water after removing gloves.
- Never cross the arms while handling a needle or other sharp objects.
- Place used needles and syringes in a puncture-resistant container. Do not recap needles after use.

b) Serological laboratories

Laboratory facilities

- It is desirable to have a laboratory (or laboratory room) devoted exclusively to working with HIV-contaminated material. If this is not possible, a secluded and clearly identified working area should be provided within the laboratory.
- Biological safety cabinets are not required for serological testing of potentially HIV-contaminated material. Safety glasses or face shields should be worn to protect the eyes and face from splashes.
- There are no specific ventilation requirements. Windows that open should be fitted with fly screens.
- The walls, ceiling and floor should be smooth, easily cleaned, impermeable to liquids and resistant to chemicals.
- The bench tops should also be impermeable to liquids and resistant to chemicals.
- The laboratory furniture should be sturdy and easily cleaned.
- Washbasins should be provided in each laboratory room, preferably near the exit.
- Laboratory room doors should be self-closing and have vision panels, and a “Biohazard - No Admittance” sign posted.
- An autoclave for the decontamination of laboratory material and waste should be available in the same building as the HIV laboratory.
- Facilities for storing clothes and items for eating, drinking and smoking should be provided outside the workroom.

Precautions for laboratory workers

- Wear gloves for all manipulations of potentially infectious materials. Discard gloves whenever they are thought to have become contaminated or damaged, wash hands with soap and water and put on new gloves.
- Wear a laboratory gown; wrap-around gowns are preferable. Remove this protective clothing and leave it in the laboratory when leaving.
- Never pipette by mouth.
- Eliminate the use of glassware as much as possible, since broken glassware may be the source of injuries.
- Perform all technical procedures in a way that minimizes the creation of aerosols, droplets, splashes or spills. People handling clinical samples should pay attention to the particular geographical origin of the samples, since other pathogens, requiring particular precautions, may be present.
- Access to the laboratory should be restricted to essential personnel. A baseline serum sample should be obtained from each of these personnel and stored for future reference.

c) *Virus isolation laboratories*

The biosafety guidelines for serological laboratories also apply to virus isolation laboratories, but they should be reinforced. All procedures involving infected cell culture manipulation should be performed in physical containment equipment, such as a biological safety cabinet and sealed centrifuge buckets or rotors.

- It is most desirable that there be a laboratory room devoted exclusively to working with HIV- contaminated material.
- Biological safety cabinets (class I or II) are the equipment of choice for HIV isolation work. A class I biological safety cabinet is recommended for laboratories where expertise and equipment are not available for the routine testing of air filters, cabinet tightness, and balanced air flow. A class II biological safety cabinet requires skilled routine testing and servicing. Biological safety cabinets must be properly installed and routinely tested and serviced; failure to do this may render the cabinet ineffective and dangerous.
 - A class I biological safety cabinet is an open-fronted work chamber which is exhaust-ventilated to provide protection for personnel and the surrounding laboratory space by means of an inward air flow away from the operator. The exhaust air is filtered through a high-efficiency particulate air (HEPA) filter before being discharged from the cabinet. The cabinet is not designed to provide protection of the material.
 - A class II biological safety cabinet is a partially open-fronted work chamber that provides protection for personnel and the surrounding laboratory space by means of a barrier air flow at the work opening. The cabinet also provides product and/or experiment protection against contamination by means of HEPA-filtered air flowing in a downward, uniform, unidirectional manner (laminar air flow).
- Sealed centrifuge buckets or rotors should be regularly checked for integrity, to prevent the accidental dispersion of any material from the centrifuge. They should be loaded and unloaded in a biological safety cabinet.
- The windows in the laboratory should be closed and sealed.

d) *Research laboratories*

These guidelines apply to laboratories working with or producing less than 10 litres of virus suspension. The biosafety guidelines for virus isolation laboratories apply to research laboratories, but they are strengthened with regard to the following:

- Entrance to the laboratory from access corridors should be through a set of two doors. This may form a double-doored clothes-changing room, preferably with an air-lock.
- The access doors should be self-closing.
- A ducted exhaust-air ventilation system should be provided, creating a directional flow that draws air into the laboratory through the entrance and maintains the laboratory under negative pressure.
- The HEPA-filtered exhaust air from biological safety cabinets should be discharged directly outside or through the building's exhaust air system.

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- A washbasin operated by foot, knee, elbow or automatically should be provided next to the exit door.

3.2 Spills and accidents

- Spills of blood or other body fluids should first be covered with paper towelling or other absorbent material. A disinfectant - a hypochlorite solution at a concentration of 1.0%(v/v) available chlorine (10 g/litre) - should be poured around the spill area and then over the absorbent material and left for 10 minutes. The solution should then be removed with absorbent material and placed in a container for contaminated waste. The surface should then be wiped again with the disinfectant. Avoid direct contact of gloved hands with the disinfected spill. Broken glass or fractured plastic should be collected with a dustpan and brush.
- Needlestick or other skin-piercing wounds, cuts and skin contaminated by spilled or splashed specimen material should be immediately disinfected (10% (v/v) polyvidone iodine solution) and thoroughly washed with soap and water. Bleeding from such wounds should be encouraged.
- All spills, accidents and overt or possible exposure to infected or potentially infected material should be reported immediately to the laboratory supervisor. A written record should be prepared and maintained.

3.3 Handling and disposal of contaminated material and waste²

- Disposable contaminated equipment, e.g. needles, syringes and other sharp instruments or objects, should be placed in a puncture-resistant metal or plastic container at the work station. The container should be autoclaved or chemically disinfected.
- Laboratory gowns, coats and other protective clothing should be placed in a separate container located within the laboratory. Before reuse, they should be autoclaved and washed.
- Incineration is the method of choice for the disposal of contaminated material and waste if the incinerator is located on laboratory premises. If the material has to be removed from the premises, it must be autoclaved or otherwise disinfected beforehand.

² For further details, see *Biosafety guidelines for diagnostic and research laboratories working with HIV*. Geneva, World Health Organization, 1991 (WHO AIDS Series, No.9).

4. Guidelines for Genetic Typing of HIV

4.1 Prevention of sample contamination

Sample contamination is a critical problem for genetic screening protocols due to the extreme sensitivity of the polymerase chain reaction (PCR). Nested PCR (see Chapter 10) can detect single molecules of the viral genome, and after amplification, a 100 µl volume can contain 10^{12} - 10^{13} viral molecules. Thus, if 1 µl is aerosolized, 10^{10} - 10^{11} molecules are released. To dilute 10^{10} molecules to less than 1 molecule per 100 µl (volume of a typical PCR reaction) requires 10^6 litres of diluent, or a space of 10m x 10m x 10m !

Sample contamination can occur in a number of ways. Carry-over contamination occurs when amplified product enters the PCR tube and then is re-amplified; it usually results from reagent contamination and is especially problematic with nested PCR. Contamination can also result from external sources such as contaminated surfaces and aerosols. Tests for contaminated reagents should be included in each PCR experiment and strict methodological guidelines should be observed to prevent contamination by experimental design.

Note: the most powerful and effective means of preventing PCR carry over contamination is good laboratory practice.

Guidelines for prevention of sample contamination:

- If possible, establish separate pre-PCR and post-PCR rooms and limit access to the pre-PCR room. The pre-PCR laboratory should not be used by anyone after they have handled PCR reactions on a given day. If, despite all efforts, contamination would occur, pipette should be cleaned by rinsing with 1M HCl or 1M NaOH as appropriate. New aliquots should be used from each reagent.

Pre-PCR:

- The pre-PCR room should be used for three procedures only - reagent preparation, nucleic acid extraction, and PCR reaction set up. Ideally, there may be two pre-PCR rooms, one for preparation of buffers and reaction mixtures that remains nucleic acid-free and another for nucleic acid extraction and PCR reaction set up.
- All buffers and reaction mixes should be prepared and aliquoted in this room.
- Pipettes and other instruments needed are always kept in this room and used exclusively for pre-PCR activities.

-
- No thermocyclers, plasmids or PCR products are allowed to enter this room, no reagents are allowed back into this room after being removed. The only exception is that positive control samples containing viral DNA templates need to be used in the pre-PCR room, but they should be prepared as dilute solutions outside the pre-PCR room.
 - Keep cleaning supplies and brooms etc. in this room and do not allow cleaning personnel in, to prevent them from tracking PCR products in from other rooms.

Laboratory clothing:

- No lab coats or gloves used in a laboratory where PCR products or plasmid preparations are handled (e.g., the post-PCR room), should enter the pre-PCR lab.
- Dedicated lab coats are used in the pre-PCR lab and should not leave the room.

Equipment and tube handling:

- All pipetting devices used should have barrier plugs (cotton plugs in disposable tips for micropipette, or in the barrel of large pipette), or positive displacement pipette tips should be used.
- Always allow tubes to thaw completely, and mix the contents well before pipetting, since the distribution of dissolved solutes becomes non-uniform during the freezing process.

Reaction setup and reagent handling:

- Use negative controls (PCR reactions without template DNA) for each PCR, and an additional negative control interspersed with patient samples for each 10-20 samples.
- Reagents used in the master mixes described below should be aliquoted in amounts suitable for one experiment and stored frozen, and then thawed only once (any left over should be discarded).
- Reagents should not be shared with anyone.
- Prepare master mixes by adding reagents in the following order to reaction tubes:
 1. 10X buffer plus dNTPs
 2. H₂O
 3. Primers
 4. DNA polymerase
 5. Template

Note: Put away each reagent before working with the next reagent on the list to rule out contamination of the preceding reagent with subsequent solutions.
- If second round PCR is to be conducted the same day as the first round, master mixes for both rounds should be prepared at the same time and the latter moved to the post-PCR room for later handling.

Post-PCR room(s):

- Ideally, two post PCR rooms or areas should be used. One for the pipetting associated with the second round of nested PCR, and a second for post-PCR steps such as gel electrophoresis.
- Wear lab coats, gloves, and overshoes.
- Wash hands and remove coats before leaving the room.
- Do not enter the pre-PCR room after working in the post-PCR room without showering (to remove PCR products that may be in hair) and changing clothes. It is highly preferable to not return to the pre-PCR room the same day after working with PCR products in the post-PCR room.
- Always centrifuge reaction tubes before opening to prevent liquid near the top of the tube or on the cap from being released.
- Use a paper towel fragment to hold and open each tube then discard the towel. This prevents gloved fingertips from getting contaminated easily and can trap droplets that are released when the tube is opened. It also creates a barrier between your glove and the next tube you handle.
- Use dilute bleach (followed by a thorough water rinse), 70% ethanol, or soap and water to decontaminate surfaces before and after each experiment, dry with towel.
- Keep cleaning supplies and brooms etc. in this room and do not allow cleaning personnel in, to prevent them from tracking PCR products out to other rooms.

4.2 Sentinel test for PCR contamination:

The sentinel test is a very simple and useful method that should be used periodically for detecting PCR fragments that may be contaminating a laboratory. Identification of contamination through the sentinel test is useful for recognizing an existing problem, and is indicative of the need for changes in laboratory procedures to prevent aerosol contamination.

- Fill several PCR tubes with 50 µl of distilled water and place them around the lab at different locations (on the laboratory bench, sink, centrifuge, etc.).
- At the end of the day close the tubes.
- The next day test 10 µl of the content of the tubes with the same primers used for fragments handled the previous day.

4.3 Reaction “sterilization”:

The most powerful means of preventing PCR carry-over contamination is good laboratory practice. However, contamination can and still does occur at times and may be extremely difficult to recognize. Multiple inactivation or sterilization protocols have been developed that use chemical, photochemical and enzymatic methods for eliminating contaminating molecules. Some of the most important methods are described in Annex 4.

Note: Since none of the methods are 100% efficient, they should not be relied upon to replace appropriate techniques and careful reagent handling.

4.4 Safe handling of phenol/ CHCl_3 used for DNA extraction:

- Always wear gloves, eye protection and a lab coat.
- Work in a fume hood and exercise extreme care when handling phenol.
- Rinse glassware carefully before putting into dirty glassware trays.

Very small amounts of skin exposure can be washed off (and into) skin with 95% ethanol without problem. Large areas of skin contact should be extensively washed with water (to avoid substantial penetration) and get to a physician for burn treatment.

5. Subject Selection

Selection of a target population for the collection of clinical samples should be based on the epidemiology of HIV in a particular country, and priority should be given to the collection of samples from potential target groups or cohorts which might eventually be considered for vaccine efficacy trials. HIV isolation from asymptomatic individuals who acquired infection within the past 1-2 years is preferable, ideally from people who recently seroconverted and for whom comprehensive epidemiological information is available. Random sampling from the general population, as well as from groups identified as being at greater risk of HIV infection, would provide information on HIV variability on a broader scale. A few individuals who are seronegative (or serologically indeterminate), but have unambiguous clinical immunodeficiency, should also be included in sample groups.

Preference should be given to incident cases of HIV infection identified as part of cohort studies. To allow extension of vaccine trials to HIV-2 endemic areas, samples collected should reflect the relative seropositivity for HIV-1 and HIV-2, and include double reactors, if possible.

Samples collected from subjects not treated with antiviral agents, particularly zidovudine (AZT) are preferable because treatment with such agents may interfere with future neutralization assays. It has to be emphasized that subject selection should be as broad-based as possible with respect to anticipated viral variability. Subjects from whom further blood samples can subsequently be collected are preferred.

Laboratories at potential sites for vaccine evaluation should have the facility to carry out serological tests for diagnosis of HIV infection. Enzyme-linked immunosorbent assay (ELISA) and Western blot analysis to identify and confirm the antibody status of infected individuals and to differentiate HIV-1 and HIV-2 infections should be in place. Participating individuals should be classified within one of the following serological categories: (a) HIV-1 positive; (b) HIV-2 positive; (c) HIV-1/HIV-2 double reactive; (d) serologically indeterminate; (e) seronegative.

For screening purposes, any appropriate ELISA kit may be used. For confirmation of a positive ELISA reaction and to identify new genetic variants of HIV, Western blot analysis should be used. A positive ELISA reaction is considered confirmed if antibodies reacting with at least two *env* bands (precursor (gp160), external glycoprotein (gp120) or transmembrane glycoprotein (gp41)) can be detected by Western blotting. Antibodies to *gag* and *pol* products may or may not be present.

Subject information should be recorded on a standardized reporting form provided by WHO. This form collects minimum essential information on epidemiological, laboratory and clinical features of the subject. It is therefore important to complete all the items on this form. The form and instructions for its completion are shown in Annex 2.

Designation of HIV isolates should be according to the convention used for naming influenza virus isolates:

Year location (two letter code for the country) serial number (three digits).

Example: 92UG001.

Tubes should be labelled according to sample type: C for cells, S for serum, P for plasma and L for lysate.

6. Specimen collection

Experience accumulated over the last 17 years indicates that blood is the best source for HIV isolation as well as genetic typing of HIV strains. Since blood samples can easily be collected and peripheral blood mononuclear cells (PBMC) can be readily separated, the most commonly used HIV isolation procedure involves the use of PBMC. HIV replicates in PBMC of human blood and has been shown to preferentially replicate in the CD4+ subset of T lymphocytes.

6.1 Collection of blood

Blood is obtained by aseptic venepuncture. Since the most sensitive virus isolation procedure makes use of PBMC, blood for virus isolation should be prevented from clotting by addition of anticoagulant (EDTA to a final concentration of 4 mM or sodium citrate to a final concentration of 12.9 mM). The use of the anticoagulant heparin should be avoided, since it inhibits the PCR reaction by binding to the DNA polymerase used for PCR, and must be removed before reverse transcription of viral RNA and PCR from plasma.

Before performing venepuncture read the safety regulations in section 3 of this Guidelines.

For venepuncture the following equipment can be used:

- a) Safety-Monovette used according to the vacuum principle. 10 ml tubes containing an appropriate anticoagulant are commercially available. Special Monovette needles with fixed guide-sleeve are also available in sizes 21G and 22G, but Monovettes are also adaptable to use with a Luer cone. Monovette tubes fulfil the criteria for blood sample transportation, since they are made of polypropylene and are equipped with a screw cap.
- b) Vacutainer tubes of different sizes with anticoagulant solution added are commercially available. The size of the most generally used needle in combination with vacutainer tubes is 21G (40mm 8/10) or 22G (40mm 7/10). To ensure the mixing of anticoagulant and blood, each vacutainer tube should be gently inverted twice, immediately after it has been detached from the needle.
- c) In the event that collection of blood is performed with a 50-ml syringe, the syringe should be prepared with 0.4 ml of anticoagulant stock solution (EDTA or sodium citrate) prior to drawing the blood. See Annex 3 for preparation of stock solutions.

For serology, the blood should be collected without anticoagulant and allowed to clot at room temperature.

To ensure that a sufficient amount of material will be available for analysis, the following blood sample volumes are recommended:

	Volume of blood sample for	
	Virus isolation (ml)	Serology (ml)
Adults	40	10
Children (age: 2-12 years)	10	5

Blood samples should be kept at 18-22°C until processing. Whole blood samples should not be frozen. Blood should be kept at 18-22°C during transport.

6.2 Sample processing

a) If the field laboratory has facilities for serological tests only:

Blood samples should be immediately transported to the laboratory/repository where the separation of PBMC will be performed promptly from the anticoagulant-treated samples. Timing of blood separation within the initial 24 hours has no major impact on HIV isolation frequency. However, separation more than 24 hours after sampling may decrease isolation frequency from the blood of asymptomatic individuals.

b) If the field laboratory has facilities for sterile work (cell culture facilities):³

Samples for virus isolation (anticoagulant-treated) should be dealt with as soon as possible after the blood is drawn. Depending on the volume of blood collected, 15-ml or 50-ml centrifuge tubes should be used for the separation of PBMC. These tubes should be of tissue culture grade, sterile, translucent, conical and should have a screw cap.

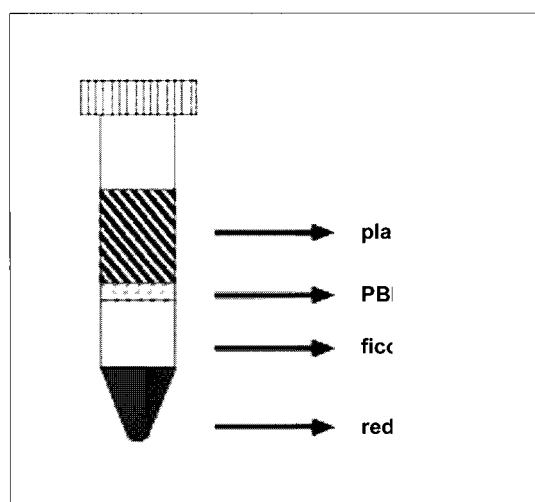
Ficoll and phosphate-buffered saline (PBS) should be prewarmed to 18-22°C before use.

Procedure I: Appropriate for smaller volumes of blood or when dilution of plasma is to be avoided.

1. Maximum 8 ml whole blood is placed on the top of 4 ml Ficoll in a 15-ml centrifuge tube (Figure 5). The tube is centrifuged at 2000 rpm (626 x g) for 10 minutes or 1500 rpm (352 x g) for 20 minutes without brake.

³ All procedures are performed under strict sterile conditions, i.e. vials, pipette-tips and solutions should be purchased sterile or adequately sterilized before use.

Figure 5: Centrifugation of whole blood using discontinuous Ficoll density gradient



Procedure II: Used for separation of 40 ml blood (one disadvantage of this procedure is that plasma is diluted 1:2).

1. Divide the 40-ml blood sample between two 50-ml centrifuge tubes. Add PBS up to a volume of 30 ml in each tube and mix by gentle inversion. Carefully layer 13 ml (or 14 or 15 ml) Ficoll under the sample using a 10-ml pipette. Centrifuge the tubes as above.

The following steps are common to procedures I and II.

2. Remove most of the upper layer (down to approximately 1 cm from the PBMC layer) with a pipette (5-ml or plastic pasteur pipette, sterile, disposable; the pipette of choice can be used throughout) and transfer the plasma to a new tube. Centrifuge at 2500 rpm (978 x g) for 10 minutes to remove remaining cells. After centrifugation, transfer the plasma (upper layer) to 1.8-ml cryotubes (10 aliquots, 1.8 ml each) and store frozen at -70°C. Dilution of plasma with PBS prior to Ficoll-separation must be noted.
3. Remove the remaining plasma from above the PBMC and collect cells carefully so they do not mix with the remaining Ficoll.
4. Transfer the PBMC to a new 15-ml tube, fill the tube with PBS and centrifuge at 1200 rpm (225 x g) for 10 minutes.
5. Decant supernatant, resuspend cells in remaining droplet and add 5 ml PBS.
6. Count cells, preferably in a disposable "KOVA Glasstic slide 10" with grid chamber (grid volume = 1 ml). Count at least 5 small grids within different quadrants of the counting grid and calculate sample cell number (average count/grid x 90 = cells/ml sample). See Annex 3 for further details.
7. Distribute cells into 15-ml tubes, 5-20 x 10⁶ cells per tube. Use a minimum of 4 tubes (maximum 10 tubes). If virus isolation is to be carried out in the same laboratory, use 2 x 10⁶ cells for this purpose (see section 8 for details). Fill all tubes with PBS and sediment cells at 1200 rpm (225 x g) for 10 minutes. Decant supernatant and resuspend cells in the remaining droplet.

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8. Resuspend cells in 1 ml fetal calf serum (approved by the Food and Drug Administration, USA) containing 10% dimethyl sulphoxide (DMSO).⁴
 9. Label the 1-ml cryotubes with date, name of site and subject identification number (see section 5 for labeling details).
 10. Transfer cells to cryotubes.
 11. Freeze at -70°C overnight: place cryotubes in a thick-walled plastic or polystyrene box at room temperature and place the box in a -70°C freezer. This will ensure slow, gradual freezing of material with minimal damage to living cells. Transfer to liquid nitrogen the next day or ship to repository.

Samples must be kept frozen in dry ice (solid carbon dioxide) during transport. For packaging instructions see section 7, below.

c) *Preparation of serum samples*

Samples for serum collection should be centrifuged at 1500 rpm (352 x g) for 10 minutes. Aliquot serum into labelled (date, name and subject identification number) 1.0-ml cryotubes (0.5 ml per tube) and freeze at -70°C. (This low temperature storage is necessary to preserve the viral genomic material (RNA and DNA) that will be subsequently analysed.)

d) *Preparation of viral genome templates for PCR: see Chapter 11*

⁴ Fetal calf serum may be used undiluted or diluted 1:2 with RPMI medium.

7. Shipment of Specimens⁵

Packaging has three main purposes: to maintain the specimen's viability; to prevent it from leaking; and to prevent cross-contamination. To satisfy these requirements, the packaging should be in three layers:

- a) The primary receptacle, containing the specimen, blood, cells or virus, which should be sealed watertight.
- b) Tubes should be individually wrapped in a sufficient amount of tissue paper or absorbent cotton to absorb the total volume of the sample. The wrapped tubes should be placed in a sealed plastic bag (secondary packaging).
- c) Outer packaging: to protect from physical damage, temperature fluctuations, etc. while in transit.

7.1 Shipment of blood

Blood collected in Safety-Monovette tubes can be shipped directly, since the polypropylene tubes do not break and have leakproof screw caps.

Blood collected in vacutainers (or syringes) should be transferred immediately into conical 50-ml plastic tubes. These will not break during transport and can be sealed properly. Never ship any glass tubes.

- 1. Tighten screw caps of plastic tubes and seal with tape of woven type (not polyvinyl chloride, which is liable to retract and separate from the tube) or parafilm.
- 2. Label the tubes appropriately.
- 3. Wrap the tubes individually in tissue paper or absorbent cotton.
- 4. Place the tubes in sealed plastic bags.
- 5. Place the plastic bags in a container that protects from physical damage and temperature and pressure fluctuations. The container should be lined with shock-absorbing padding (loosely packed paper or absorbing cotton wool) and should have a tight-fitting lid (screw-on or push-on) that is taped shut or clipped.

⁵ For further details, see Madeley, C.R. Guide to the collection and transport of virological specimens (including chlamydial and rickettsial specimens).

6. In order to prevent delays in transit, all sample labelling must include:

- name and address of sender
- name and address of recipient
- customs declaration
- warning label

Postal, governmental and carriers' regulations should be consulted in advance as appropriate.

7. A subject report form, providing clinical and epidemiological information, must accompany each blood sample. Blood should be kept at 18-22°C during transport, even inside aircraft. Please inform air-carrier accordingly.

7.2 Shipment of cells, serum samples and virus

The primary receptacle for cells, serum and virus should be sterile cryotubes of appropriate size with screw caps.

1-5. As described above for shipment of blood samples.

Since these samples must be kept frozen (-70°C) during transport, the outer package described above will need to be enclosed in an insulated box together with dry ice (solid carbon dioxide). The outermost packaging must permit the release of CO₂ gas. The samples must be supported in such a way as to prevent them becoming loose as the dry ice evaporates.

Liquid nitrogen is not suitable unless specimens are personally accompanied.

6. As described above for blood samples.

7. Cell and serum samples must be accompanied by a subject report form. To provide additional information on virus isolation, virus samples must be accompanied by an HIV isolate data sheet.

8. HIV Isolation

The recommended HIV isolation procedure involves coculture of subject PBMC with phytohaemagglutinin (PHC)-stimulated PBMC from seronegative donors, usually blood donors. In order to impose the smallest possible selective pressure on the viral isolates, isolation must be carried out in primary cultures of cells and not in cell lines. The role of donor PBMC is potentially three-fold. First, they provide antigenic stimulation (allogenic stimulation) of the subject's PBMC and thereby induce expression of the interleukin-2 (IL-2) receptor on the surface of the subject's T-lymphocytes. As a consequence, such cells will become susceptible to the growth stimulatory effect of exogenous IL-2 added to the medium during virus isolation. DNA synthesis and cell division may stimulate latently infected T-lymphocytes to virus production and increase the chance of successful virus isolation. Second, donor PBMC may provide susceptible target cells for HIV replication. Since different donor PBMC may vary in susceptibility to HIV infection *in vitro*, whenever possible the use of a mixture of PBMC obtained from at least two donors is recommended. A third effect is that when using donor PBMC in excess, the potentially inhibitory effects of the subject's CD8 positive cells in the sample are diluted out. This may enhance virus isolation, at least in some cases.

8.1 Preparation of PBMC from a seronegative donor

Ficoll separation

If whole blood is used, follow procedure II for Ficoll separation of subject's PBMC described in section 6.

If buffy coat (leukocyte-enriched whole blood, from which 80-90% of red cells and plasma have been removed) is the starting material, separate the donor PBMC according to the following procedure:

1. Divide the 40-ml buffy coat sample between two 50-ml tubes. Add PBS (prewarmed to 18-22°C) up to a volume of 30 ml in each tube.
2. Carefully layer 15 ml Ficoll under the sample using a 10-ml pipette.
3. Centrifuge the tubes at 1500 rpm (352 x g) for 30 minutes without brake.
4. Remove most of the upper layer. Collect the PBMC carefully, without disturbing the Ficoll.
5. Transfer the PBMC to a new 50-ml tube and wash cells twice in 45 ml PBS by resuspension and centrifugation at 1200 rpm (225 x g) for 10 minutes.
6. Cells can then be frozen in aliquots of 10×10^6 cells/cryotube or used directly for phytohaemagglutinin (PHA) stimulation.

Stimulation of donor PBMC with phytohaemagglutinin

PHA will stimulate DNA synthesis in T lymphocytes and induce expression of the IL-2 receptor, thereby rendering the cells susceptible to the growth stimulatory effect of IL-2 added to the medium during virus isolation.

1. After the second PBS wash (see 5 above) resuspend cells in 10 ml RPMI supplemented with 10% fetal calf serum (RPMI 10%) (for details of tissue culture media see Annex 3).
2. Divide cells between three 75-cm² tissue culture flasks (sterile, translucent, screw cap). Add 40-100 ml RPMI medium to adjust cell concentration to 1×10^6 cells/ml. Add 100 μ l PHA to each flask (to a final concentration of 2.5 μ g PHA/ml).⁶ Cells from different donors should not be mixed at this point; use separate flasks for each donor. Cultures should be kept at 37°C in a humidified atmosphere (5% CO₂), in an upright position and with flask caps slightly loosened.
3. Donor PBMC can be used for coculture after 2-5 days (optimum 3-4 days) of PHA stimulation.
4. Prior to use, decant most of the medium and count the cells as described in section 6. No centrifugation is necessary. If possible, mix cells from two donors and use in coculture with subjects' PBMC for virus isolation.

PHA stimulation of frozen donor PBMC

1. Thaw cryotubes rapidly in lukewarm water. Thaw twice as many cells as needed.
2. Transfer cells to a 50-ml centrifuge tube. Add 10ml RPMI 10% medium, prewarmed to 18-22°C.
3. Add RPMI 10% to 45 ml and centrifuge at 1000 rpm(156 x g) for 10 minutes.
4. Resuspend cells in 10 ml RPMI medium and follow the procedure for PHA stimulation described above.

8.2 Virus isolation

- Day 1: Mix 2×10^6 subject PBMC with 10×10^6 PHA-stimulated blood donor PBMC (preferably from two donors) in a 25-cm² tissue culture flask and bring to 10 ml with IL-2 medium. Flasks should be incubated as described above. For each batch of donor PBMC a control culture containing donor PBMC alone should be included. For this purpose, 10×10^6 PHA-stimulated donor PBMC (preferably from two donors) is cultured in IL-2 medium and culture fluids are regularly harvested and tested for p24 antigen.
- Day 4: Half of the volume of culture medium is exchanged for fresh medium.

⁶ the final concentration may be increased up to 10 μ g PHA/ml. The optimal concentration of PHA used may be determined in each laboratory.

Method A:

Day 7: Harvest cultures as follows:

1. 4 ml (4 x 1-ml aliquots) supernatant culture fluid for storage at -70°C (virus stock).
0.9 ml supernatant culture fluid for p24 antigen ELISA.
2. 2.5 ml medium containing cultured cells to be frozen in liquid nitrogen (add 2.5 ml FCS with 20% DMSO and freeze in three aliquots in liquid nitrogen).
3. Add 3 x 10⁶ fresh blood donor PBMC (PHA-stimulated) to the remaining culture.
4. Restore culture volume to approximately 10 ml with IL-2 medium.

Day 10: Repeat steps 1, 2 and 4 from day 7 procedure.

Day 14 & 21: As day 7.

Day 17 & 24: As day 10. Note: p24 antigen-positive cultures can be discontinued on day 17 and thereafter if antigen levels are >2000 pg/ml in two sequential ELISA tests. Whenever possible, a reverse transcriptase assay on high-speed centrifugation pellets of culture fluids should be carried out before culture is discontinued. Alternatively, immunofluorescence on fixed cells with known HIV antibody-positive serum may be performed.

Day 28: Isolation attempts are terminated. p24 antigen-negative cultures and corresponding frozen supernatant should then be discarded.

Method B:

Day 7: Harvest 0.9 ml supernatant culture fluid and analyse using a p24 antigen ELISA. If culture is antigen-positive, harvest as described above for Method A. If culture is antigen-negative:

- gently swirl the flask to resuspend cells and remove 4 ml (split ratio = 1:2)
- add 3 x 10⁶ fresh blood donor PBMC (PHA-stimulated).
- restore culture volume to 10 ml with IL-2 medium.

Day 10: Harvest 0.9 ml supernatant culture fluid and analyse using p24 antigen ELISA. If culture is antigen-positive, harvest as described for Method A. If culture is antigen-negative, repeat steps 1 and 3 from day 7 procedure.

Day 14 & 21: As day 7.

Day 17 & 24: As day 10. Note: p24 antigen-positive cultures can be discontinued on day 17 and thereafter if antigen levels are >2000 pg/ml in two sequential ELISA tests. Whenever possible, a reverse transcriptase assay on high-speed pellets of culture fluids should be carried out before culture is discontinued. Alternatively, immunofluorescence on fixed cells with known HIV antibody- positive serum may be performed.

Day 28: Isolation attempts are terminated and p24 antigen-negative cultures should be discarded.

A complete specimen should yield a minimum of five frozen vials of PBMC coculture and 10 x 1-ml aliquots of p24 antigen-positive supernatant culture fluid, designated as primary virus stock.

Practical considerations

Since flasks are stored standing up in the incubator the cells will sediment on the bottom of the flask. Handle flasks carefully; cell-free medium can then be conveniently harvested and there is no need to remove cells by centrifugation. Change the pipette between each flask.

For p24 antigen ELISA, add the 0.9 ml medium sample to tubes containing 0.1 ml 5% Triton-X 100. Samples may be stored at -20°C prior to testing.

Following the harvest of medium, cultures can be observed microscopically.

Note: Keep flask in the horizontal position for as short a time as possible during microscopic observation.

Subsequently, cells can be resuspended in the remaining medium by gentle movement. 2.5 ml of culture fluid containing half of the cells can then be removed. Storage of cells is important for future polymerase chain reaction (PCR) studies as well as for re-culturing if necessary.

Note: Prepare protocols and label tubes prior to the harvest of medium and cells. During harvest, repeatedly cross-check the labels of flask and tubes against the protocol.

For details of the p24 antigen ELISA, reverse transcriptase assay and immunofluorescence on fixed cells see Annex 3.

9. Biological Characterization of HIV Isolates

Knowledge about the biological characteristics, replicative capacity, cytopathogenicity and receptor usage of the virus isolates facilitates antigenic characterization by virus neutralization. Two types of biological characterization will be distinguished: a) primary characterization that involves systematic monitoring of the primary PBMC cultures for virus content and signs of cytopathic effects; and b) extended biological characterization that assesses viral replication in established cell lines. Primary characterization can be carried out by all laboratories that isolate HIV, whereas extended biological characterization has to be performed by laboratories with appropriate facilities.

It is recommended that virus isolation and characterization involving cell lines be physically separated, that is, carried out in two different laboratory rooms. This will minimize the risk of contamination of primary isolates by a virus strain replicating in cell lines. In addition, it will prevent contamination by mycoplasma - a problem often encountered when working with cell lines. It is mandatory that laboratories working with cell lines regularly carry out mycoplasma testing of all cell lines used and, when positive, take steps to remove the mycoplasma.

9.1 Primary characterization.

The virus isolation procedure outlined in section 8 includes the possibility of systematic monitoring of virus replication as well as cytopathic changes in the primary PBMC cultures. Since the time of appearance, as well as the amount, of virus are important, cultures should be observed regularly according to a standard protocol (see Annex 3). The same protocol incorporates information about the culture's condition, occurrence of cytopathic effect(s) such as syncytia formation, ballooning and/or cell death, the addition of new cells, harvests and the results of p24 antigen ELISA. The isolation protocol should give a complete account of the process of virus isolation including biological properties of the virus as it appears in the primary culture.

PBMC cultures derived from HIV-1 infected individuals with normal CD4+ lymphocyte counts in blood, and usually no (or mild) symptoms of HIV infection, are likely to yield virus after several weeks *in vitro*. Not only is virus production delayed in such cultures, but viral titres remain low, even after a prolonged period of cultivation. In contrast, PBMC from severely immunodeficient subjects with low CD4 counts in blood, often yield high amounts of virus, even during the first two weeks *in vitro*. The designations slow/low and rapid/high, respectively, emphasize the characteristic differences in replicative capacity of HIV isolates in PBMC cultures [28]. We know today that these differences are due to the differential use of chemokine receptors as coreceptors to CD4 for entry of cells [29].

Activated CD4+ T-lymphocytes express high levels of the CXCR4 molecule resulting in the rapid and high level replication of viruses using this molecule as receptor. Appearance of the CCR5 molecule is much slower, it needs not only PHA activation but prolonged exposure to IL-2. In addition, the replication of viruses using CCR5 may be further inhibited by β -chemokines produced by the PBMC cultures. Since the majority of HIV-1 isolates use CCR5 (designated R5 viruses) and are sensitive to inhibition by β -chemokines [30], they will replicate slowly in PBMC cultures. This makes it important that virus-negative cultures be continued for four weeks; they may be exceptionally extended to five weeks.

Viruses replicating in PBMC cultures display distinct cytopathic characteristics. Accordingly, slowly replicating viruses may not display any cytopathic effect at all. Some R5 isolates form small syncytia that are sparsely present in PBMC cultures (Figure 6a), other isolates kill single cells (Figure 6b); Syncytia formation and single cell killing may occur in the same cultures. Extensive syncytium formation accompanies replication of CXCR4-using viruses (Figure 6c, for figure 6 see end of section 9). Syncytial cells are variable in size but may encompass an entire clump of cells. Ballooning of cells is particularly easy to see at the edge of clumps. Based on these cytopathic characteristics, HIV-1 isolates have also been named non-syncytium-inducing (NSI) and syncytium-inducing (SI), respectively. Again, whether a virus behaves as NSI or SI in PBMC cultures depends on its coreceptor usage. High level expression of CXCR4 in PHA-stimulated PBMC allows abundant replication of viruses that use this receptor, with efficient syncytium induction as a consequence. In contrast, the delayed and low level expression of the CCR5 receptor will limit replication and syncytium induction of R5 viruses in the PBMC cultures. Since the cytopathic effects may be slight and in some cases transient, cultures should be observed twice a week for the entire isolation period.

9.2 Extended biological characterization.

This involves cell-free transfer of viruses or cocultivation of infected donor PBMC with cell lines. The virus dose can be standardized on the basis of p24 antigen content, 1 ng/10⁶ cells, reverse transcriptase activity, 20.000 cpm/10⁶ cells or infectious virus, measured as median Tissue Culture Infectious Dose 50% (TCID₅₀) on PBMC. When the virus dose is based on infectious virus titres, titration of virus stocks is ideally performed in connection with infection of PBMC or cell lines. TCID₅₀ titres older than two months are not reliable, even if the virus stock has been stored at -70°C. It is therefore recommended that re-titration of stocks is performed at two-monthly intervals.

9.2.1 Infection of donor PBMC.

A productively infected culture will result 6-10 days after infection of PBMC with most isolates. It is appropriate to use PBMC from two donors, as this may decrease variation in sensitivity to viral infection. Some donors - with varying frequency in different populations - may be homozygous for the 32 base-pair mutation in the CCR5 gene and, as a consequence, be devoid of cell surface CCR5 expression [31, 32]. Others may be heterozygous for this mutation and show varying levels of CCR5 expression. Infection of PBMC from these individuals may, therefore, vary in sensitivity or be completely resistant to infection with R5 viruses. However, use of PBMC from two donors minimizes the risk of this variation.

Cultures should be thoroughly observed for cytopathic changes, which may be more easily observed at first passage than in the primary culture. In fact, the classification based on cytopathic characteristics of HIV-1 isolates, SI and NSI, has been performed in donor PBMC following cell-free transmission of virus. It should be kept in mind, however, that virus populations may undergo changes, due to selection during *in vitro* passage [8]. For example, the syncytium inducing capacity may appear or disappear. The first peak of virus production is also the time when cocultivation with cell lines should be performed. This procedure, will to some extent circumvent the difficulties in quantifying infectious virus and will allow classification of HIV isolates into rapid/high (=replication in cell lines, including MT-2 cells) and slow/low (=no or transient replication in cell lines) [1].

Day 1: Infection of PBMC cultures carried in 25-cm² flasks.

1. Virus stocks (the supernatant culture fluids from primary PBMC cultures) are stored below -70°C or in liquid nitrogen. Select the virus to be used for infection of PBMC and allow it to thaw in the working hood. The virus can be thawed more quickly by placing the tube in cold water.
2. Count the donor PBMC (pooled PBMC from two donors) and calculate how many cells will be needed in total. 3 x 10⁶ PBMC is optimal for a starting culture in 10 ml IL-2 medium using a 25-cm² flask. (If necessary, cell number per culture may be increased to 5 x 10⁶ cells; increase also the volume of medium to 15 ml).
3. Transfer 3 x 10⁶ PBMC to a 10-ml round-bottom tissue culture tube and centrifuge at 1200 rpm (225 x g) for 10 minutes. Remove the supernatant and resuspend the cells in the remaining droplet by gently tapping the tube.
4. Add virus: 20 000 cpm reverse transcriptase activity per 1 x 10⁶ cells in a volume of 0.5-1 ml will give optimal results. (Amount of virus added may, however, vary between 5000 and 50 000 cpm per 10⁶ PBMC.) Amount of virus can be calculated on basis of p24 antigen content of the virus stock. 1 ng of p24 antigen per 1 x 10⁶ cells may reproducibly yield a virus positive culture of most primary HIV-1 isolates.

Mix cells and virus thoroughly by gently tapping the tube.

5. Incubate tubes for a minimum time of one hour in a humidified 5% CO₂ atmosphere at 37°C. Incubation may be prolonged overnight, but in this case increase volume by addition of 1 or 2 ml (depending on cell number) IL-2 medium to each tube. Slow/low viruses using CCR5 as co-receptor may benefit from overnight incubation.

Day 1 or 2:

6. Following incubation, pellet the cells by centrifugation at 1200 rpm (225 x g), remove the supernatant containing virus and wash cells once in 5 ml IL-2 medium.
7. Resuspend the cells in 10 ml IL-2 medium and transfer to a 25-cm² tissue culture flask. Place flasks upright in a humidified 5% CO₂ atmosphere at 37°C.

Day 4: Exchange half of the volume of culture medium for fresh medium.

Day 6 or 7:

1. Harvest culture medium as follows:
 - 3.5 ml for storage at -70°C (expanded virus stock)
 - 1.2 ml for reverse transcriptase assay
 - 0.9 ml for p24 antigen ELISA.
2. Depending on cell number and magnitude of the cytopathic effect, cultures may be split 1:2 or 1:3 according to the method described for virus isolation (see Chapter 7, Method B, 7-day procedure).
If necessary, add 3 x 10⁶ fresh blood donor PBMC.
3. Restore culture volume to approximately 10 ml with IL-2 medium.

Day 10: Repeat steps 1 and 3 from day 7 procedure.

Day 14: Repeat step 1 from day 7 procedure.

Note: p24 antigen positive cultures can be discontinued. Negative cultures may be continued until they become p24 antigen positive, but no longer than day 35 post-infection. Score for cytopathic effects (syncytia or cell death) twice a week using a light microscope.

9.2.2 *Cocultivation of PBMC with cell lines*

If infected in the standard way, PBMC cultures become productively infected with most HIV isolates 7-10 days postinfection. Cocultivation with cell lines can then be performed. Previously it has been recommended that three cell lines be used for each virus: a T-lymphoid cell (HuT-78, H9, CEM, Jurkat etc.); a monocytoïd cell line (U937 including its well-characterized clonal sublines, THP-1 etc.); and the Jurkat-tat cell line as a control. The Jurkat-tat cell line (with constitutive expression of Tat, the transactivator protein of HIV-1) supports replication of all HIV isolates that also replicate in PBMC. Jurkat-tat was, therefore, an essential control for slow/low viruses that otherwise did not replicate in cell lines. The details of cocultivation are the same as cocultivation with MT-2 cells and will be described below.

The present recommendation is to use the MT-2 cell line for fast and simple phenotyping of HIV-1 [33]. The MT-2 cell line has been established from cord blood cells transformed by the human T-leukemia virus type I (HTLV-I) and has been shown to be highly susceptible to infection with some HIV-1 strains. Using MT-2 cells is the most efficient way to define the SI/NSI phenotype. Similar to other immortalized CD4+ T-cell lines, MT-2 cells express CXCR4 and are susceptible to viruses using CXCR4. It is recommended that when using MT-2 cells, viruses be named either corresponding to the assay, MT-2 negative or MT-2 positive, or in a more descriptive way, syncytium inducing (SI) and non-syncytium inducing (NSI).

MT-2 cells can be infected by co-cultivation with infected PBMC or directly by cell-free virus.

Co-cultivation of PBMC with the MT-2 cell line.

Day 1: Ideally, PBMC cultures are used 7 days post-infection.

1. Harvest medium from PBMC cultures according to the day 7 procedure (section 8.2.1). In the remaining culture volume the approximate cell concentration will be 10^6 cells/0.5 ml.
2. Count cells in MT-2 cultures. Dispense 3×10^6 cells into labelled 25-cm² flasks.
3. Transfer 0.5 ml of the virus-infected PBMC cultures (approximately 10^6 cells) to the appropriate flasks containing the cell lines.
4. Add RPMI medium up to a volume of 10 ml.
5. Place flasks in a humidified 5% CO₂ atmosphere at 37°C.

Day 4, 7, 10, 14, & 17:

1. Harvest culture fluids and test for presence of virus by p24 antigen ELISA and score for syncytia using a light microscope.
2. Split cultures 1:5 (1:2-1:10 depending on the cytopathic effect), as necessary. Gently swirl the flask to resuspend the cells and remove 8 ml.
3. Add RPMI medium to restore culture volume to 10-12 ml.

Evaluation: Cultures score virus-positive when syncytia appear and p24 antigen tests show increasing values on three consecutive testings and can then be discontinued. In some cases, vigorous syncytia formation by day 4 might kill off the MT-2 cells before substantial p24 antigen production. Virus-negative cultures should be regularly tested for at least 21 days (preferably 28 days) and then discarded (see “practical considerations” in section 8).

Cell-free infection of MT-2 cells

1. Count cells in MT-2 cultures. Dispense 3×10^6 cells into labelled 10-ml round-bottom tissue culture tubes and centrifuge at 1200 rpm (225 x g) for 10 minutes. Remove the supernatant and re-suspend the cells in the remaining droplet by gently tapping the tube.
2. Add virus in 1 ml volume: 20 000 cpm reverse transcriptase activity or 1 ng of p24 antigen per 1×10^6 cells may give optimal results.

Mix cells and virus thoroughly by gently tapping the tube.

3. Tubes should then be incubated for a minimum time of one hour in a humidified 5% CO₂ atmosphere at 37°C. Incubation may be prolonged overnight, but in this case increase volume by addition of 1 or 2 ml (depending on cell number) RPMI medium to each tube.
4. Following incubation, pellet the cells by centrifugation at 1200 rpm (225 x g) and remove the supernatant, containing virus.
5. Resuspend the cells in 10 ml RPMI medium and transfer to 25-cm² tissue culture flasks. Place flasks upright in a humidified 5% CO₂ atmosphere at 37°C.

Day 4, 7, 10, 14, & 17 and evaluation:

as described for co-cultivation of PBMC with the MT-2 cell lines

9.3 Test for coreceptor usage

Human cell lines engineered to stably express CD4 and one of the chemokine receptors CCR1, CCR2b, CCR3, CCR5 and CXCR4 or the orphan receptors Bonzo and BOB are used for testing of HIV coreceptor usage [34, 35]. Since these cell lines are derived from human glioma (U87) or osteosarcoma (HOS) cells they form monolayer cultures and can be maintained in Dulbecco's Modified Eagle's medium (DMEM). The cultures are grown in 25-cm² tissue culture flasks and split at a ratio of 1:5 (range 1:3 - 1:10) twice a week by treatment with 5 mM EDTA (pH 8.0). It is essential that these indicator cell lines are not cultured for longer than 2 months, because they may lose expression of different receptors. The faster a cell line grows the higher the risk for the disappearance of the markers. For example, GHOST(3) cells, a derivative of HOS cells carrying CD4, grow faster and are more prone to change than U87.CD4 cells. It is important that even during the two-month period in culture, the cell lines be subjected to regular control of the different markers. Details of culture medium, supplements and re-selection of the different markers are described in Annex 3.

The indicator cell lines can be infected by co-cultivation with infected PBMC or directly by cell-free virus. Co-cultivation is an effective and fast way of qualitative testing of co-receptor usage since syncytia in the U87.CD4 cell series and activation of the green fluorescent protein (GFP) marker in GHOST(3) cells may be detected 2-3 days after infection.

9.3.1 *Co-culture of infected PBMC with indicator cell lines*

Infect donor PBMC (PHA-stimulated as described in section 8.1) with virus as described above and use the cells for co-cultivation 7-10 days postinfection.

Co-cultivation with U87.CD4 cells

Seed in 24-well plates at a concentration of 5×10^4 cells per well in 1-2 ml DMEM. Incubate plates at 37°C with 5% CO₂ in a humidified atmosphere, until cultures reach half confluence (after 1-3 days).

- Day 1:
1. Remove medium from the 24-well plates containing the U87.CD4 cells, and add one ml fresh medium.
 2. Add 1×10^5 infected PBMC/well in one ml (total volume in wells is 2 ml). Mock-infected PHA-stimulated PBMC and medium only should be included as controls.

Note: Care must be taken that the U87.CD4 monolayers do not dry out during this procedure.

- Day 2:
1. Harvest culture fluid and test for presence of virus by p24 antigen ELISA. The sample withdrawn before the washing procedure at day 2 contains virus produced by the PBMC and can be considered as a “virus control”.
 2. Rinse plates two times with PBS and add 2 ml fresh DMEM.

Note: Monitor cell cultures daily for 7 days for appearance of cytopathic effects (syncytium formation) using a light microscope. Cultures may become confluent between days 3 and 5 and may then be treated with 5 mM EDTA, split 1:3 and fresh medium added.

Culture supernatants may be tested for p24 antigen, however, interpretation of data must be done with care. Increasing p24 values following the wash or particularly the split of cultures is indicative of productive infection of U87.CD4 cells. In order to follow the kinetics of p24 antigen production, at least three samples must be obtained; for example: day 2 “virus control”, day 4 and day 7. Samples from different time-points within one experiment should be tested simultaneously in the same p24 antigen ELISA assay.

Co-cultivation with GHOST(3) cells

Seed in 24-well plates at a concentration of 2×10^4 cells per well in 1-2 ml DMEM (7.5% FCS). Incubate plates at 37°C with 5% CO₂ in a humidified atmosphere overnight.

- Day 1:
1. Remove medium from the 24-well plates containing the GHOST(3) cells, and add 1 ml fresh medium.
 2. Add 1×10^5 infected PBMC/well in 1 ml (total volume in wells is 2 ml). Prepare duplicate wells. Mock-infected PHA-stimulated PBMC and medium only should be included as controls.

Note: Care must be taken that the GHOST(3) monolayers do not dry out during this procedure.

Day 2: 1. Harvest culture fluid and test for presence of virus by p24 antigen ELISA. The sample withdrawn before the washing procedure at day 2 contains virus produced by the PBMC and can be considered as a “virus control”.

2. Rinse plates two times with PBS and add 2 ml fresh DMEM.

Note: GHOST(3) cells carry the HIV-2 LTR driven green fluorescent protein (GFP) marker, which becomes activated upon infection with HIV-1 (HIV-2 or SIV). GFP expression can easily be detected in a fluorescence microscope and quantitative data obtained by flow cytometry analysis. Monitor cultures for appearance of fluorescence by observing daily under a fluorescence microscope. The fraction of fluorescent cells can then be estimated. Clearcut data can be obtained this way for viruses not using CXCR4. Since the GHOST(3) cells show a background expression of CXCR4, virus isolates able to use this receptor will induce GFP expression at various levels more or less across the entire panel of GHOST(3) cells. It is, therefore, recommended that GHOST(3) cultures infected with viruses that use CXCR4 are tested by flow cytometry. Fluorescence can be observed 48 hours after infection, peaks at 72 hours and decays thereafter.

Day 4: 1. Prepare cells from one of the duplicate wells for analysis by flow cytometry. For preparation protocol see Annex 3.

2. Split cells 1:3 in the parallel wells and maintain cultures for another 3 days for p24 antigen detection.

Day 7: Remove 0.2 ml culture fluid for p24 antigen ELISA. Cultures can then be discontinued.

9.3.2 *Cell-free infection of indicator cell lines*

This procedure has the advantage that the kinetics of virus replication may be followed more accurately.

Infection of U87.CD4 cells

Seed cells in 48-well plates at a concentration of 1×10^5 cells per well in 1 ml DMEM (10% FCS, antibiotics and 2 µg polybrene/ml). Incubate plates at 37°C with 5% CO₂ in a humidified incubator, until cultures reach half confluence (after 1-3 days).

Day 1: Prior to infection, rinse the monolayer cells once with PBS and add virus in 0.3 - 0.5 ml final volume. Use 100-1000 TCID₅₀ (refers to titration on PBMC and corresponds to 2.5-30 ng of p24 antigen) for infection of U87.CD4 cells. See Chapter 9 for titration of virus stocks on PHA-stimulated PBMC.

Note: Care must be taken that the U87.CD4 monolayers do not dry out during this procedure. Remove medium from one row at the time and add virus immediately.

Incubate 4 - 6 hours. Depending on the infectious virus titre, incubation may be prolonged over night. If so, add 0.5 ml fresh medium.

-
- Day 2:
1. Harvest 0.2 ml culture supernatant from each well and store at -20°C for future p24 antigen ELISA. These samples are used as the virus controls.
 2. Wash plates by rinsing twice with PBS, and add 1 ml fresh DMEM.

Day 3, 7 & 10:

1. Remove 0.2 ml culture fluid for p24 antigen ELISA. Store frozen at -20°C until testing. Samples from different time-points within one experiment should be tested simultaneously in the same p24 antigen ELISA.
2. Restore culture volume to 1 ml.

Note: Monitor cell cultures daily for 7 days for the presence of cytopathic effects (syncytia) using a light microscope. Between days 3 and 5, wells with a confluent cell layer may be treated with 5 mM EDTA, split 1:3 and fresh medium added.

Infection of GHOST(3) cells

Seed cells in 48-well plates at a concentration of 1×10^4 cells/well in 1ml D-MEM (7.5% FCS, antibiotics and 2µg polybrene/ml) and place in an incubator (at 37°C with 5% CO₂ and humidified atmosphere) overnight. Each sample should be tested in duplicates (at least), particularly if flow cytometry analysis is performed.

- Day 1:
- Prior to infection, rinse the monolayer cells once with PBS and add virus in 0.3 - 0.5 ml volume. Use 100-1000 TCID₅₀ (refers to titration on PBMC and corresponds to 2.5-30 ng of p24 antigen) for infection of GHOST(3) cells. See section 8.2 for titration of virus stocks on PHA-stimulated PBMC.

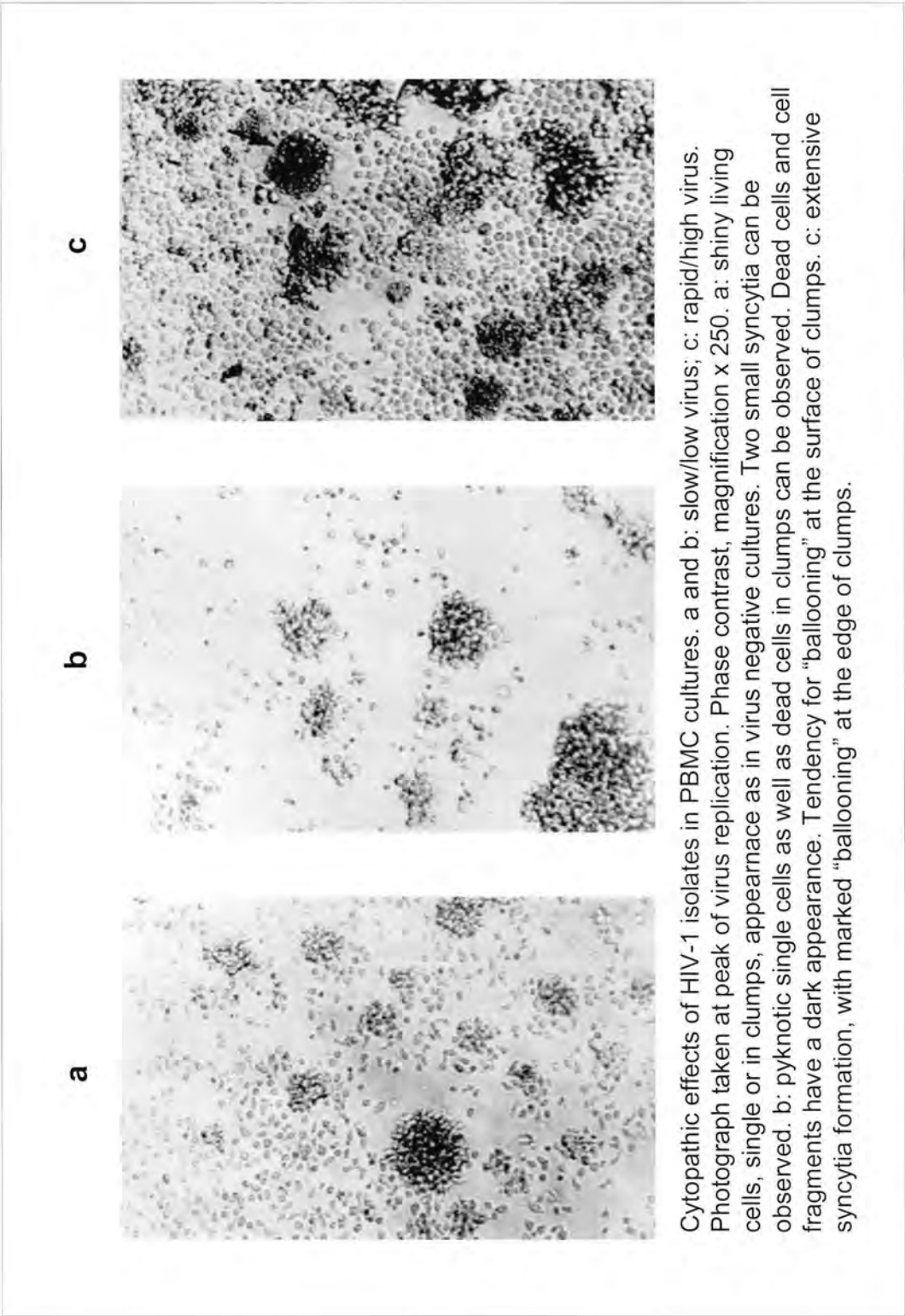
Note: Care must be taken that the GHOST(3) monolayers do not dry out during this procedure. Remove medium from one row at the time and add virus immediately.

Incubate overnight.

- Day 2:
- Wash plates by rinsing twice with PBS, and add 1 ml fresh DMEM.

Further procedure and notes: as described for co-cultivation with GHOST(3) cells.

Figure 6: Cytopathic effects of HIV-1 isolates in PBMC cultures



10. HIV-1 Neutralization

Virus neutralization using blood donor PBMC as target cells will be described here. Assays using the human cell lines U87 and HOS, engineered to stably express human CD4 and human chemokine receptors (see section 9.3 for details), are in progress. These assays will be standardized as part of a WHO-UNAIDS collaborative effort and reported later.

10.1 Separation and characterisation of donor peripheral blood mononuclear cells (PBMC)

PBMC should be separated from donor blood or buffy coats according to the procedures described in section 8.1. PBMC may be frozen in aliquots of approximately 20×10^6 cells/cryotube for later use or used fresh. PBMC cultures, stimulated with PHA for 3 days, may be used directly for virus titration and neutralization assays. While the use of fresh PBMC tends to give higher virus titres, the use of the same large batch of PBMC for the same series of experiments should help to minimize experimental variation. The CCR5 genotype of the batched PBMC can be determined by polymerase chain reaction (PCR), as described in Annex 3.

Note: Similarly to virus isolation, the use of pooled PBMC (from 2 or more donors) is recommended to reduce batch-specific variation. PBMC from different donors should be PHA-stimulated separately and pooled only shortly before infection. See section 8.1 for details.

10.2 Preparation of virus stocks from primary HIV-1 isolates

Virus stocks are prepared by infecting PHA-activated donor PBMC with supernatants from primary isolation cultures. For details of infection see section 9.2. If infected in the standard way, PBMC cultures become productively infected with most HIV-1 isolates 7-10 days postinfection. While culture supernatants continue to be reverse transcriptase and p24 antigen positive, virus infectivity (measured as median Tissue Culture Infectious Dose 50%, TCID₅₀) may decrease after this time. For preparation of high-titred virus stocks (high TCID₅₀) it is recommended that supernatants of infected donor PBMC are harvested as soon as the cultures become virus positive, preferably day 6-7, but no later than day 10 postinfection. In cases where the level of infectious virus is low further passage in PBMC is necessary to increase the titre to a level sufficient for use in neutralization assays. In such cases, one may add 10×10^6 fresh PHA-stimulated donor PBMC to 7-10 day old cultures and harvest supernatant culture fluids 3 days later. Virus stocks are clarified by centrifugation ($800 \times g$ 5 min) or by filtration through a $0.45 \mu m$ filter, aliquoted and stored frozen below $-70^\circ C$ or in liquid nitrogen.

Note: Some primary isolates are not suitable for preparation of frozen virus stocks, because freezing, no matter how carefully done, will decrease infectious virus titres below the level used in neutralization assays. In these cases PBMC supernatants may be used directly in the neutralization assay (see further comments below in section 10.3).

10.3 Determination of tissue culture infectious dose-50 (TCID₅₀) titre

For neutralization tests performed using the *Endpoint Neutralization Assay* format with a single virus dose, it is essential to determine the infectious virus titre for a particular virus stock on a particular batch of PBMC. In some cases, however, when virus with low replicative capacity is tested, fresh virus harvests on fresh PBMC may be used to achieve maximum virus titres. Use of several virus doses is then recommended (see further section 10.4.1). For the *Infectivity Reduction Assay*, it is also prudent to establish that the virus stock to be used has a TCID₅₀ which exceeds 100 per 75 µl (the unit volume used in the assay).

Day 1: Infection of PBMC cultures directly in 96-well microtitre plates.

1. Virus stocks (the supernatant culture fluids from primary or expanded PBMC cultures) are stored below -70°C. Select the virus to be used for infection and allow it to thaw in the working hood. Viruses can be thawed more quickly by placing tubes in cold water.
2. Prepare serial virus dilutions in IL-2 medium using sterile capped tubes or a 24-well tray. Make at least six 5-fold dilutions in the ranges 1/5 to 1/15625 or 1/25 to 1/78125.

Example of virus dilution:

- Make dilutions in a 24-well tray. Start with the 1:5 dilution in the following way:
- Add 800µl of IL-2 medium to six wells.
- Add 200µl of virus stock to the first well (1/5 dilution)
- Change tip, mix and transfer 200µl to the adjacent well containing 800µl of IL-2 medium (a 1/25 dilution), and so on. Continue as far as necessary.

Note: Ensure thorough and consistent mixing of each well and replace tips between dilutions.

3. Transfer 75 µl of each virus dilution to five replicate wells of a round bottom 96-well tray with lid. Use of the outer rows and columns of the tray should be avoided. These outer wells will serve for titration of the standard p24 antigen in the readout ELISA (Figure 7a-c). For figure 7 see end of section 10.4.1.

Alternatively, each titration block can be limited to 24 wells of a round bottomed 96-well tray (for example: 4 replicates x 6 dilution steps). Titration blocks may then be physically separated on the tray.

Include negative controls: five wells with virus only (75 µl of the lowest virus dilution), serves as a washing control, and five wells with cells only. See template depicted in Figure 7a.

4. To the designated TCID₅₀ wells, add 150µl of IL-2 medium containing 1×10^5 (0.667×10^6 cells/ml) of PHA-activated PBMC from at least two donors. Finally, to wells with cells only, add 75 µl IL-2 medium.

The final volume in each well should now be 225 µl.

5. Fill in all redundant wells with sterile water or RPMI medium supplemented with antibiotics (shaded areas in Figure 7a); and wrap the plate in plastic foil to avoid evaporation. Incubate overnight at 37°C in a humidified atmosphere (5% CO₂).

Day 2 - 4: Wash plates to remove excess virus that may interfere with the p24 antigen ELISA readout. Different washing procedures have been systematically evaluated by Albert et al. [36]. The recommendations are as follows:

Method A:

Change the culture medium on day 2 and 4: centrifuge the tray (10 min, 1200 rpm (225 x g), *low* brake to avoid stirring) in a tray holder equipped with a safety bucket. The plates may be wrapped in plastic foil to further minimize aerosol formation. As soon as the centrifuge stops (and while the cell pellet is still firmly attached to the bottom of wells) remove the safety buckets from the centrifuge and open inside of a safety cabinet. Discard the culture medium into a waste container in the safety cabinet by turning the plate upside down with a quick move of your hand. Add 200 µl of fresh IL-2 medium to each well.

Method B:

If the centrifuge is not equipped with tray holder, one may remove 180µl of culture medium and replace it with fresh IL-2 medium on three sequential days (day 2, 3 & 4). Although the PBMC form a discrete pellet at the bottom of each well, extreme care should be taken during these wash steps to prevent disturbance or removal of the cells.

Note: Each wash procedure results in a 9-fold (Method A) or 5-fold (Method B) dilution of the previous culture fluid. In general, two washes (A) on days 2 and 4, or three washes (B) on day 2, 3 & 4, are sufficient to reduce input p24 antigen to below detectable levels.

After the last wash continue incubation at 37°C, in a humidified 5% CO₂ incubator until day 7.

Day 7: Harvest 100-150µl supernatant from each culture well into a 96-well tray. A multichannel pipette with the same set of tips may be used starting with the highest virus dilution moving to the lowest. Samples may be stored frozen at -20°C for a few weeks. Samples must be treated with detergent (for example, a final concentration of 0.5% Empigen or 0.5% Triton-X 100 must be reached) prior to removal from the virus isolation laboratory for p24 antigen determination by ELISA. There are several commercially available ELISAs that can be used. The antigen detection test should be carried out according to the manufacturer's recommendations.

Note: Replace medium in the *master tray* (neutralization test tray) and keep it in the incubator until ELISA results are ready.

Evaluation of the results: Each well is scored positive or negative according to the assay cut-off point. In general, this is determined as twice the mean optical density (+3SD) of 4-6 replicate wells containing the lowest dilution of virus without added PBMC. The TCID₅₀ is then calculated using standard Reed-Meunch or Spearman-Kärber formulae and represents the reciprocal of the virus dilution which was calculated to give 50% positive wells (for details of calculation see Figure 8 at the end of section 10).

Note: the assigned TCID₅₀ value is valid only for a given virus stock on a particular batch of PBMC that is treated under the exact conditions as the procedure for its determination. If the virus stock is kept frozen at -70°C for longer than 2 months, re-titration is recommended, since decrease in titres may occur over time.

10.4 Virus neutralization assays

Virus neutralization assays may be performed with primary HIV-1 isolates and the patients' own sera (autologous neutralization) or with sera from other HIV-1 infected individuals (heterologous neutralization). According to format we distinguish End-point Neutralization Assay and Infectivity Reduction Assay.

10.4.1 End-point Neutralization Assay

This assay estimates the end-point titre of neutralizing antibodies in serum using a fixed virus input, usually 10-50 TCID₅₀. The neutralizing antibody titre is determined at the 90% level of inhibition of virus infection, as measured by p24 antigen output, compared to the growth of the same input virus in the presence of medium or HIV-1 seronegative serum [11].

Note: The 50% level of inhibition should only be used in conjunction with the 90% level of inhibition, because results might be highly variable at the 50% level.

Day 1: Begin preparation by planning the protocol using the ordinary ELISA (paper) protocols. Calculate the exact amount of virus, serum and PBMC that is required for the test. Mark the position of each serum to be tested on the lids of the 96-well round bottomed trays. The outer wells of each plate should not be used (see comments above and Figure 7a and 7b for templates).

1. Heat-inactivate the sera for 30 min at 56°C (complement inactivation).
2. Make five serial two-fold dilutions of serum in IL-2 medium, for instance from 1/10 to 1/160, ensuring that the volume is 75µl or its multiples, for each dilution. Transfer 75µl volumes of each serum dilution to duplicate or triplicate rows of wells.

Example of serum dilution: five 2-fold dilution steps in triplicates

- Make dilutions directly in the 96-well round bottom test tray to save material. Start with the 1:10 dilution in the following way:
- Add 15 µl of serum to the first well in each of the three parallel rows. Add 135 µl of medium to the three wells (containing serum) and 75 µl of medium to all remaining wells in the three rows.
- Use three tips on a multichannel pipette, to be able to simultaneously titrate the three parallel rows. Mix well and transfer 75 µl from the first column to the second column. Change tips, mix well and continue transferring 75 µl to the next column. Discard 75 µl from the last three wells.
- You will now have 75 µl of serum dilutions 1:10, 1:20, 1:40, 1:80 and 1:160 in three parallel rows.

3. Add 75µl of each virus dilution to the designated wells and place the tray in a 37°C incubator for 1 hour.

Note: Duplicate or triplicate rows of serial serum dilutions may be used for two or three different virus dilutions. The row with the optimal virus dose(s) (10-50 TCID₅₀) will then be evaluated for virus neutralization. This way one may minimize the effect of the inter-assay variation in virus titres on the outcome of neutralization assay.

4. Add 75µl IL-2 medium containing 1x10⁵ (1.34x10⁶ cells/ml) PHA-activated PBMC.

The total volume in each well is now 225µl.

Controls:

- virus only (p24 antigen background wells as “washing control”): 4-6 replicate wells containing 150µl of IL-2 medium and 75µl of virus (the same concentrations as used);
- PBMC only: 4-6 replicate wells containing 150µl of IL-2 medium and 75µl of 1×10^5 PBMC;
- titration of virus stock in order to confirm the input $TCID_{50}$. If the intra-assay $TCID_{50}$ is outside the range of 10-200 the assay should be repeated. For details of titration see section 10.2;
- positive serum control: selected sera from HIV-1 seropositive asymptomatic individuals with known neutralizing capacity (one low and one high titred neutralizing serum); and
- positive endpoint wells (optional): 4-6 replicate wells containing 75µl of a 1/10 dilution of seronegative serum, 75µl of virus dilution, and 75µl of 1×10^5 PBMC.

5. Fill in all redundant wells with sterile water or RPMI medium supplemented with antibiotics; and wrap the plate in plastic foil to avoid evaporation. Incubate overnight at 37°C in a humidified atmosphere (5% CO_2).

Days 2-4: Wash all wells, as described in section 10.3. Washing will result in a final serum dilution factor of 1:6163 by method A, and 1:3750 by method B. Both methods reduce anti-gag activity of sera with low or medium anti-gag content. For the relatively rare sera that contain high anti-gag levels, centrifugation seems to be the washing procedure of choice [36]. If necessary, an estimate of the potential for p24 antibody interference for each serum can be made (see Annex 3).

Day 7: Harvest and detergent-treat supernatants containing virus, as described above in section 10.3 and carry out the p24 antigen ELISA. Plates can be replenished with an appropriate volume of IL-2 medium and continued to day 10 for slowly replicating primary isolates.

Evaluation of the results: The mean p24 antigen of the background, virus only, wells should be subtracted from all samples. The reciprocal of the highest test serum dilution to inhibit virus growth, relative to the mean of positive endpoint control wells, by 90% is the endpoint neutralization titre for that serum sample. It is the serum dilutions at the stage of virus-serum added (for instance, 1/20 to 1/320) which are used to calculate the endpoint neutralization titres for each serum. Neutralization titres of 20 are considered weak, while titres of 40-80 and =160 are considered medium and strong, respectively.

Note: To reduce the potential for non-antibody interference in control wells, for example by b-chemokines, a pool of seronegative serum is recommended.

Figure 7: Template 96-well trays for neutralization assay.

A)											
<-----Virus titration----->						<-----Neutralization----->					
	1:5	—>	—>	—>	—>	1:5 1:20	—>	—>	—>	—>	
	1:25	—>	—>	—>	—>	1:25 1:20	—>	—>	—>	—>	
	1:125	—>	—>	—>	—>	1:125 1:20	—>	—>	—>	—>	
	1:625	—>	—>	—>	—>						
	1:3125	—>	—>	—>	—>		<—	Controls		—>	
	1:15625	—>	—>	—>	—>						
B)											
<-----Serum 1----->						<-----Serum 2----->					
	1:5 1:20	—>	—>	—>	—>	1:5 1:20	—>	—>	—>	—>	
	1:25 1:20	—>	—>	—>	—>	1:25 1:20	—>	—>	—>	—>	
	1:125 1:20	—>	—>	—>	—>	1:125 1:20	—>	—>	—>	—>	
	1:5 1:20	—>	—>	—>	—>	1:5 1:20	—>	—>	—>	—>	
	1:25 1:20	—>	—>	—>	—>	1:25 1:20	—>	—>	—>	—>	
	1:125 1:20	—>	—>	—>	—>	1:125 1:20	—>	—>	—>	—>	
<-----Serum 3----->						<-----Serum 4----->					

C) ELISA assay, all plates

Blank	← Serum 1 →					← Serum 2 →					
Blank	1:5 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	1:5 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	
Antigen 2 µg	1:25 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	1:25 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	
Antigen 2 µg	1:125 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	1:125 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	
Antigen 1 µg	1:5 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	1:5 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	
Antigen 1 µg	1:25 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	1:25 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	
Antigen 0,5 µg	1:125 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	1:125 1:20	→ 1:40	→ 1:80	→ 1:160	→ 1:320	
Antigen 0,5 µg	Antigen 0,25 µg		Antigen 0,125 µg								

Legend to Figure 7

Template 96-well tray for neutralization assay: A) Plate 1: virus titration and controls; controls comprise positive serum control arranged in a 3x5 format, virus only, PBMC only and medium only. Positive endpoint wells containing virus, PBMC and a pool of HIV seronegative sera, may also be included (see text for details). B) All subsequent trays, arrangement of four sera/ tray. Shaded areas in trays A) and B) are filled with sterile water or RPMI medium supplemented with antibiotics. C) Template tray for p24 antigen ELISA.

10.4.2 Infectivity Reduction Assay

This assay estimates the neutralizing potential of a given serum from an HIV-1 positive individual using a fixed input of serum antibodies in the presence of serial virus dilutions. The ratio of TCID₅₀ determined in the presence of seronegative serum, over that obtained in the presence of test serum, is scored as the virus neutralizing index, or VN_I.

Day 1: Begin preparation by planning the protocol using the ordinary ELISA (paper) protocols. Calculate the exact amount of virus, serum and PBMC that is required for the test. Mark the positions of each serum to be tested on the lids of the 96-well round bottomed trays. Each test serum will require a titration block of 24 wells. The outer wells of each plate should not be used (see comments in section 9.4.1 and Figure 7 for templates).

1. Heat-inactivate the sera for 30 min at 56°C (complement inactivation).
2. In sterile capped tubes, or wells of a 24-well tray, dilute seronegative control serum and test serum to 1/20 in IL-2 medium and add 75µl to each well of the designated titration block.
3. Make serial 5-fold dilutions of the virus stock (from 1/5 to 1/15625 or 1/25 to 1/78125, dependent on the approximate TCID₅₀ range) in sterile tubes, ensuring adequate mixing of each dilution. Transfer 75µl of each dilution to quadruplicate wells for each serum to be tested, including seronegative control serum, and place in a 37°C incubator for 1 hour.
4. At the end of this period, add 75µl IL-2 medium containing 1x10⁵ (1.34x10⁶ cells/ml) PHA-activated PBMC and return to the 37°C incubator.

On the tray containing the virus titration block in seronegative serum, also include (i) 4-6 replicate p24 antigen background wells, containing 150µl of IL-2 medium and 75µl of starting virus dilution (1/5 or 1/25); and (ii) 4-6 replicate PBMC only wells, containing 150µl of IL-2 medium and 75µl of 1x10⁵ PBMC (see also previous section for controls).

Days 2-4: Wash all wells, as described in section 10.3 and 10.4.1.

Day 7: Harvest and detergent-treat supernatants containing virus, as described above in section 9.3 and carry out the p24 antigen ELISA.

Evaluation of the results: Calculate the assay cut-off point, based on the residual p24 antigen, and TCID₅₀ for each titration block, as for the determination of TCID₅₀, described above in section 10.3. In short, individual cultures are scored positive or negative according to the assay cut-off point (twice the mean + 3D of 1:5 diluted virus only wells) and a TCID₅₀ value is assigned to each titration block based on the Spearman-Kärber formula (Figure 8). The virus neutralizing index (VN_i) is expressed as a ratio of the TCID₅₀ obtained in the presence of HIV-1 negative serum over that obtained in the presence of test serum. A VN_i of ≤3 (i.e. equivalent to a half log₁₀ reduction in virus titre) is considered negative, while a serum with a VN_i of 3-9, 10-100 or >100 is considered as having weak, medium or strong neutralizing activity, respectively.

Note: Since virus titres in the presence of test serum are compared to those in negative control serum, it is particularly important to minimize the potential for non-antibody interference in control wells, for example by β-chemokines. The use of a pool of seronegative serum is recommended.

Figure 8: Determination of TCID_{50} by Reed-Meunch and Spearman-Kärber formulae

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Virus dilution	Number of wells		Cumulative wells		Infection	
	Positive	Negative	Positive	Negative	Ratio	%
5 ¹ (1/5)	4	0	13	0	13/13	100
5 ² (1/25)	4	0	9	0	9/9	100 ¹
5 ³ (1/125)	3	1	5	1	5/6	83.3
5 ⁴ (1/625)	2	2	2	3	2/5	40
5 ⁵ (1/3125)	0	4	0	7	0/7	0

¹ Highest dilution giving 100% positive wells

Reed-Meunch:

$$\begin{aligned} \text{Proportionate distance} &= \frac{(\% \text{ positive above } 50\%) - 50\%}{(\% + \text{above } 50\%) - (\% + \text{below } 50\%)} \\ &= \frac{83.3\% - 50\%}{83.3\% - 40\%} = -0.769 \end{aligned}$$

Endpoint dilution = $5^{-3 + (-0.769)}$

$$\text{TCID}_{50} = 1/5^{-3.769} = 431$$

Spearman-Kärber:

$$\text{TCID}_{50} = \frac{\text{Last dilution of 100\% positive wells} + \frac{1}{2} - \text{Total number of test units showing positive wells}}{\text{Number of test units per dilution}}$$

$$\text{TCID}_{50} = \frac{5^{-2 + 1/2 - (9/4)}}{1} = 1/5^{-3.75} = 418$$

11. Genetic Typing of HIV Strains

This chapter describes methods for the genetic typing of HIV strains from infected blood specimens. It will be divided into three parts, covering nucleic acid isolation methods, PCR of viral genome segments, and analysis of PCR products, focussing on the heteroduplex mobility assay (HMA) for genetic subtyping. Begin planning of work by reading section 4, which provides guidelines for laboratory set up and methods to prevent sample contamination.

11.1 Preparation of viral genome templates for PCR

Three protocols for cellular DNA preparation and one for viral RNA preparation are provided below. Each is recommended for different samples and laboratory situations. For DNA preparation, the “Puregene” protocol is recommended in situations in which supply costs are available for kit purchase and a cold chain can be maintained between blood collection and DNA extraction. The cell lysate method is the least expensive and thus is recommended when a cold chain can be maintained but supply funds are more limited. The Dried Blood Spot (DBS) method is recommended when a cold chain cannot be maintained after sample collection prior to extraction. In all cases, manipulation of infected blood should be performed using basic biosafety precautions and in an environment free of contaminating HIV DNA (i.e., in a pre-PCR facility, see section 4 for details).

11.1.1 *DNA purification using the Puregene DNA isolation kit*

This is the method of choice, since it is fast, results in highly pure DNA, and does not lead to significant sample loss. However, it requires purchase of an extraction kit from Gentra Systems, Inc.⁵

1. Add 300 µl of whole blood to a 1.5ml-microcentrifuge tube containing 900µl “RBC lysis solution”. Invert to mix, incubate for 10 min, at room temperature. Invert once during incubation.
2. Centrifuge for 30 in a microcentrifuge at full speed.
3. Remove supernatant with a micropipette, leaving the pellet and 10-20 µl of liquid behind.
4. Vortex vigorously to resuspend cells.

⁵ Minneapolis, USA, phone: 800-866-3039 (from the USA), or 612-476-5858, fax: 612-476-5850; <http://www.gentra.com/>. A list for purchase of alternative DNA extraction kits is provided in Annex 4.

-
5. Add 600 µl “cell lysis solution” to the tube, triturate to lyse the cells. This and subsequent volumes correspond to twice the volumes required for the Puregene protocol.
 6. Add 1.5 µl “RNase A solution” and incubate at 37°C for 15 min. Samples are then stable at room temperature (18-22°C) for at least 18 months.
 7. Cool sample to room temperature and add 200 µl “Protein precipitation solution” to the lysate, vortex vigorously for 20 seconds to mix.
 8. Microcentrifuge at full speed for 3 min, precipitated proteins will form a dark brown pellet.
 9. Pour the supernatant into a fresh 1.5 ml tube containing 600 µl of 100% isopropanol, being careful to leave the precipitate behind. If <300,000 white blood cells are expected, add 1 µl of 20 mg/ml glycogen to enhance DNA precipitation.
 10. Mix by gently inverting the tube 50 times to completely form a white fibrous clump of DNA.
 11. Centrifuge at full speed for 1 min.
 12. Pour off supernatant and drain tube on clean absorbent paper, taking care not to pour off the small white DNA pellet.
 13. Add 600 µl cold 70% ethanol, and mix by inverting the tube several times.
 14. Centrifuge at full speed then carefully pour off supernatant (be careful, the pellet will be looser than after the first precipitation) and drain on absorbent paper.
 15. Air dry for 15 min do not over-dry in a vacuum dessicator.
 16. Add 100 µl “DNA hydration buffer” and allow rehydrating overnight at room temperature.
 17. Store at +4°C to –20°C.

11.1.2 Extraction of DNA from cells lysates:

Dr. Marcia L. Kalish from the Centers for Disease Control and Prevention, Atlanta, USA provided this method. It is very fast and inexpensive, but does leave behind inhibitors of the PCR reaction, and thus while samples are typically PCR positive, the amount of virus detected may be low.

Prepare lymphocytes by Ficoll-Hypaque separation (see section 6 for method). Cells can then be processed immediately or stored frozen at –70°C or in liquid nitrogen as a dry pellet for subsequent DNA preparation. If cells are frozen in DMSO to retain cell viability, wash twice with cold PBS before proceeding to the next step. PBS detailed in Annex 3.

-
1. Add lysis buffer to washed cells or frozen pellet, final concentration should be $\sim 6 \times 10^6$ cells/ml. Lysis buffer detailed in Annex 4.
 2. Incubate at 56°C for 1h.
 3. Heat to 95°C for 10 minutes to inactivate the proteinase K.
 4. Use 10 μ l of the preparation ($\sim 60,000$ cells) for a 100 μ l PCR reaction, store remainder at -70°C or in liquid nitrogen for future use.

Note: If the PCR reaction is negative, titrate the amount of lysate in a separate experiment. Getting a positive result could require less lysate if the problem is an inhibitor, or it could require more lysate if the problem is a scarcity of templates in the reaction. We recommend the routine use of “Gene-Releaser” according to the procedures recommended by the manufacturer⁶ to remove inhibitors prior to the first round of PCR.

11.1.3 Extraction of DNA from Dried Blood Spots

Blood collection:

1. Collect blood from one patient onto all 4 tips of a “IsoCode Stix” collection device⁷. Each collection device has 4 triangular tips that absorb blood, either from a finger puncture, or by dipping the tips into blood. Each tip holds about 10-12 μ l of blood.
2. Dry samples for 15-20 min at 80°C, or place in a clean dry container with dessicant for a minimum of 3 hours (it is critical that the sample be dried for storage and subsequent use).
3. Place each sample device in its own storage bag along with dessicant, at room temperature (18-22°C) and protected from light. Dried blood spots can be stored this way for months to years.

DNA extraction:

1. Detach one triangular tip by placing over a sterile microcentrifuge tube and closing the lid. Store remainder at room temperature.
2. Rinse tip with 500 μ l of distilled water, vortex 3 times for 5 seconds each.
3. Pellet tip in a microcentrifuge, remove the water with a pipette.
4. Repeat steps 2 and 3 twice.
5. Add 100 μ l of distilled water to the tube containing the tip and incubate at 95°C for 30 min. Vortex twice during incubation.
6. Pellet paper tip in a microcentrifuge, and then transfer the supernatant to a clean microcentrifuge tube.
7. Use 10 μ l for PCR and store the remainder at -20°C.

⁶ BioVentures, Murfreesboro, TN; <http://www.bioventures.com>.

⁷ Schleicher & Schuell; (www.s-and-s.com)

11.1.4 RNA extraction and cDNA synthesis

RNA is substantially less stable in solution than DNA, thus the first step in analyzing viral RNA sequences usually involves reverse transcription into complementary DNA (cDNA). Also, due to a high concentration of the ubiquitous RNases, the half life of viral RNA after virion disruption in the blood is measured in seconds. Thus all viral RNA capable of evaluation is protected either within cells or within virions prior to RNA extraction. Several methods are available for viral RNA extraction from plasma and serum virions. The method we employ involves ultracentrifugation, since recovery seems to be greater than direct lysis procedures. However, viral RNA can even be isolated with good sensitivity from dried blood spots [37]. The amount of nested-PCR amplifiable viral RNA in a sample (see below) is dependent on the length of the desired fragment. In general, we have found the sensitivity of 800 bp env fragment amplification to be ~5 fold lower than the concentration determined by DNA or the Roche Amplicor™ method.

Method:

1. 200 µl of plasma is diluted to 1.2 ml with PBS and ultracentrifuged at 45,000 rpm 4°C for 1 hour.
2. RNA extraction is done with the RNAzol™ B reagent (Biotecx, Houston, TX) according to the procedures provided by the manufacturer.
3. cDNA synthesis is performed using the primer BH2 or ED12 (see below) and SuperScript™ II RNase H free reverse transcriptase (Life Technologies, Gaithersburg, VA) at 42°C for 1 hour, according to the procedure provided by Life Technologies.
4. cDNA should be stored at -20°C or preferably phenol extracted and stored at -20°C until used.

11.2 Polymerase chain reaction (PCR) amplification of HIV-1 env segments

The following protocols describe PCR conditions used for amplification of HIV-1 envelope (env) sequences for subsequent characterization by DNA sequencing, or a genetic typing method such as heteroduplex mobility analysis (HMA). PCR primers and reference plasmids containing env genes from reference subtypes are available as part of an HMA kit provided by the NIH NIAID AIDS Reagent Program in the USA [18] and by the NIBSC AIDS Reagent Project in the UK [27]. The primers included in this kit have been chosen because of their success in amplifying HIV-1 env sequences from each of the known subtypes (A-H) within the Main (M) group. They are not expected to function, however, with sequences of the recently described Outlier (O) group. Furthermore, they do not function well for amplification of HIV-1 strains recently detected in certain regions of West Africa, such as Nigeria.

Any combination of the following primers can be used for nested PCR, as long as the second round primers amplify a fragment wholly within the first round product. The primers are grouped according to commonly used primer sets and comments on their use is provided. In general, the smaller the PCR fragment amplified in the first round, the more sensitive the PCR reaction. Figure 9 illustrates the placement of each of the primers in the HIV-1 env gene region.

Figure 9:

	1	11	21	31	41	51	61	71	81	91	100
	>————— ED3 —————>										
RW20-A											
SF170-A											
BR20-B											
TH14-B											
SF162-B	ttaggcatct	cctatggcag	gaagaagcgg	agacagcgac	gaagagctcc	tcagacagct	gaggttcac	aagtttctct	accaaagcag	taagtagtat	100
MA959-C											
ZM18-C											
IND868-C											
BR25-C											
UG21-D											
UG38-D											
UG46-D											
TH22-E											
TH06-E											
BZ162-F											
BZ163-Fa.....g.	acc..a....	..agag...c	c..a.a....	...a.ct.g.	100
RU131-G											
LBV21-7-G											
VI525-G											
CA13-H											
VI557-H											
	101	111	121	131	141	151	161	171	181	191	200
RW20-A											
SF170-A											
BR20-B											
TH14-B											
SF162-B	atttaatgca	acctcttcaa	atattagcaa	tagtagcatt	agtagtagca	gcaataatag	caatagttgt	gtggaccata	gtgtacatag	aatacaggaa	200
MA959-C											
ZM18-C											
IND868-C											
BR25-C											
UG21-D											
UG38-D											
UG46-D											
TH22-E											
TH06-E											
BZ162-F											
BZ163-F	..g.....tc	tga.t.gtt.	gc.a..agtg	..ac.....	.a.....	ct.....t...	..a.t....t.....	200
RU131-G											
LBV21-7-G											
VI525-G											
CA13-H											
VI557-H											

Figure 9 (cont'd)

	201	211	221	231	241	251	261	271	281	291	300
								→env start			
RW20-A								gagtgagg	gggatacaga	cgagttggca	28
SF170-A								at ga.....t	t..ac...t..	32
BR20-B											
TH14-B							c.cag..	a..a..at.g	28
SF162-B	aatattaaga	caaagaaaaa	tagataggtt	aattgataga	ataacagaaa	gagcagaaga	cagtgccaat	ga.....a.cag..	a..a..at..	300
MA959-C							c.c	g..a.....	28
ZM18-C								at ga.....	..a.....t..	g..a.....	32
IND868-C											
BR25-C											
UG21-D							a...gag..	g..a...t..	28
UG38-D											
UG46-D								at ga.....tg...	g..a..at..	32
TH22-E							a..c....	t..a.....c	28
TH06-E							a..c....	t..a.....c	28
BZ162-F								at ga.....g....	g..a.....	32
BZ163-F	.c.g.....ga.....	..ta...ag..	...g.....at	ga.....g....	g..ac.....	300
RU131-G											
LBV21-7-G								at ga...c..a.t..	g..a.....	32
VI525-G								at ga.....a.....	32
CA13-H											
VI557-H											
	301	311	321	331	341	351	361	371	381	391	400
RW20-A	aaactgtgg	aga——	———	———tgg	ggaactatga	tctggggat	gctagtgaat	tatagtctg	cagaa—aa	ctgtgggtt	101
SF170-Aa.t	...——	———	———...a..	..t..a..a..c	.g.....a	..agc—..	a.....	105
BR20-B											
TH14-B	gc.a..a...	...——	———	———...	...c..c...c	..c.t....	at.ga....c	.g.....—..	g.....c	101
SF162-B	gc.....	...——	———	———g..	...c..ct..c	..c.t....	..t.ga....c	.g.....	t....—..	a.....c	373
MA959-C	.c.a.g....	.t.——	———	———...	...c.tct.ag	g...tt...	..t.ca....	.g.....tgt	gg.gg—..c	101
ZM18-C	.c.a.g....	.t.——	———	———...	...c.tct.ag	g...tt...	..g..a...ac	...a..tg.	t..g.gac..c	108
IND868-C											
BR25-C						...—.. a.....c	15				
UG21-D	gc.t.....	g..——	———	———...	...c..ct..c	..c.t.a...	..t.ga....aa..a	...g.—..	t.....c	101
UG38-D											
UG46-D	gc.tc.....	gagaggaatc	agcactcgtt	gtggaga...	...c.tc...c	..c.t....	..t.a....a	.g..a——	~.g.—g.	a.....c	123
TH22-Ea.——	———	———...	..g...t...	..c.t...t	..g.ga.a...	..g..a...ct	...c—..	101
TH06-Ea.——	———	———...	..g...t...	..c.t...t	..g.ga.a...	..g.....ct	...c—..	101
BZ162-F	gc.....g..	..ag——	———	———...	...cct.t.at	..c.....	at.ca.a..c	..g..a.....	a...c—..c	105
BZ163-F	gc.....g..	..a.——	———	———...	...cct.t.at	..c.....	at.gt.a..c	..g..a.....	a.———..c	370
RU131-G											
LBV21-7-G	.c.....	..a.——	———	———...	...t.t...	..c.t...t	..g..a.a...	..g..a...ct	...gt—..c	105
VI525-G at——	———	———...	...g...t...	..c.t...t	..g.ga.a...	ag.....ct	..a.t—..c	105	
CA13-H											
VI557-H											

Figure 9 (cont'd)

	401	411	421	431	441	451	461	471	481	491	500
RW20-A	gctgtctact	atgggggtacc	tgtgtggaaa	gacgcagaga	ccaccottatt	tgtgtcatca	gatgctaaag	cttatgatac	agaagtgcac	aatgtatggg	201
SF170-A	a.....t.a.....g.gc....	205
BR20-B											
TH14-B	a.a.....t.a.....	..a...acc.tc....a.....	...g..a...t....	201
SF162-B	a.a.....t.a...acc.tc....c.....c..	...g..a...c....	473
MA959-C	a.a.....t.a.a.	.t..tc....	c.....a.....g.gc....	201
ZM18-C	a.a.....t.a...a.a.	.t..t....	c.....a.....g.gc....	208
IND868-C											
BR25-C	a.a.....t.a...a.a.	.t..tc....c.....	.a.....c....	115
UG21-D	a.a..t..t.a...acc.	.t..tc....t	.a....ag.	...g.ca...	...a.c....	201
UG38-D	a.a.....t.a...a.c.tc....t	.a...ata.a	...g.ca...	...a.c....	100
UG46-D	a.a..t..t.a...acc.	.t..tc....	..c.....t	.a....ac.	...g.ca...	...a.c....	223
TH22-E	a.a..t..t.t.t....t.c....c....	.ac...g..ct....	201
TH06-E	a.a..t..t.t.g.	..t....t.c....c....	.ac...g..	...a...cc....	201
BZ162-F	a.a..a..t.a...acc.	.t..tc....	c.....t	.a....c.g	...g..a...c....	205
BZ163-F	a.a.....t.t...acc.	.t..tc....	c.....t	.a....g.g	...g..a...c....	470
RU131-G		g..	..t....t.tc....tc	.a...ag...	t...agc...c....	79
LBV21-7-G	a.a.....t.c...g..t.	...gc....ta...ag...	t...agc...c....	205
VI525-G	a.a.....g..	..t....a.	..c.tc....tt	.a...ag...	t...agc...c....	205
CA13-H											
VI557-H											
	501	511	521	531	541	551	561	571	581	591	600
RW20-A	aaacacatgc	ctgtgtaccc	acagaccccca	accacacaaga	aatacatttg	gaaaatgtga	cagaagattt	taacatgtgg	agaaataaca	tggtagagca	301
SF170-A	cg.....gt....gg..a.....	305
BR20-B											
TH14-B	cc.....g..gta...	.g.....a.c..a.....a..	301
SF162-B	cc.....t.gta...a....a.....a..	573
MA959-C	ct.....gt..g.a.a....a...g...	.a....t..	301
ZM18-C	ct.....gt....	.g.....a.a....a...g...	...g..t..	308
IND868-C											
BR25-C	ct.....t.....	...ggt....a.a....	ga...g...	...g..a..	215
UG21-D	ct.....agtac.ac.	...ga.c..c...	.a.....	...g....	301
UG38-D	ct....c..ag...	...g.ac..c.a.c..a.....	...g....	200
UG46-D	ct.....g..ag...	...a.ac..c.a.c..a...g...	...g....	323
TH22-E	cc.....a..t.t.cc..a.a....a.....	301
TH06-E	cc.....g....cc..a.a....a.....	301
BZ162-F	ct.....t....	.t.....	...agaa..	..c....a.	t...a....	.g.t.....	.a.....a..	305
BZ163-F	ct.....t....	.t.....	.g..gt.c..a.a....	.g.t.....	.a...g..a..	570
RU131-G	ct.....c...g..ac.c..a.a....	..t.....	.a.....a..	179
LBV21-7-G	ct.....t...t.....tc.c..	a.c....a.a....a.....a..	305
VI525-G	ct.....g..tc.c..a.a....a....a.a..	305
CA13-H											
VI557-H											

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Figure 9 (cont'd)

	801	811	821	831	841	851	861	871	881	891	900
									V2>		
RW20-A	gctcttca	tataaccaca	gaattaaagg	ataagaaaca	gcaagtgtat	tcactttttt	ataagcttga	tgtagtacaa	attaaatgaaa	agaatgaaac	526
SF170-Aa...	...g.....	...c...g..a.....a....c.	...g..a...	.t...gt.a	569
BR20-B											
TH14-Bc....g	agca....aa	c....gt—	aa...ac...	g.....a....c.	..ag..a.tg	.t...a—	540
SF162-B	gg.c.....	agca...gaatg..	.a...aa...	g.....a....c.	..ag..a.tg	.t...a—	827
MA959-C	.t.....	.gc.....ga.g...	.a.....	g.....a....	.a.....c.	c.....g.	..g...a.c.g	556
ZM18-Cgc.....	...c...a.	...a....	.ag.....	g.....a....	.a.....c.g.	.t.ca.ct.	545
IND868-Cgc.....	..a...ga.	..g...g..	.ac.....	g.....	...ga....	.a.....c.	c...g..a.t	...ga.g.a	224
BR25-C	.t.....	...g.....	...g...ga.g.g.	.a.g..ac..	g.....	...ga....	.a.....c.	c...ga.tg	...g.agt.a	461
UG21-D—	——...	...g...ga.ga.ca...	g.....g.....	..g...tg	.t—a.t.g	532
UG38-Dg....	at.g...ga.c.ga.	.g..a.aa..	g..a....	...g.....	...g.....	..g.c.a.t-	—a.t.g	455
UG46-Dg.....	a.g...ta.ga.aac..	g.....a....	...g.....	..gg...t-	—a.t.g	566
TH22-E	.ta....t..	...g.....	...a...ga.ac.g..	.a.g..cc..	g.....	...g.....	.a.....	..gc...a.tg	.a...agt.g	568
TH06-E	.t....t..	...g.....	...C...ga.g..	.a.g..cc..	g.....a.....	g.——	—...agt.g	556
BZ162-F	.t.....	...g.....	...g...ga.c.ga.	.ag...ac..	g.....a....	.a.....c.	.c.——	——.tg	542
BZ163-F	.t.....	.g.g...tg	...g...ga.c.g..	.a...ac..	g...g...-	...ga....	.a.....c.	...gca.tg	ga.gaa.tg	824
RU131-Gg...t...	...a...ga.a.	.ac..aa..c	g.g...c.a....c.tg	.a.g.agt.a	440
LBV21-7-Gg...ga.gta.	.a...aa..c	g.t...c.	...ga....	...g...c.gt.	.tg.aa.t.g	587
VI525-Gg.....	...C...ga.c.ga.	.gc..aa..c	g.g...c.a....c.	...g..a.t	.t.gaa.tga	605
CA13-H											
VI557-H											
	901	911	921	931	941	951	961	971	981	991	1000
			V2>	>—————	ED31	—————	>				
RW20-A	agacaa—	——gt	atagattaat	aaattgtaat	acctcagcca	ttacacaggc	ttgtccaaag	gtatccttgg	agccaattcc	catacattat	614
SF170-A	ta....tata	aggactca..a	669
BR20-B											
TH14-B	———	——ccagc.	...g...g..ct..a..	626
SF162-B	———	——caagc.	...a...g..ct..	c.....a.....	913
MA959-C	cag——c	tctggagaa.c.g.a.tg	.a....a..	c.....	.c..t...	.c.....	t.....	650
ZM18-C	tagtg.ggac	tctagtga..a....a..	c.....	..ca.t.g.	.t.....	t.....	645
IND868-C	ca..tcta—	-gtgagtat.a....a..	c.....	.cca.t...	.t.....	t....c...	321
BR25-C	cacttctg—	——gtgat.g.a....a..	c.....	.c..t...	.c.....	t.....	555
UG21-D	tact..tacc	a——gt.	g....a...	g.....	a.....	626
UG38-D	ta.tg.t—	———	...g....	.C...C...a...	a..a...	543
UG46-D	tact..——	—taccaac.t..	g.....	..a...	660
TH22-E	t..gt.——	———	...g.....t....t..	...ag....	a.....	.t.....	t.....	653
TH06-E	t..gt.——	———	...c.g....t....t..	...ag....	a.....	.t.....	t.....	641
BZ162-F	cagt.gaaca	t——ca.	.c..gc....a...t.gg.	.t..t....	636
BZ163-F	cagtggagac	t——ac.	.c..gc....c...a...at.gg.	gt.....	918
RU131-G	tagt.c——	——t.	...gc....	gt...a...	...a.....gaat...	.c.....	528
LBV21-7-G	tagt.caact	g——at.	...gc....	g....a...	...a...a..ga.t...	.c.....	681
VI525-G	tagt——	———	...gc....	.c.....	gt...a...	...a.....c...t...	.a.....	690
CA13-H											
VI557-H											

Figure 9 (cont'd)

	1001	1011	1021	1031	1041	1051	1061	1071	1081	1091	1100
RW20-A	tgtgccccag	ctggctttgc	gattctaaag	tgtaaggata	cagagttcaa	tggaacaggg	ccatgcaaga	atgtcagcac	agtacaatgc	acacatggaa	714
SF170-At....t....t..g	769
BR20-B											
TH14-B	...a...g.t....C..ca...	a.a.....at.ca.t..t	726
SF162-Bg.t....t...	aga.....t...at.ca.t	1013
MA959-C	...a.t....t.a...ta...	agaca.....	...g...ac.t.g..t	750
ZM18-Ct....	...a.a...ta...	agaca.....	...g...ac.t.tc....	745
IND868-Ct....t....	...a.....ta...	agaca.....	...g...agt.t	421
BR25-Ct....	...g..t.a...ta...	agaca.....g..a	..t....t.a...g..t	655
UG21-Da.....	a.....at...	a.a.....	...g....tt.a.g..tg.	726
UG38-D	a.....	...a....	aga.a.....ca.t	643
UG46-Dt....	...a.....t...	aga.....	...g..g..ta.	.C.....	...g..g..tg.	760
TH22-E	...a.t....t.a...t....t..C.	aga.t..t.c	...g.....t..a.t.	753
TH06-Et....t.a...t....t...	a.a.c....	...g.....t..a.t.	741
BZ162-Ft....t.a.a.	a.a.....	...g.....g...t	736
BZ163-Ft....t.a...t....t...	a.a.....	...g.....-....g..t	1017
RU131-Gt....t....t....	...g....	aga.t...cat..a.t..t	628
LBV21-7-G	..c..t....	...g.....ca	...g....	aga.....a	..g..t..a.t..	...g....t	781
VI525-Gt....t....	a..t....	...g...g.	aga.t...ca	..t..t..a.t..t	790
CA13-H											
VI557-H											
	1101	1111	1121	1131	1141	1151	1161	1171	1181	1191	1200
				>—— ES7 ——>							
RW20-A	tcaggccagt	aatatcaact	caactgctgt	taaatggtag	tctagcagaa	gaagggatgc	agattagatc	tgaaaatgc	acaacaatg	ccaaaaccat	814
SF170-Acc..g..a..g..a	869
BR20-B						...g	ta.....c..t.....	44
TH14-B	.t.....	.g.....c..	.t.....	...a..g..g	ta.....	..agc...t..	..gg.....	.t.g.gt...	826
SF162-B	.t.....	.g.g.....	...t....c..g..g..g	ta.....t..	...g.....	.t....t..	1113
MA959-C	.t.a.....	gg.....	...a....	c.....	..g..aa...a	ta.....c.g	t....a..	850
ZM18-C	...a.....	gg.....	...a....	c.....	...a...a	ta...g..c.aa..	845
IND868-C	.t.a.....	gg.....	...a....	..g.....	c.....a	ta.....c.gta..	521
BR25-C	.t.a.....	...g.....	..c..a....a...a	ta.....	..a....c.a	...g.....	t....a..	755
UG21-D	.t.a.....	.g.g.....t..	.g....c..a...a	ta.....c..t...	.t...tt..	826
UG38-D	.t.....	.g.g.....C..	cg....C..	.t.....	...a...a	ta.....	t.....	.t.....	743
UG46-D	.t.c.....	.g.g....ct..	.g....C..a...a	ta.....c..t....	.t.....	860
TH22-E	.t.a.....	gg.....	...t....c..a...a	ta..c....c..	853
TH06-E	.t.a.....	gg.....	...t....c..a...a	ta..c....c..	841
BZ162-F	.t.aa....	gg.....	...t..t..c..	c.....	...a.t...g	ta..c....	.c.....	t.g.t....	.a...t...	836
BZ163-F	.t.aa....	gg.....	...t..t..c..	c.....	...at...a	ta..c....	.c.....	t.g.t....	.a.....	1117
RU131-G	.t.a.....	gg.....	...t.at..c	.g....C..	ct.....	...aa...a	tag.....ct..	...g.....	...gt...	728
LBV21-7-G	.t.a.....	gg.....	...t.a...c	.g....C..	.t.....	...aa...a	t.....ct..a...	881
VI525-G	.t.a.....	gg.....	...a...c	.g....C..	.t.....	a...aa.ta	ta.....ct..gt...	890
CA13-H						...aa.g..a	t...c....g....aa...	50
VI557-H						a.ccatg..a	ta.....	.a.....	...g....aa...	50

Figure 9 (cont'd)

	1201	1211	1221	1231	1241	1251	1261	1271	1281	1291	1300
						<V3					
RW20-A	aatagtacaa	cttgataagg	ctgtaaaaat	taattgtacc	agacctaac	acaatac—	aagaaaaggt	gt——aa	ggataggacc	aggacaagca	905
SF170-Aa.c...ag....—a..c	.t.....	960
BR20-Bg.g	..aa.gg.ccg.c..ac....—ga..	a.——c	at.....	...gag....	135
TH14-Bg	..ga.g.atg....ac....—a..	a.——c	atc.....	...g.g....	917
SF162-Bg	..ga.gg.atg....at.....—a..	a.——.	ct.....	g.gag....	1204
MA959-Cg.c	...a.g.atg....	gtg....ac....	.t.....—g.a..	a.——.	.a.....t.	941
ZM18-Ct	...a.g.atg....	gtg....ac.g..	.t.....—a..	a.——.	936
IND868-Ctc.acg....	gtgc....aa....	.t.....—a..	a.——.a..	612
BR25-Ct	...a.cg.atg.g.cgc....	.t.....—a..	a.——.	846
UG21-Dt	...a.g.tcc...c.a	.g.ct.g	.t.ggt—	...ct.ca.g	ac——c	ct.....gt	...g.g....	917
UG38-Dg	...a.g.tc....	.g...g.a	.g.ctt.t	.tgc..taga	...c.aaa	ac——c	ct.....a	...g...t.	837
UG46-Dg	...a.g.tcc...ct.a	.g.ct.g	.a...a—	...cg.c..	ac——c	ct.....t	...g...g	951
TH22-Eg.c	...a....atg....	c.....ctc..—c.a..	a.——.	ct.....t.	944
TH06-Egt.c	...a....atg....	c.....ctc..—c.a..	a.——.	ct.....t.	932
BZ162-Fc	...a.g.atc....ac....—a..	a.——c	at.....	...gg...	927
BZ163-Fc	t.a.g.atc.g..ac....—	a.——c	at.....	...g....	1208
RU131-Gg.g	...a....atg....	.ca.....t.—a..	a.——.	ctt.t.c..g	819
LBV21-7-Gg...	t.a....at	.a.g.t..	gtg....c....—a..	a.——c	ac.....	t.....	972
VI525-Gg.g	...a.g.aag....c....—a..	a.——.	aat.c...a.	...g..tg	981
CA13-Hg	...a.a.tcgt...	...c....c....	.t.c.—	g...g.a.	a.gcgat.g	.t.....ga..	147
VI557-Hg	...a.a.gtccc...	...c....c....	.t.....—	g.....a..	a.——.	.t....g..	141
	1301	1311	1321	1331	1341	1351	1361	1371	1381	1391	1400
				V3>							
RW20-A	ttctatgcaa	caggttggtat	aataggggat	ataagacagg	cacattgtca	tgtcagtaga	gcaaaaatgga	acgacacttt	acgaggggta	gccaaaaaac	1005
SF170-Aac..a.	.t....a.g.c....	.ta.a....	.a.....cc..t	1060
BR20-B	.t.....	...a.ac..a...a.a.	cc.t.c...	...c....	.t.....	.n.caa.a..	.tt.c...t	235
TH14-B	.gg.a...	...acaa..a...a.a.	cc.t...c	a.....	.ta.....	.a.ca.a..	a.tg....t	1017
SF162-B	.t.....	...a.ac..a...a.a.	ca.t...g..	.a.....	.ta.....	.aa.ca.a..	.tt.c...t	1304
MA959-C	ataa..ac..a..ca.ca.	ca.t...ag	.atgic....	.agt....	.a.aa....	.gt.....t	1041
ZM18-Cc..aa.ca.a.	ca.t...a.	.ag.c....	.ta.a....	.a.aa....	.ga.....t	1036
IND868-Ca.ac..a..cga.	.t....a.	ca.t...ga.	aac.....	.t.a....	.a.a...g	agt.....t	712
BR25-Ca.aa..a...a.a.	ca.t....	a.gc....	.a.a...c.	.a..a....	.gt.....t	946
UG21-D	.c....a...	.a.a—.	.a..a...a.a.	ca.t...g..	.a.....	.ta.a....	.a.ca....	.tgcg...t	1014
UG38-D	c....a...	.aag—aaagg	...g...a.a.	a.t.caga.	...g.t...	.ga.a....	.a.ca....	.t.....t	934
UG46-D	.a...a...	.aaa—t.	.a..at.ca.a.	.a.t...g..	...g....	.ta.a....	.a.ca....	.t.....t	1048
TH22-Eag..	...a.ac..a...aga.	.t....g.	ga.t.a.g..	a.....	.ta.ag....	.aa.ca....	a.tg....t	1044
TH06-Eag..	...a.ac..a...a.	.t....g.	ga.t.a.ga.	a.....	.t.a....	.aa.ca....	a.t.....t	1032
BZ162-F	c.t.....	...a.ac..a..c	.c...a...a.	c.t.....	a.c....	.ta.g.ag..	.gacca...	aaggc.g.gt	1027
BZ163-F	.t.....	...a.ac..a..c	.c...a...	.c....a.	.t...g..	a.c....	.ta.a.a..	.ga.caa...	agggc...gt	1308
RU131-G	c.....aa..a...a.a.	.t.....	aa.g.t...	.t.g.tg..	g.agaat.g	a.t.c....	919
LBV21-7-G	.a.....c.cc..c...a.a.	.t...ga.	a.g.c....	ga...tg..	.agaa....	aaagc.c...	1072
VI525-G	.a.....cc..aa..a.a.	.t...c.	.a.....g	gta.g.tg..	.agaat...	.tgc....	1081
CA13-H	...c....	t...c...a...a.a.	.a.t...g..g...	.t...tg..	.accaa....	.t.c.c..t	247
VI557-Hac..	c....a...a.a.	.t....a.	.a.t.c...g	.a.g.t....	.aagg....	.at.a....	.ttc.gc.t	241

Figure 9 (cont'd)

	1401	1411	1421	1431	1441	1451	1461	1471	1481	1491	1500
										<—————	ED33
RW20-A	tacgagaaca	ctttaaaaac	aaaacaataa	tcttgagaa	atcctcagga	ggggatatag	aaattacaac	acatagcttt	atttgtggag	gagaattttt	1105
SF170-A	..aa.agtt.gt...ct.g	c.....t...t...	.a.....	1160
BR20-B	..a.....	a...g.g.t	ag...c.c							273
TH14-B	..a.....	a...ggg...ga.tc.ccc..	.g...gt..t	g...c..t...	.a.....	.g.....	1117
SF162-B	..a.c...	a...ggg..tga.c.ccc..gt..t	g...c..t...	.a.....	.g.....	1404
MA959-C	..aa.....	...ccct..t	ca....acc	.ca.....	.a...c...t...	.a....a...	1141
ZM18-C	..gc...g..	...ccct..t	aa....cc.	.ca.....cc...a....a...	1136
IND868-C	..gc...t.	...ccct..tc	aa....catccc...a....a...	812
BR25-C	..gc...g..	...ccct..t	..g.....	aa...ca..	gca.....cc...a....a...	1046
UG21-D	..a...c.t	tc...cc.a	.c.g.....	.t...a.accg...ccc..c.....	.a.....	.g.....	1114
UG38-D	..a...c.t	tc...c.ta	.c.g.g...	.t...a.accccc..	..C.....	...C.....	.a.....	.g.....	1034
UG46-D	..g...c.t	t...c.cc.g	.c.....	.t...c.acc	.ca...g...ccc..C...c..	.a.....	.g.....	1148
TH22-E	..aa...g..	..—tt.t	.g.....c.acc	.c.....	.a...C...t	g...cat...	.a....a...	.g.....	1141
TH06-E	..aa...g..t..t	.g.....c.ccc	.c.....	.a...C...t...	g...cat...	.a....a...	.g.....	1132
BZ162-F	..aagtct..	t..ccct..t	gc.....c	aa...a.ctccc...	.g.....t	g...cat...aa..	1127
BZ163-F	..aagtct..	t...cct..t	gc.....	aa...a.ttccc...t	g...t...	.a....a...	1408
RU131-G	..aa.g.atc...	.c.ac...	ca.a.a.ctc	.g.tg....	.a...C...t...	.a....a...	1019
LBV21-7-G	..a.g.at	..—c...	...gc...	c...a.ctccc...g.t...	.a....a...	1169
VI525-G	..a.a.g.	a—tt.t	...ac...	c...a.ctccc...t...	.a....a...	1178
CA13-H	..g.a....	.c.g.c.g	.gc.a....c	aa...a.acca.c....t...	...c.tg...	.ac....C..	347
VI557-H	..a.....t..t	c.....	.t.c..accc.g.t	g...ct...	.a....a...	341
	1501	1511	1521	1531	1541	1551	1561	1571	1581	1591	1600
	————<			<V4							
RW20-A	ctattgtaat	acatcaggcc	tgttcaatag	cacctgggag	tcaaacagca	ca————	—gagtcac	aacaacacaa	cgtc aaatga	c————	1184
SF170-A	t.....g..t....	..a...g.	gg...t...	.tgacagcat	acag.....	.t.g...g	a.....	.———	1251
TH14-B	...c.....	..a.caa.	...t....	t...a.t	gat.ct.t.	.ttggaataa	taataca—	gga.tggc.	———	———	1194
SF162-B	...c.....	t.a.cag.	.t.t....	t...a.t	aat.ct.tag	ggccaaataa	cact———	—..tgg..	———	———	1475
MA959-Cc...g.	...t....	t.a.tta—	——.tgat-	——acag	aaggt.—.	tt...tt.	a———	———	1206
ZM18-Cc...aa..	...t....	t.a.ata..	c.t.tgat-	——acaa	atagt.c—.	t.t.tc.t.	atga———	———t	1212
IND868-Cc...aa..	...t...g.	t.a.acat.	—..tg.t-	——acaa	aaagt.—.	tt...gctc.	a———	———	880
BR25-Cc...a...	...t....	t.a.acaca	c.t.t.t.t.	..gt					1100
UG21-D	...c.c...a.	...t..c.a	t.gtgtatg.	a—tcaa.	t.glacg.at	aggg.——	-ca.tggg.	———	———	1185
UG38-D	..tc.c...caa.	...t....	t.a...g.	agt.tgat.	.tcaaatag	tacat——	-t..t.ac.	———	———	1108
UG46-D	...c.cg..	g....a.a.	...t....	g..tata.t	a—tcagg	t.gtaca.gg	ggtaa——	-t...gt.	———	———	1219
TH22-Ec...	..a.c.a.	...t.g.a	t.t.cata	aa...tg—	——aaac	catg.c.ggg	tgt...ggc.	———	———	1212
TH06-Ec...	...c.aaat	...t...g.	a...at....	gggcct.—	——ataa	cacg...ggg	cct..t.ac.	.t———	———	1205
BZ162-F	...c.cg..a.a.g.a	..agaatc-	—c..tg..-	——acag	a———.	tc...tggc.	———	———	1189
BZ163-F	...c.c...a.	...t——	———	——.ga.-	——acag	g———.	tc...tggc.	———	———	1455
RU131-Gc...a.	...t...a	t.gt———	———	——gagaa	taactcca.t	.t.g.g.at.	ac..———	———c	1083
LBV21-7-Ga.	...t....	t.gtataffa	..t———	——aacia	taat.cac..	tct..t.gc.	gtaa———	———g	1242
VI525-G	t.....gag.	...t....	t...t.t.	.tt———	——aatag	tact.g.ctg	glt..t.gt.	.t.gg.....	tacacgaaaa	1266
CA13-Ha.aaa.	...t....	t....ac.	a.t...c.-	——aatgg	cagct.tgtg	.g...t.ac.	.aga.....-	———gt	432
VI557-Hc...aaat	...t.c..	t...t.t	aac.gt.c—	——atcaa	atgat.tctc	—..tggc.	.aa...———	———	418

Figure 9 (cont'd)

	1601	1611	1621	1631	1641	1651	1661	1671	1681	1691	1700
		V4>									
RW20-A	actataactc	ttacatgcag	aataaagcaa	attataaata	tgtggcagaa	agtaggacaa	gcaatgtatc	cccctcccat	t—caagga	gtaataaggt	1281
SF170-At..	.cc.....gg	c—.....a..	1348
TH14-B	-....c.g.	.cc.t....a...	..g...C.g.a..g	c—ag....	ca...t.a.	1290
SF162-B	-....c.a.	.cc.....a...C.	g.....g.a..g	c—ag....	ca...t.a.	1571
MA959-C	-gc....c.	.cc.....a...C.g.	g.....g.g	—g....	aac....ca.	1302
ZM18-C	..a.c.a.	.cc.....a...C.gg	g.....g	—gc....	aac....ca.	1309
IND868-C	-c...c.aa	.cct.....a...	g.g...c.g.	g.....g.g	—g....	aac....ca.	976
UG21-D	-a..C.a.	.cc.....a...gga..a	—g....	ca...c.ac.	1281
UG38-D	-a..c.aaa	.cc.....ga...Cg.a..ga	c—g....	c....c.at.	1204
UG46-D	-a..c.aa.	.cc.....a...C.gga..g	—g....	c....c.aa.	1315
TH22-E	-...c.ta.	..c.....a	g.....C.gg	..C.....g	.t.....	—agg...	ag...t.at.	1308
TH06-Ec.ta.	..c.....a	g.....	..g...c.gg	..C.....g	.t.....	c—agt...	ag...t.at.	1302
BZ162-F	-....c....	.cc...tc.a...	..gg...c.g.g.	a.....g	..g.....	c—gc....	aac..t.cc.	1285
BZ163-F	-....c....	.cct...tc.a...	..gg...c.g.	..g...g.g	..g.....	c—tc..a.	aac..t.cc.	1551
RU131-Gc.a.	.cc...t..	g....a...ta.g	..g..g...g	c—gc....	aac..c.ca.	1180
LBV21-7-G	..a..c.a.	.cc...t..a...	..g...ga.g	g..g.....g	—gc....	aac..t.ca.	1339
VI525-G	ga...t.a.	.gca.....aa...	..g.g.ga.g	..g.....g	cgcagc...	aac..tgaa.	1366
CA13-Ht.a.	.gca.....a...	..g...gc.g	..C.....g	c—a.t...	aag..t.ca.	529
VI557-H	-ta..tgaa.	.gc.....a...C.gg	c—a....	aa...t.ca.	514
	1701	1711	1721	1731	1741	1751	1761	1771	1781	1791	1800
					<V5					V5>	
RW20-A	gtgaatcaaa	cattacagga	ctactattaa	caagagatgg	agggaaat—	aacagtaca-	————	——aat	—gaaatct	tcagacctgg	1357
SF170-A	..at.....	t.....	..t.....	t..g..aatg..-	————	——...	—....c..ac	1427
TH14-B	..tc.....	t.....g	t.g.....	t..t.——	————	——tagtga	gaacaag.cc	acc..g.c..	1372
SF162-B	..ctc.....	t.....	..g.....	t..t.——	————	——agagat	cagtaac.cc	acc..g....	1653
MA959-C	..a.....	t..c.....g.	..c.t.g...	...tccaactca——	————	——aag.ca	—..g.ca.	...g....	1381
ZM18-C	..a.....	t..c.....g.	..c.g.....	...tcaaact	g.t.cc——	————	——aca..c	aca..g.ca.	1391
IND868-C	..a.....	t..c.....gg	..c.t.....	...a.ca——	g.gt——	————	——caaatc..	aca..g..a.	1055
UG21-D	..tc.....c	t.....	...t.g..g.	t..tgt——	————	——gaaaaataa	tagtcag..c	—..g.c..	1363
UG38-D	..ac...g..	t.....gc.g.	t..t.tagg.	————	——gaaaaataa	tagtcag...	aat..t.c..	1292
UG46-D	..tc.....t.g..g.	g..——	————	——aataa	tactaggc.g	aat....c..	...g....	1394
TH22-E	...t.....	t.....	a.....g.	...g....	t..tgc.aat	..tg——	————	——cg	aac..g.c..	1385
TH06-E	...t.....	t.....	a.....	t..tgc.aat	..t——	————	——cg.c.	aac..g.c..	1381
BZ162-F	..a.c....	t.....tg.c..	t...ttagat	g.tg...gga	atactagtca	gactaatcgg	act..g....	1385
BZ163-F	..a.c..g..	t.....tg.	t..tc.gaat	..t——	————	——aatc.g	act..g.c..	1630
RU131-G	..att....	t....caat	..t.ac.gc-	————	——aca...	aat..g.c..	1265
LBV21-7-G	..a.....a.....	t.....agt	..c..a.gg—	————	——a	aat..g.c..c	1418
VI525-G	..ag....ga.....	t.....aat	..t.a.——	————	——c	aat..g.c..	1445
CA13-H	...t.....	t.....	..a....g.	...t.cca	t..tt...cgg	ttctgcaga—	————	——g	gag.tg.c..	608
VI557-H	t.....g	..a....g.	...t...ca	t..tt...tct	gcagaa.g—	————	——.	aat.tt.c..	593

Figure 9 (cont'd)

	1801	1811	1821	1831	1841	1851	1861	1871	1881	1891	1900
			← ES8 →	←							
RW20-A	aggaggaaat	atgagggaca	attggagaag	tgaattatat	aagtataaag	tagtaaaaaat	tgaaccacta	ggagtagcac	cctccagggc	aaagagaaga	1457
SF170-Ag..a.....g....t..a...c...	1527
TH14-B	...t...g..a.....g....g...t..a...a.c.	1472
SF162-B	...t...g..a.....g....g...t..a...a...	1753
MA959-Cg..g...g....	..a.g...t.ga.t.a...	...a..g...	1481
ZM18-Cg..c....	..a.....	..g...g....	..a....t.g	..ca.....	..a.tgc...	...a..g...	1494
IND868-Cg..a...a.c....	..g...g....	..a.g...t.gt..	..a.t.ca...	...a..g...	1155
UG21-D	...g...g..a....	.c.....ac	..a.....g...tc.....	..a...a...	..g.....	1463
UG38-Dg..a....c	..a.....g...a.....	..a...t...	1392
UG46-Dgg..a....c	..a.....g...tc.....	..a.tga...	1494
TH22-Ea.a....a.....c....a.....	..a.....	1484
TH06-Ea.a....a.....c....a.....	..a.....	1481
BZ162-F	g.....	...aa...a.....g....a...a...	...a...ca.	1485
BZ163-F	g..t.tg..	...aa...tt.....g....a.....	...a...ca.	1730
RU131-Gg..g....	..a.....a.....	1353
LBV21-7-Gg.ca.....	..a.....a...a...	..g.....	1518
VI525-Gg.cg..	ca..t....a.....	..g.....	1545
CA13-Hg..a.....g....a.t.a...	708
VI557-Hg..a.....g.g.	c.....	...t....	..a.....	..g.....	693
	1901	1911	1921	1931	1941	1951	1961	1971	1981	1991	2000
							← ED12 →	←			
RW20-A	gtgggtggag-	—agagaaaa	aagagcagtt	gga—atag	gagctgtctt	ccttgggttc	ttaggagcag	ctggaagcac	tatgggcgcg	gcgtcaataa	1551
SF170-Ac.-	—.....—...a..	..a.....c.....	1621
TH14-Bc.-	—.....g	...acg...a.g..g...a...	..a.....a	1569
SF162-Bc.-	—.....g	—acgc...a.g..g.....	..a.....a	cg...c.g.	1847
MA959-C-	—.....g	...—...g..g.....	..a.....g.g.	1575
ZM18-Ca	cg.....g	...—...g..g.....	..a.....	1588
IND868-C-	—.....g	...—g.g..g.....	..a...c...	1230
UG21-Da-	—.....a.a	...—c.g.t.g..g.....	..a.....	g.....agc.g.	1557
UG38-Da-	—.....	1413
UG46-Da-	—.....a.a	...—c...a.g..g.....	..a.....	g.....ag.	1588
TH22-E-	—.....g	...—...a.ga.	..t.....a.....	1578
TH06-E-	—.....g	...—...a.ga.	..t.....a.....	1575
BZ162-Fa.-	—.....g	...—...a.g..g.....	..a....t.a	1579
BZ163-Fag.-	—.....g	...—g.t.g..ga.c..g.	..a.....	1824
LBV21-7-Gg.-	—.....a.	act——.g....c..g	1609
VI525-G-	—.....a.	...—c.g.a.....	1639
CA13-Hg.-	—.....—c...a.....	802
VI557-Ha-	—.....t.g	...—c.g.	a.....t.g.....	..a.g.....g.g.	787

Figure 9 (cont'd)

	2001	2011	2021	2031	2041	2051	2061	2071	2081	2091	2100
RW20-A	cgctgacggc	acaggccaga	caattattgt	ctggtalatg	gcaacagcaa	agcaatttgc	tgagggctat	agaggctcaa	cagcatatgt	tgaagctcac	1651
SF170-Att...c....a...c...a....	1721
TH14-Btg....g	..a.....	t....g...c...	..c.a....	1669
SF162-Btg	..a.....a....	t....g...c...	..c.a....	1947
MA959-Ct	...a.....	...c.g....	t...c....g...	..t...c..c	..c.a....	1675
ZM18-Cg.g....a..c..g...c...	..c.a....	1688
UG21-Dtg...c...a.....g...t...	..c.a....	1657
UG46-Dt	..									1600
TH22-Etg..gc.a....	1678
TH06-Etg..gc...	..c.a....	1675
BZ162-Fta....a.....	...a.....	t..a..g...c...	..c.....	1679
BZ163-Fta....g	..t...c...	t..a..g...c...	..c.....	1924
LBV21-7-Gtt...c....a.....g...	..a...c...	..c.a....	1709
VI525-Gtt...c....g..gc...	..c.a....	1739
CA13-Htt...c....t.....c...a....	902
VI557-Htcaa....	..c....a...	887
	2101	2111	2121	2131							
	<----- ED14 -----<										
RW20-A	ggtctggggc	attaaacagc	tccaggcacg	a							1682
SF170-Aa.	.							1752
TH14-B	a.....	..c..g....a.	.							1700
SF162-B	a.....	..c..g....a.	.							1987
MA959-Cg....a..a.	.							1706
ZM18-Cg....a..a.	.							1719
UG21-D	a..t.....									1673
TH22-E	a.....a.	.							1709
TH06-E	a.....a.	.							1706
BZ162-F	a.....	..c..g....a.	.							1710
BZ163-F	a.....a.	.							1955
LBV21-7-G	a.....a.	.							1740
VI525-G	a.....a.	.							1770
CA13-H	a										903
VI557-H	.										888

11.2.1 PCR Primers

ED3 5'-TTAGGCATCTCCTATGGCAGGAAGAAGCGG (5956-5985*)

ED14 5'-TCTTGCCTGGAGCTGTTTGATGCCCCAGAC (7960-7931*)

These amplify a ~2.0 Kb fragment spanning from the first exon of *rev* to the transmembrane protein gp41 coding region of *env*.

Nef3 5'-TAAGTCATTGGTCTTAAAGGTACC (9037-9014)

Combined with ED3 to produce a ~3.1 kb product, this primer is substituted for ED14 when a full length (gp160) *env* open reading frame is desired for subsequent cloning and expression studies.

ED5 5'-ATGGGATCAAAGCCTAAAGCCATGTG (6556-6581*)

ED12 5'-AGTGCTTCCTGCTGCTCCCAAGAACCCAAG (7822-7792*)

These amplify a fragment of ~1.25 Kb spanning the V1-V5 coding region of gp120. We have had good experience using these fragments for HMA when quantitative mobility data is desired for deriving phylogenetic relationships. They are also used as an alternative to the two sets below for genetic subtyping by HMA.

BH2 5'-CCTTGGTGGGTGCTACTCCTAATGGTTCA (7724-7695)

This primer is often used instead of ED12 in first round PCR reactions since it amplifies a smaller product. It typically shows better sensitivity than ED12.

ES7 5'-tgtaaacgacggccagtCTGTTAAATGGCAGTCTAGC (7001-7020*)

ES8 5'-caggaaacagctatgaccCACTTCTCCAATTGTCCCTCA (7667-7647*)

These amplify an internal fragment of ~0.65 Kb spanning the C2-V5 coding region of gp120. This is the most commonly used second round primer set for DNA sequencing studies. Lower case letters are complementary to the universal M13-primer (ES7) or the reverse M13-primer (ES8). These allow direct sequencing of the PCR product.

DR7 5'-TCAACTCAACTGCTGTTAAATGGCAGTCTAGC (6989-7020*)

DR8 5'-CACTTCTCCAATTGTCCCTCATATCTCCTCC (7667-7637*)

These primers correspond to ES7 and ES8 but with the 5' terminal nucleotides corresponding to HIV sequences rather than the M13 universal primer sequences. These primers are, therefore, useful for cloning fragments into phage or plasmid vectors (such vectors typically contain the universal primer sequences and thus would double-prime in sequencing reactions and not provide useful sequence data). For routine use, DR7 and DR8 usually show better sensitivity than the ES7 and ES8 combination.

ED31 5'-CCTCAGCCATTACACAGGCCTGTCCAAAG (6816-6844*)

ED33 5'-TTACAGTAGAAAAATTCCCCTC (7359-7380*)

These amplify a fragment of ~0.5 Kb spanning the C2-V3-C3 coding region of gp120. This region is more conserved and has less length variation than the fragments amplified with the above primer sets. This results in overall faster fragment mobility in HMA and therefore is useful when comparing more distantly related sequences.⁸

11.2.2 Polymerase Chain Reaction

Nested PCR reactions are required for the preparation of sufficient quantities of HIV *env* DNA fragments from infected body fluids and cells to perform efficient cloning, direct sequencing or HMA analyses. See section 11.2.4 for a discussion of number of HIV templates in typical PCR reactions and the impact of viral copy number on viral diversity measurements. However, reference fragments for HMA (see section 11.2.3) can easily be amplified from 10 ng of plasmid DNA using 2nd round primers only (note that not all reference plasmids contain annealing sites for the ED3 and ED14 primers, used in the example below). Positive and negative control PCR reactions (10 copies of pNL4-3 and reaction mix alone, respectively) should be carried through both rounds of amplification.

First Round PCR

The amounts indicated are for a single 50 µl reaction. When setting up multiple reactions, prepare a “master mix” of reagents minus template DNA. The volume should be a little larger than the calculated amount (e.g., $n + 1$) to allow for pipetting errors). For preparation of PCR mix and dNTP mix see Annex 4.

1. Mix, in this order:

5 µl 10X	PCR mix (stored as frozen aliquots)
6.25 µl	10 mM MgCl ₂
28.25 µl	H ₂ O
5 µl 10X	dNTP mixture (stored as frozen aliquots)
2 µl ED3	(5 pmoles/µl) (stored as frozen aliquots)
2 µl ED14	(5 pmoles/µl) (stored as frozen aliquots)
0.5 µl	Taq polymerase (2.5 U)

Note: The order of addition of reagents should be considered and adhered to in each experiment to avoid contamination of reagents.

⁸ Coordinates from the HIV1-HXB2 genome (GenBank accession no. K03455).

-
2. Overlay reaction mixture with 30 µl mineral oil or an Ampliwax (Perkin-Elmer) bead.

Note: Mineral oil or paraffin beads (Ampliwax) are used to prevent sample evaporation and are not required if a thermocycler with a heated lid is used. Beads can also be used to separate reaction components prior to reaching an elevated temperature. This is referred to as the “hot start” procedure and is helpful in preventing amplification of spurious products from cellular DNA. In this procedure, a master mix is prepared without template DNA and aliquoted into PCR tubes. The tubes are heated to 80°C to melt the wax and then cooled to solidify the wax. To begin the reaction at an elevated temperature, DNA template is added to the tube above the Ampliwax plug, and the tube is capped and placed in the thermocycler to begin the PCR. Once the wax melts, the DNA mixes with the rest of the reagents and DNA synthesis begins. Hot start can be accomplished most easily using the “AmpliTaq Gold” enzyme from Perkin-Elmer, which requires heat for activation and which obviates the cumbersome hot start wax procedure.

3. Add 0.1-2.0 µg of infected cell DNA or an equivalent amount of lysate (the higher range indicated should only be used with highly purified DNA, such as with the phenol or Puregene methods described above, otherwise substantial inhibition due to contaminating substances could result).
4. Amplification conditions:
3 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min.
32 cycles of 94°C for 15, 55°C for 45, 72°C for 1 min.
Final extension at 72°C for 5 min.
5. When completed, store samples at -20°C.

Second Round PCR (Prepare a 100 µl reaction **per** sample):

1. Mix, in this order:

10 µl	10X PCR mix (stored as frozen aliquots)
12.5 µl	10 mM MgCl ₂ ^{*1}
57 µl	H ₂ O
10 µl	10X dNTP mixture (stored as frozen aliquots)
4 µl ED5	(5 pmoles/µl) (stored as frozen aliquots)
4 µl ED12	(5 pmoles/µl) (stored as frozen aliquots)
0.5 µl	Taq polymerase (5 U/µl)

Note: The optimum MgCl₂ concentration for primer pair ES7/ES8=1.8mM; ED31/ED33=1.4mM; ED5/ED12=1.4mM.

2. Overlay reaction mixture with 30 µl mineral oil or an Ampliwax pellet, or use a thermocycler with a heated lid. Hot start is typically not helpful in second round PCR reactions.

-
3. Add 2 µl of the 1st round reaction, or 1 µl reference plasmid.

Note: There appears to be an optimal amount of first round reaction mixture to use in second round PCR reactions, corresponding to a 50-fold dilution of the first round material. The use of larger amounts may result in substantial inhibition of DNA synthesis.

4. Amplification conditions:

3 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min.

32 cycles of 94°C for 15, 55°C for 45, 72°C for 1 min.

Final extension at 72°C for 5 min.

Note: In some instances, standard amplification conditions (55°C annealing) do not yield a product visible following agarose gel electrophoresis and ethidium bromide staining (see below). In these instances, a 'step-up amplification' is carried out with 5 initial cycles at lower annealing temperatures (from 52°C to 37°C), followed by 30 cycles under standard conditions. If samples are still negative after step-up amplification, another amplification can be attempted using the above conditions, this time using the alternative set of second round primers (e.g., ES7-ES8 to amplify a 0.7kb product spanning the V3 to V5 region or ED31-ED33 to amplify the 0.5kb C2-C3 region).

5. When completed, store samples at -20°C.
6. Check second round reactions for amplification product by loading 5 µl mixed with 1 µl loading dye (see Annex 4) on a 1% agarose gel in 1X TBE or 1X TAE. Electrophorese at 100 V for 1 hour. The gel is stained with ethidium bromide (0.5 µg/ml in H₂O) for 30 min and the DNA detected by UV transillumination.

11.2.3 Subtype References for HMA

The following set of reference sequences were chosen based on clone availability in 1994, those currently available in the HMA kit are shown in Table 2. Since that time, additional HIV-1 subtypes, the HIV-1 O and N groups were identified, and HIV-2 subtypes defined. Furthermore, the overall diversity of HIV in the env sequence continues to grow at about 1% per year. Thus, as will be discussed below, reference sequences for HMA are often chosen based on the target population. These use expected subtypes and local strains as references in the first set of analyses, and are expanded to include other references only if a sample is not unambiguously typable in the first pass. A recent set of reference sequences for phylogenetic studies has been put forward, although not all have yet been evaluated for use by HMA. A new set of standards were made available by the AIDS Reagent Programme in 2000.

Table 2: Reference sequences in 1999 HMA kit

Subtype Reference	HIV-1 Strain	Accession Number	Cloned Fragment	Plasmid Vector	Insert size (bp)	EcoRI Sites	HindIII Sites	Antibiotic ^R
A2	IC144	n/a	ED5/ED12	pCR1	4381	1	2	Kan
A3	SF170	M66533	gp160	pCR2	6550	2	1	Kan/Amp
B1	BR20	U08797	gp160	pCR2	6772	2	1	Kan/Amp
B2	TH14	U08801	gp160	pCR2	6750	2	2	Kan/Amp
B3	SF162	M65024	6.6kb 3'end	pUC19	3952			Amp
C1	MA959	U08453	gp160	pCR2	6783	2	1	Kan/Amp
C2	ZM18	L22954	ED5/ED12	pCR1	5466	2	2	Kan
C3	IN868	U07103	ED5/ED12	pCR1	4150	1	1	Kan
D1	UG21	U08804	gp120	pCR2	5621	2	2	Kan/Amp
D3	UG46	U08737	gp120	pGEM-T	273			Amp
E1	TH22	U09131	gp160	pCR2	6778	2	2	Kan/Amp
E2	TH06	U08810	gp160	pCR2	6294	2	2	Kan/Amp
E3	CAR7	n/a	ED5/ED12	pCR2	5197	2	1	Kan/Amp
F1	BZ162	L22084	gp120	pCR2	5664	2	1	Kan
F2	BZ163	L22085	gp120	pCR1	6226	1	1	Kan
G1	RU131	U08364	ED5/ED12	pUC18	1353			Amp
G2	LBV21-7	U096647	gp160	pUC19	2595			Amp
G3	VI525	U09665	gp160	pUC19	2604			Amp
H2	VI557	U09666	gp160	pCR2	4836	2	1	Kan/Amp

Note: Each plasmid described in Table 2 is supplied as plasmid DNA at a concentration of 100 ng/µl in 10 mM Tris HCl (pH 7.4) plus 0.1 mM EDTA (pH 8.0). Transformation and growth of these plasmids is possible. However, it is recommended that after plasmid purification, all reference fragments be PCR amplified and heteroduplexes formed as illustrated in Figures 10-12. This will ensure the detection of cross-contamination (>1 heteroduplex pattern present per lane) or mislabeling of plasmid preps (erroneous heteroduplex pattern for that fragment). An alignment of each of the available sequences is shown in figure 9 and the sequence files are provided on a computer disk with the HMA kit (for figure 9 see end of section 11).

11.2.4 *Template Quantitation*

HIV consists of an evolving population of related variants, or quasispecies, in infected individuals. When characterizing the populations of genetic variants comprising this quasispecies, it is common practice to obtain a number of sequences by amplifying a region of the viral genome from a small amount of infected tissue (typically 1 µg of DNA) using PCR, and then to select and sequence a number of clones, or to use the amplified genome in HMA analysis. Uncultured PBMC typically contain 1-100 copies of proviral templates per µg of DNA. Thus, a PCR reaction may contain only a few amplifiable templates and if analyzed by DNA sequencing, the probability of re-sequencing products from the same template is high, especially for cases in which the copy number is low. For example, if 10 clones are to be sequenced, 25 input templates would give 88% probability of resampling at least one of the original templates. The dilemma of resampling is also evident when other techniques, such as HMA, are applied to HIV and other biological samples with low copy numbers of target molecules. One might detect a low level of heteroduplexes by HMA and conclude that the sample had little sequence heterogeneity, when in fact only one or few different molecules of what may actually be a highly heterogeneous mixture was sampled.

To avoid the resampling problem, each clone to be sequenced could be obtained from an independent PCR, a preferable but expensive and laborious approach. Alternatively, endpoint dilution or other methods can be used to quantitate target templates, and appropriate caution applied to the interpretation of data.

To determine amplifiable template numbers in PCR experiments we developed the program QUALITY, a variant of the minimum Chi-squared (MC) method described by Taswell [38] for limiting dilution assays. Taswell demonstrated by simulation desirable properties of minimum variance (i.e., high precision) and minimum bias. The QUALITY method also allows the user to specify the probabilities of a false negative and false positive PCR reactions. The program, and the methods used, are described in detail in Rodrigo et al. [39]. On-line information and updated versions are available at <http://ubik.microbiol.washington.edu/cbu/quality/>.

11.2.5 *Agarose gel electrophoresis*

PCR products are typically analyzed on a 1% agarose gel to determine if:

- DNA fragments of the desired size were produced
- nonspecific products were produced
- the amount of DNA is sufficient to perform HMA (100-250 ng/reaction are needed)

Method:

For reagents see Annex 4.

1. Prepare a 1% agarose gel: Weigh 1g agarose and add 100 ml of TAE (1X). Boil the mixture in a microwave oven or on a hot plate for a few minutes, swirling occasionally to prevent boil-over, until all the agarose is dissolved. Let the solution cool to approximately 60°C before pouring (until it is cool enough to hold the flask in your hand), and insert the comb. After the gel has become solid, put it in the agarose gel box filled with TAE (1X). It is advisable to wait 1 hour before removing the comb to allow the gel to set fully.
2. Load samples on the gel: Remove 5 µl of (typically 2nd round PCR product), add to it 2 µl loading dye, mix and load the gel. One well is used for a DNA size marker (100 bp ladder) to provide a migration reference to other gels.
3. Electrophorese for approximately 1 h at 90V (the time and voltage may need to be changed and will depend on the gel thickness and size).
4. After electrophoresis, stain the gel with ethidium bromide (0.5 µg/ml in H₂O) for 20 to 30 minutes and destain in H₂O for 10 minutes. Photograph with a Polaroid camera with UV illumination and Wratten 1 filter.

11.3 Analysis of PCR products

11.3.1 Strategies for *env* subtype determination using HMA

Once PCR fragments are generated, viral genetic analysis can be undertaken using a variety of methods, including DNA sequencing. However, we will focus the remainder of this section on HMA [2], since it is usually more rapid, and can be performed without use of the expensive equipment and/or reagents required for DNA sequencing. As described above, nested PCR is used to generate 0.5 -1.2 Kb *env* gene fragments from uncharacterized strains of HIV-1. The 1.2 Kb fragments encompass the V1 through V5 regions of the gp120 coding sequence, while the ~0.7 Kb fragments encompass an internal V3-V5 fragment, and the 0.5 Kb fragment covers the regions between V2 and V4 from C2-C3. The variability of the two larger fragments are similar and in general, the highest within the HIV-1 genome, whereas the C2-C3 region is more conserved and has less length variation. We currently recommend performing subtyping experiments with the 0.7 kb fragment and use the larger or smaller fragments if required, that is, if PCR reactions are initially negative or if a subtype cannot be unambiguously assigned using the 0.7kb fragment. In particular, if highly divergent samples are being compared, the 0.5 kb fragment may be preferable.

Method Overview:

Using the chosen second round primers, the corresponding fragment is amplified from different reference plasmids containing *env* genes of known subtypes, as well as from the patient specimens to be examined.

- PCR reactions are checked for appropriate product length and yields on an agarose gel.
- Heteroduplexes are formed by melting amplified DNA from the unknowns with amplified DNA from the reference strains, and analyzed on polyacrylamide gels.
- Heteroduplexes formed between the unknown sample and the most closely related sequences exhibit the fastest mobilities. If the closest relatives are all from a single genetic subtype, the likely subtype of that strain is thus determined. The goal should always be to positively identify the subtype of the unknown. Therefore, heteroduplexes formed with a set of references from the assigned subtype should have markedly faster mobilities than with other subtypes, although the degree of this distinction is subjective.
- We recommend that the first few samples analyzed be compared to all of the reference strains provided, in order to confirm reagent identity, to obtain unambiguous results, and to gain familiarity with the assay.
- When analyzing a geographic region suspected of having only a single or a few subtypes (e.g. subtype B in North America, subtypes B and E in Thailand) it is possible to reduce the number of reference strains used for initial subtyping. In Thailand, for example, where both the subtype B and E strains have to date not diversified greatly, it is possible to positively identify the subtype of an unknown by comparison to references from only the B and E clades. The low degree of diversity within these two subtypes in individuals infected in Thailand, and consequently the very fast mobility of their heteroduplexes, provides a high degree of confidence in assigning subtypes.
- Even though the B subtype predominates in the United States, subtype determination is more complex because this virus population, having been introduced earlier, has diversified for a longer time. Therefore, subtype B *env* genes are not as clearly related to each other (and form slower migrating heteroduplexes) as the Thai B or E *env* genes are to each other. We, therefore, recommend that for subtyping of North American-derived viral sequences, these be compared to multiple B reference strains in addition to at least one strain from a few other subtypes (as negative controls).
- Where there is the likely presence of multiple and highly diversified subtypes, such as in Central Africa, Russia, Brazil, etc., it is often necessary to compare each unknown to a panel of multiple reference sequences from multiple available subtypes.
- Comparison to only a single reference strain from a given subtype does not always provide an unambiguous result. Thus it would always be best to use a panel of multiple reference sequences that are expected to be closely related to your unknowns.

- There is great flexibility in the choice of subtype reference strains that can be used. The use of references derived from local cohorts is recommended, since within a given geographic region and cohort the resident HIV strains are more likely to be closely related to each other than to geographically more distant reference strains. This permits more rapid and confident assignments of subtype and intrasubtype relationships to be drawn. If *env* genes of known sequence from the region under study are available, they may also be used for generation of the standard curves required for inference of numerical estimates of sequence divergence.
- Some samples may be difficult to subtype with confidence by HMA using the fragments described above. Such results generally indicate detection of a genetic outlier within a known subtype, the detection of a new subtype, or a recombinant virus derived from parental viruses from different subtypes (e.g. Zam184 analyzed corresponds to a subtype A/C recombinant in *env*). It is also possible, however, that the difficulty stems from a large deletion or insertion in the V1-V2 or V4-V5 region, or less likely, another region of *env*.
- The next step in characterizing an initially non-subtypable strain would be to try another fragment of *env* in an HMA, or determine a portion of the DNA sequence. Use of the ED31-33 fragments covering the C2-V3-C3 region, which normally has little length variation, typically obviates this problem [40]. There is, of course, no barrier to performing HMA with other regions of the HIV-1 genome and Heyndrickx and van der Groen and colleagues have recently developed a *gag* gene HMA [40a].
- The degree of variation required for good discrimination of heteroduplexes in neutral polyacrylamide gels is comfortably within the range of 3-20%, hence the expected degree of mismatch should determine the fragment to be used for subtyping. Note that this mismatch figure counts mismatches only; for detection of length variation by HMA, normally only single base gaps are required.

11.3.2 Production of heteroduplexes

1. Assess the genetic diversity of each PCR-amplified sample.

This analysis provides a baseline heteroduplex pattern (due to quasispecies diversity *in vivo*) with which to compare deliberately formed heteroduplexes. It is useful if this sample is run on the same gel as the heteroduplexes formed between references and the unknown, so that bands present due to intra-quasispecies heteroduplexes can be identified.

- Mix, in a 500 µl Eppendorf PCR tube:
 - 5 µl second round PCR reaction (~100-250 ng of DNA)
 - 5 µl H₂O
 - 1.1 µl 10X heteroduplex annealing buffer
- Heat to 94°C for 2 min in a thermocycler (or in a boiling water bath).
- Cool tubes rapidly by transferring to wet ice.

Rapid cooling facilitates stable formation of heteroduplexes between highly divergent sequences, but is less important for creating heteroduplexes between closely related sequences. You will note that the apparent heteroduplex yield will, in any case, be greater between more closely related sequences (c.f. enclosed figures).

- Heteroduplexes can be kept at room temperature before loading, or stored at -20°C.
2. Form heteroduplexes using approximately equal amounts of amplified fragments from the unknown and each reference.
 - Mix, in a 500 µl Eppendorf PCR tube:
 - 5 µl second round PCR reaction from the unknown (~100-250ng of DNA)
 - 5 µl second round PCR reaction from the analogous fragment of *env* derived from a reference strain
 - 1.1 µl 10X heteroduplex annealing buffer
 - Heat to 94°C for 2 min in a thermocycler (or in a boiling water bath).
 - Cool tubes rapidly by transferring to wet ice.
 3. Mix heteroduplex reaction with 3 µl 5X Ficoll/loading dye and load onto a 5% non-denaturing polyacrylamide gel.

11.3.3 Polyacrylamide gel electrophoresis

General considerations: The mobility of heteroduplexes is noticeably affected by changes in temperature during electrophoresis, particularly when the reannealed DNA strands are from highly divergent viral strains. The higher the temperature in the gel (i.e. the faster the gel is run) the slower the mobility of the heteroduplexes. Temperature increases result in local increases in duplex melting and thus slow heteroduplex mobility. In order to compare data acquired across experiments, it is, therefore, important to **consistently reproduce** the electrophoresis conditions as closely as possible. For this purpose the gel units, plates, acrylamide concentration, voltage/current and buffer conditions should be **carefully adhered to** in each experiment. Given a particular apparatus and conditions, an equation can be derived to estimate the genetic distance between two DNA fragments from their heteroduplex mobility. The estimated genetic distances can then be used to derive fairly reliable phylogenetic relationships between multiple sequences without analyzing all of the pairwise heteroduplexes possible [41]. If the electrophoresis apparatus or conditions are changed, the given equation is no longer valid and therefore must be reestablished using newly determined heteroduplex mobilities with DNA fragments from known sequences. To date, we have typically evaluated 25-33% of the $[N \times (N-1)]/2$ possible comparisons when deriving data for phylogenetic tree construction (where N = number of sequences being compared) [2]. However, the degree of effort required in making all of these comparisons often equates with or exceeds the effort required to derive DNA sequence data, and hence is not generally recommended.

Electrophoresis conditions:

The following conditions are routinely employed:

Figure 10: Agarose gel electrophoresis:
1.2 kb fragments (ED5-ED12), 200 V for 6 hours

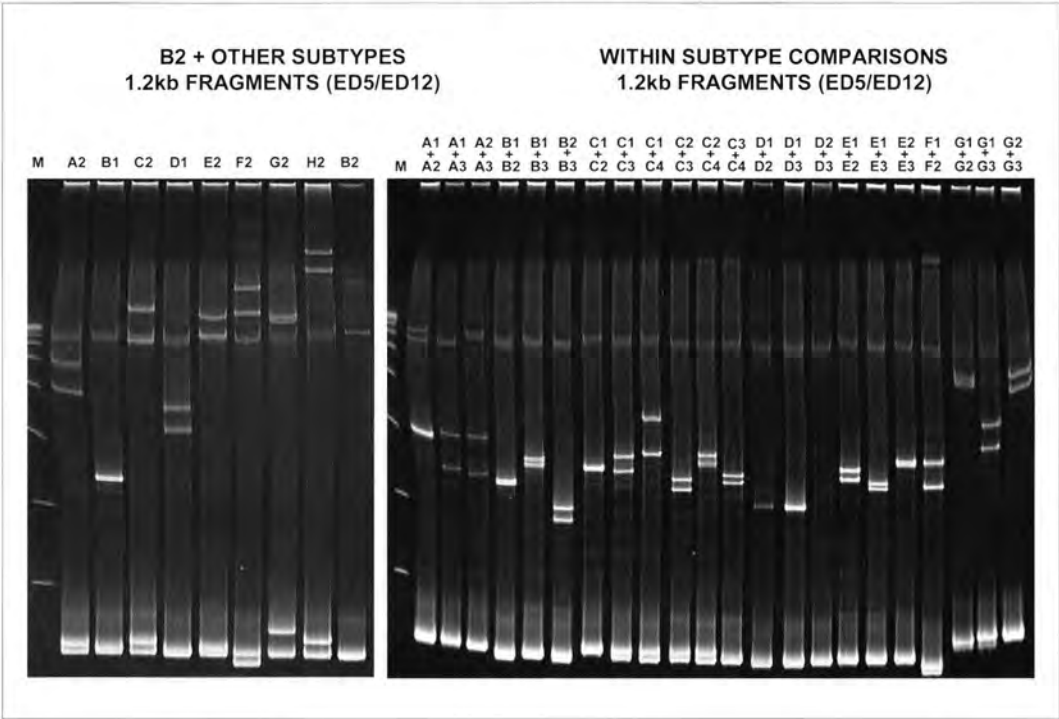


Figure 11: Agarose gel electrophoresis:
0.7 kb fragments (ES7-ES8 or DR7-DR8), 250 V, 3 hours

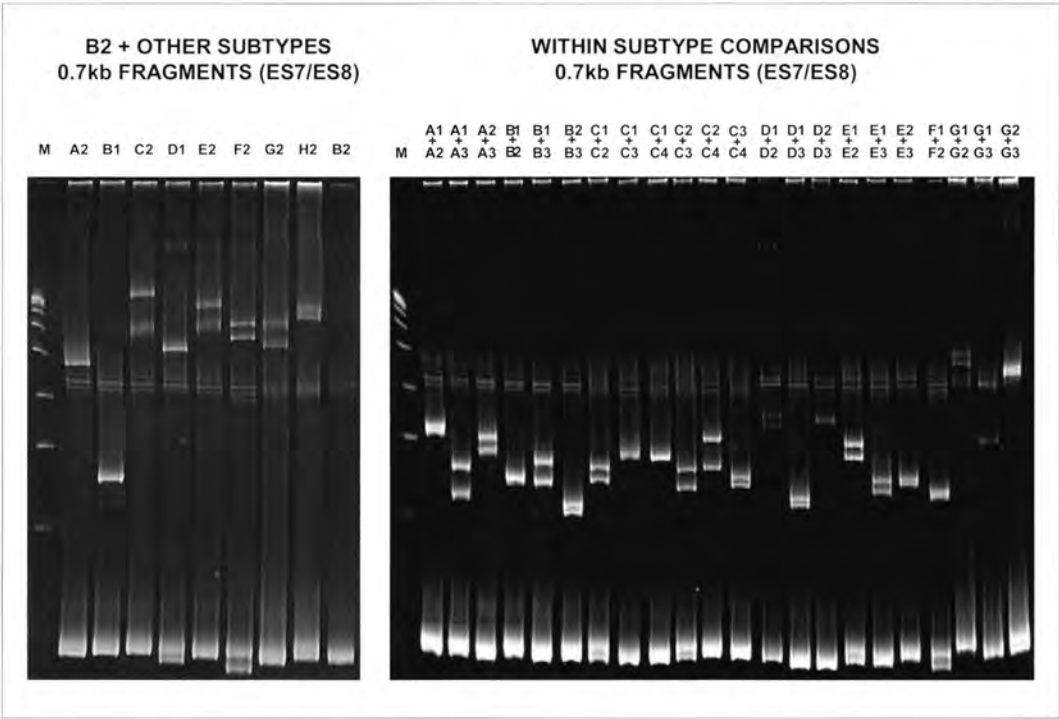
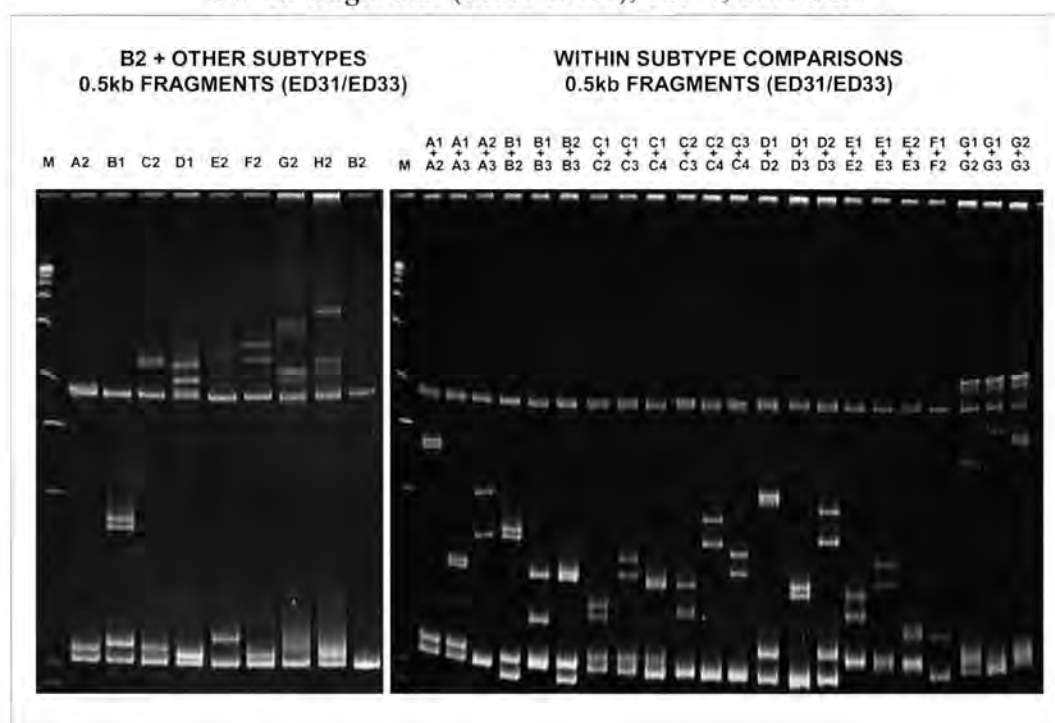


Figure 12: Agarose gel electrophoresis:
0.5 kb fragments (ED31-ED33), 250 V, 2.5 hours



11.3.4 Analysis of heteroduplexes

The problem of inherent quasispecies complexity

When amplifying viral sequences from a plasmid or single provirus or RNA template, only homoduplexes are seen on the polyacrylamide gel. When amplifying sequences from a viral quasispecies, heteroduplexes can form between different, simultaneously amplified variants within the mixture and multiple heteroduplexes are seen on polyacrylamide gels. Heteroduplexes can take the form of sharp bands or of a smear-like pattern. The complexity of the heteroduplex pattern in a single sample can vary widely, with complex quasispecies usually seen as both homoduplexes and as heteroduplexes with reduced mobility. Thus, if a single variant or a collection of highly related variants is amplified from the unknown sample, a single homoduplex band is seen in the gel. When such products are reannealed with a reference sequence the two fast migrating homoduplexes (with indistinguishable or similar mobilities) and only two sharp heteroduplex bands are seen (occasionally migrating at the same position).

When a complex quasispecies is reannealed with a reference sequence, the heteroduplexes formed between the reference and the multiple variants can appear of a series of bands or a diffuse smear. Occasionally, difficulties in identifying the inter-strain heteroduplexes may be encountered. We have found it useful to serially dilute the DNA preparation prior to nested PCR, in order to generate products derived from a less complex mixture or a single variant. Assigning subtypes with less complex quasispecies is easier because of the simpler pattern of heteroduplexes formed with reference sequences. Quasispecies complexity is typically lower for *in vitro* cultured isolates compared to their PBMC of origin [42] and, if available, the use of co-cultured PBMC as a source of proviral DNA can decrease the problem of complex quasispecies.

Discrete single or multiple bands migrating with a mobility of about 40% that of homoduplexes are sometimes seen when examining PCR products (see figures 10-12). The position of these signals varies only slightly in location between different samples, and corresponds to single stranded DNA fragments that failed to reanneal with a complementary strand. They occur due to slight imbalances of primer concentrations in the PCR and an overabundance of one strand product. However, the uniform positioning of these bands makes them useful for visual comparisons of heteroduplex mobilities in different lanes.

Subtype determination

To assign an uncharacterized strain to a known subtype, the use of an equation that converts heteroduplex mobility into estimated DNA distance and subsequent phylogenetic analysis is not essential. Rather, the PCR fragment derived from the unknown strain is reannealed with the corresponding fragment from multiple representatives from each of the previously identified subtypes (or from any other sequence suspected to be related to the unknown strain). The heteroduplexes exhibiting the fastest mobilities between the unknown and the most related subtype indicate the likely subtype of that strain. In general, the closer the relationship between an unknown and a given reference, the fewer references that need to be compared to comfortably assign a subtype.

12. HIV Sequence Data Management

12.1 HIV sequence repositories

As of writing, there are close to 37,000 HIV-1 sequences in the public DNA sequence databases. All HIV sequences are referenced with a unique accession number provided by the international DNA sequence databases. A compilation of sequences is edited by the HIV Database, who also provide phylogenetic analyses and associated information on protein structure, immunology, and drug resistance. Hard copies of the HIV Sequence Compendium are available free of charge to HIV researchers upon request.

12.1.1 International DNA sequence databases

The international DNA sequence databases are GenBank in the USA, European Bioinformatics Institute (EBI) in UK, and the DNA Database of Japan (DDBJ). They store all public DNA sequence data from any organism and provide accession numbers that reference these sequence data in publications. Each of the three databases collects a portion of the total sequence data reported worldwide and all the new and updated sequence records are exchanged between the databases on a daily basis.

The HIV-1 records are accessible in GenBank through the Web-based browser Entrez, using keywords such as the author's name, publication date, gene name, sequence length. Only the information entered by the submitter in the sequence record can be searched, as no additional information is added to the sequence records by the international databases unless requested by the submitter. Accordingly, HIV records with incomplete information may not be found during the search process and may not appear in the results. It is, therefore, crucial to maintain the database to a high standard with well-annotated HIV records.

12.1.2 The HIV database

The HIV Database located in the Los Alamos National Laboratory (NM) is a specialized research resource database for compilation, analysis, and dissemination of genetic sequences and associated data for HIV-1 and related lentiviruses. The HIV records are obtained from GenBank and also from unpublished sources. The information associated with the HIV records in the DNA sequence databases is often insufficient for in-depth analysis, the HIV Database staff enhances the annotation of the HIV records by adding pertinent data extracted from publications. In addition, a multitude of tools are provided on the database web site for HIV-1 sequence analysis, assessing sequence contamination, aligning multiple sequences and determining the

subtype of a HIV-1 sequence (see Annex 5 for an overview). The HIV database also provides access to several specialized databases such as the immunology database, the drug resistance database, a coreceptor usage database, and an overview of SIV/SHIV vaccine reagents.

12.2 HIV sequence submission to the international DNA sequence databases

12.2.1 Accession numbers, release and update

Most journals now require accession numbers to reference DNA sequences in publications. These can be obtained the same day the sequence data are submitted to one of the three DNA sequence databases. Continuous accession numbers are provided for a collection of HIV sequences when they are packaged together in one submission (see section 12.2.3). The sequences are released only after a successful validation procedure performed by the database staff (usually 5 days) or after publication in cases where the HUP (Hold until Publication) flag was set at the time of the submission. Updates, such as the correction of a DNA sequence and additional descriptive information, can be sent to the databases by notifying the accession number of the sequence.

12.2.2 HIV sequence annotation

Generally, little information is required for the public release of HIV records. The nucleotide sequence, the protein translation and the author's names are often the only information found in a HIV-1 record. Submitters are, however, encouraged to complete the annotation of their HIV records with biological information (e.g. "proviral" to qualify genomic DNA, "virion" for non-integrated genomic RNA), the country of sampling (see section 12.2.2), and the subtype of the strain (see section 12.3), etc.

The annotation of a large number of sequences can be long and tedious. Now Sequin (see section 12.2.3), the program for data submission used for all international databases, allows the annotation to be added to all sequences at once. For instance, when all the HIV-1 nucleotide sequences encompass the same genomic region, they have to be annotated with the same gene feature, the same coding region feature and the corresponding translation product. This procedure of global sequence annotation is automated in Sequin.

Isolate information

In order to make each HIV-1 entry unique in the databases, a minimum amount of source information is required to describe the sequences distinctly. Ideally, each record should contain at least a distinct isolate, strain, or clone name, and these names should appear in the title of the record (named DEFINITION line). The DEFINITION line contains a short description of the record's content. Unique DEFINITION lines are important for identification purposes because they are displayed initially when browsing the HIV-1 database or searching for sequence similarities (by BLAST programs for instance). The general format of the DEFINITION line for an HIV-1 record is as follow: "HIV-1 isolate/strain/clone from country, product (gene), partial or complete cds".

It would be helpful if a simplified version of the WHO style nomenclature could be used to name all newly derived strains and included the following information: country and year of identification, unique laboratory identification and clone number. For example, “US99.labID.5” corresponds to a strain sampled from a person living in the US in 1999, labID to the in-house laboratory designation, and 5 to the clone number. A list of two letter country codes (derived from the International Organization for Standardization, ISO) can be found at the Los Alamos HIV Database (<http://hiv-web.lanl.gov/HTML/databasecountrycode.html>). The two letter code “00” will be used for strains identified in the year 2000. For the labID the first two characters should, if possible, associate the sequence with the individual from whom the sequence was derived. Again, GenBank and other international databases do not require the use of the WHO isolate/sequence nomenclature, but it would be helpful for greater consistency within the field.

Standard gene and protein names

In an attempt at standardization, a list of preferred names for the genes and associated proteins was established in accordance with the reference genomic map published in Retroviruses [44]). A reference sequence of the complete HIV-1 genome (accession number AF033819) was annotated following these conventions. It was generated from modifying the HIVHXB2CG entry (K03455) such that the sequence begins at the transcription initiation site and ends at the polyadenylation site.

Country of origin

A country name is now required for all HIV-1 records submitted to GenBank. It allows specific data sorting. Preferably, it should correspond to the country of residence; if the country of infection is not the same, for example if the infected individual is an immigrant, this should be noted in a separate comment. This is not required in GenBank, but it is part of the recommended WHO nomenclature, adopted in 1994. Since the country name was added to the GenBank submission guidelines 4 years ago, the GenBank database has accumulated over 20,000 HIV-1 entries from more than 80 countries. The HIV database has manually confirmed that most of the entries correspond to country of residence, and added country names to approximately 5000 older entries in the database.

12.2.3 Computer resources for sequence submission

A new generation of sophisticated sequence submission tools is now available. These guide the user through a succession of forms to enter sequence data and descriptive information in an interactive way. They allow authors to submit data to one of the DNA sequence databases either via Web-based forms (BankIt, Webin) or via a multi-platform (Mac/PC/Unix) stand-alone software tool (Sequin).

Web-based sequence submission tools

The Web-based tools have quickly become the preferred data submission tool, BankIt for GenBank, WebIn for EBI. They can be used to submit single mRNA or genomic sequence, associated coding sequences and simple biological information. All the information required to create a database entry will be collected during this process: (1) submitter information, (2) release date information, (3) sequence data, description and source information, (4) reference citation information, (5) feature information (e.g. coding regions, regulatory signals etc.).

However, the Web-based tools are not designed to handle large collections of related sequences frequently generated by studies of molecular epidemiology of HIV. Submissions of large collections of sequences are easily processed by Sequin.

Sequin

Sequin is the latest multi-platform (Mac/PC/Unix) stand-alone software tool developed by the NCBI for submitting entries to one of the DNA sequence databases, GenBank, EBI and DDBJ. The Sequin program, along with detailed downloading and installation instructions plus general information is available from NCBI via Web browser, anonymous FTP and from the file server.

One powerful feature of Sequin is the automated global annotation of multiple sequences (see section 12.2.2). Another useful feature is the validation function that helps the user to check the accuracy of the submission. Mistakes such as incorrect coding region length, internal stop codon in coding regions, or non-consensus splice sites are reported as error messages and can be corrected by double clicking on the messages.

12.2.4 Submission and retrieval of sequence alignments

Full-length alignments of nucleic acid or protein HIV-1 sequences from large population studies are no longer published in most journals. Archives of alignments exist at the National Center for Biotechnology Information (NCBI) and the European Bioinformatics Institute (EBI) allowing the preservation of phylogenetic information generated by the authors for their population studies. Further analyses by other investigators are possible by retrieving the alignments from these databases.

Alignments of population studies at the NCBI are accessible using the Entrez retrieval system. In the Entrez system, the nucleic acid database (GenBank), the protein database, the literature database (PubMed) and the alignment database are linked, and the alignments are accessible given the accession number of any sequence within the alignment or the literature citation. Note that one can update existing GenBank records by submitting an alignment of the sequences to the GenBank staff. Alignments may include both newly submitted sequences and existing HIV-1 entries already in GenBank. In order to avoid issuing a new accession number to an existing database entry, it must be labeled with the prefix “acc”, followed by the GenBank accession number of the sequence. For instance, the sequence name accAF033819 means that the sequence that follows in the alignment is the full-length or a partial segment of the HIV-1 sequence of accession number AF033819.

12.3 HIV-1 sequence subtyping

12.3.1 *HVI-1 groups and subtypes*

Three main genetic groups characterize the genetic diversity of internationally collected HIV-1 isolates: M (for major), O (for outlier) and N (non M/non O). Members of the 3 groups differ by more than 40% in the amino acid sequences of their envelope proteins. The group M can be subdivided into at least nine distinct subtypes (A, B, C, D, F, G, H, J, K) between which there is more than 25% variation in the amino acid sequence of the envelope protein, and four circulating recombinant forms (CRFs; a CRF is a mosaic genome that has caused at least three epidemiologically unrelated infections; these can be dominant epidemic strains). The occurrence of mosaic genomes resulting from inter-subtype recombination further complicates the problem of vaccine development and highlights the need for subtype monitoring for tracking the epidemics. The methods widely used for HIV-1 subtyping are based on the comparison of the sequence to be subtyped and a panel of reference sequences representative of the subtypes. The methods differ mainly by the quantitative measure used to compute the sequence similarity.

12.3.2 *Reference sequences representative of the subtypes*

The determination of the subtype of a sequence, and the delineation of potential breakpoints relies on the availability of full-length, non-recombinant, reference genomes. Reference genomes are available for the subtypes and CRFs; alignments of the reference sequences can be found on the HIV database web site. Subtype E has been designated a CRF, although whether it is really a recombinant is a subject of debate. Subtype I has been removed from the nomenclature, as recent analysis of full-length genomes showed it to be a complex mosaic. See the 1999 HIV Sequence Compendium and web site for a detailed nomenclature discussion and criteria for designation of a new subtype or CRF. Maps of CRF mosaics reference strains are also provided.

The HIV Database also provides reference alignments for subtyping, consisting of three representative sequences for each subtype and circulating recombinant form. In addition, complete gene and protein alignments representative of the global diversity of the virus, and whole genome alignments of HIV-1, HIV-2 and SIV, are made available and updated yearly. Alignments for specific regions of the genome, either of all available sequences or a selection based on subtype, country of sampling, or a set of accession numbers or sequence names can be generated automatically on the website.

12.3.3 *Intersubtype recombinants*

Intersubtype recombinants show interspersed segments of genetic material from two or more subtypes. Recombinant genomes may not be determined by classical phylogenetic tree analyses because the comparison of the full-length sequences masks the local differences of relative similarity to the subtypes. In phylogenetic analyses, recombinant forms may appear as distant members of the subtypes and could wrongly be identified as new subtypes. They may also appear well clustered in a subtype.

The HIV-1 subtyping methods can be used for mapping recombinant genomes by employing a sliding window along the query sequence. In each window, the method compiles a measure of similarity between the query sequence and reference sequences representative of the subtypes. The use of the sliding window highlights the local differences of parental subtype.

12.3.4 Computer resources for HIV-1 subtyping

Similarity plot

A simple method (used by the programs Recombination Inference Program or RIP, and DIVERT) first requires aligning the query sequence with a panel of reference genomes representative of the subtypes. The programs then compute the similarity between the query sequence and the reference sequences in a sliding window that moves along the sequence alignment by defined step. RIP produces both a printed version of the alignment, with subtype associations highlighted, and a graphical plot of the similarities between the query and the reference sequences. Consensus sequences for the subtypes are often used as reference sequences. A simple measure of similarity is the percent identity between two sequences. Both DIVERT and RIP incorporate statistical tests which indicate the significance of the breakpoints and/or subtype associations.

BLAST-based subtyping resource

This web-based subtyping tool employs a BLASTN comparison (fast similarity searching program developed at NCBI) between the HIV-1 sequence to be subtyped and a panel of reference sequences. The subtyping panel includes complete genomic references for the 9 subtypes of group M and the groups O and N. During the subtyping process multiple BLASTN comparisons are performed over a sliding window of user-defined size and step value. A color-coded graph is generated showing of the BLASTN sequence similarity scores between the query sequence and each reference sequence in the panel against window location.

To begin the subtyping process, the query sequence is pasted into the text area in the query web page; no alignment is required. The window size and step value are entered; alternatively the defaults (window size=330 nucleotides, step value=100 nucleotides) may be used. The subtyping procedure generates a plot featuring the BLASTN similarity scores and a color-coded bar indicating the subtype with the highest similarity score within the corresponding sequence window. Full-length subtypes show uniformly high scores for the reference sequences of their subtype. Recombinant forms are easily recognized by breakpoints in the similarity bar.

Both distance and BLAST-based methods should be used for screening for recombination, and followed up by bootscanning and other phylogenetic methods for verification if a recombinant is suggested.

Bootscanning method

The “bootscreening” method is based on the observation that traditional methods of phylogenetic analysis are not sufficient to distinguish recombinant forms from full-length subtype sequences. This method generates a series of phylogenetic trees from sequential segments of the query sequence aligned to representatives of the subtypes. Query sequences made of one subtype only join the corresponding subtype in all the phylogenetic trees, and recombinant query sequences alternately cluster with the parental subtypes.

The first step in the subtyping procedure is to align the query sequence with the genomes representative of the subtypes. The alignment is divided into sequential overlapping segments of 200-500 bases and each segment is used to generate a set of phylogenetic trees, using the bootstrapping method and different tree construction methods. The subtyping procedure generates a plot of the bootstrap values with which the query sequence is clustered with the subtypes. High bootstrap values are found in association with the segments encompassing genome region of one subtype only. Low bootstrap values are found in the transition regions where recombination breakpoints are localized.

The program SimPlot can be used to automate the bootscreening procedure; it works in conjunction with the PHYLIP programs. At present, SimPlot is available only for PC-Windows.

12.3.5 Localization of breakpoints in intersubtype recombinants

In all the methods, the breakpoints in recombinants are localized by a sudden change of the subtype most similar to the query sequence. The precision of their mapping depends on the size of the window used to scan the sequence. Also, depending on the size of the window are (i) the time of the computation: for some programs (though not for RIP) short windows divide the query sequence into numerous segments to be analyzed, (ii) the significance of the result (loss of confidence of the similarity measure computed on short region) and (iii) the chance of detecting short recombinant regions (long windows may mask shorter recombinant regions). Ideally, the size of the window matches the shortest genome region of “pure” subtype. Observations show that window size of 200-300 bases is a good starting place to detect significant recombinant regions.

Once possible breakpoints have been identified, it is vital to try to confirm their significance using a statistical test. Testing the significance is not a trivial problem, please refer to the literature for an accurate description of the potential tests and the problems associated with them. As more recombinant viruses are characterized, it may be possible to define consistent patterns of recombination and hotspots for breakpoints in the HIV-1 genome, as well as the frequency of recombination event.

Table 3: URL addresses of sequence databases and tools

Database	Tool	Adresses (URL, email, ftp)
GenBank (US)	Help email Browser Entrez Sequin BankIt Submission email Alignment(PopSet)	http://www.ncbi.nlm.nih.gov/ info@ncbi.nlm.nih.gov http://www.ncbi.nlm.nih.gov/Entrez/ http://www.ncbi.nlm.nih.gov/Sequin/ http://www.ncbi.nlm.nih.gov/BankIt/ gb_sub@ncbi.nlm.nih.gov http://www.ncbi.nlm.nih.gov/entrez/query.fcgi
EBI (Europe)	Help email Sequence Retrieval WebIn Submission email Alignment	http://www.ebi.ac.uk/ support@ebi.ac.uk http://srs.ebi.ac.uk/ http://www.ebi.ac.uk/submission/webin.html datasubs@ebi.ac.uk ftp://ftp.ebi.ac.uk/pub/databases/embl/align/
DDBJ (Japan)	Submission email	http://www.ddbj.nig.ac.jp/ ddbjsub@ddbj.nig.ac.jp
HIV Database ⁹		http://hiv-web.lanl.gov/
HIV immunology db		http://hiv-web.lanl.gov/immunology/

Table 4: HIV-1 sequence subtyping programs

Subtyping method	Input data	Output data	New subtype	URL (or publication)
Simplot	global alignment	% identity plot	equidistance	http://www.med.jhu.edu/deptmed/sray/download/
RIP	global alignment	% identity plot	equidistance	http://linker.lanl.gov/RIP/RIPsubmit.html
DIVERT	global alignment	% identity plot	equidistance	http://igs-server.cnrs-mrs.fr/anrs/phylogenetics/
Bootscanning [45]	global alignment	bootstrap value	low bootstrap	Simplot (for MS-windows): http://www.med.jhu.edu/deptmed/sray/download/ Sun Unix version: http://www.ktl.fi/hiv/mirrors/pub/programs/
BLAST-based subtyping	local similarity	similarity score	equidistance	http://www.ncbi.nlm.nih.gov/retroviruses/HIV1/subtypeHIV1.html

⁹ For sequence analysis tools at the HIV database see Annex 5.

Annex 1:

List of equipment for a virus isolation laboratory

Virus isolation laboratory (Biological level 2, BL-2):

- Biosafety cabinet, class II

Care must be taken in locating the biological safety cabinet within the laboratory. Air currents across the working front of the cabinet can interfere with the protective air flow and this may allow microorganisms to escape from the cabinet. The cabinets should, therefore, not be located near doors, windows or near the supply or exhaust grilles of mechanical ventilation systems, and should be away from traffic patterns within the room. **Skilled servicing of cabinets is mandatory.**
- Refrigerator
- Freezer, -20°C
- CO₂ incubator
- Centrifuge, for low-speed centrifugation, with sealed buckets or rotors
- Balance
- Microscope, inverted
- Water bath
- Vortex mixer
- Pipette-aid or equivalent
- Mobile carriage
- Mobile shelves

Equipment available in the same building:

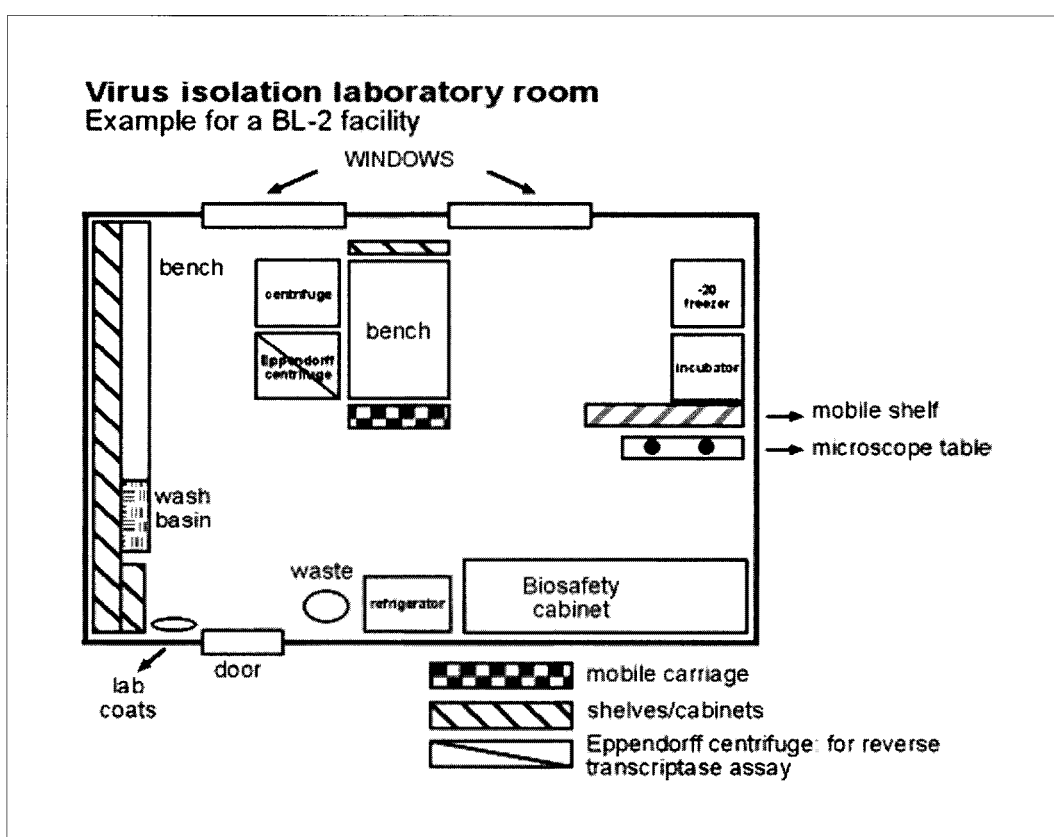
- Autoclave
- Freezer, -70°C
- Freezer, -140°C or liquid nitrogen
- ELISA reading apparatus
- Fluorescence microscope
- Supplementary equipment for research laboratories:
- Ultracentrifuge, with 3 rotors (2 fixed-angle, 1 swing-out)

- Centrifuge, for high-speed centrifugation, refrigerated (2 rotors minimum)
- Centrifuge, for Eppendorf tubes, sealed rotor, refrigerated
- Equipment for reverse transcriptase assay

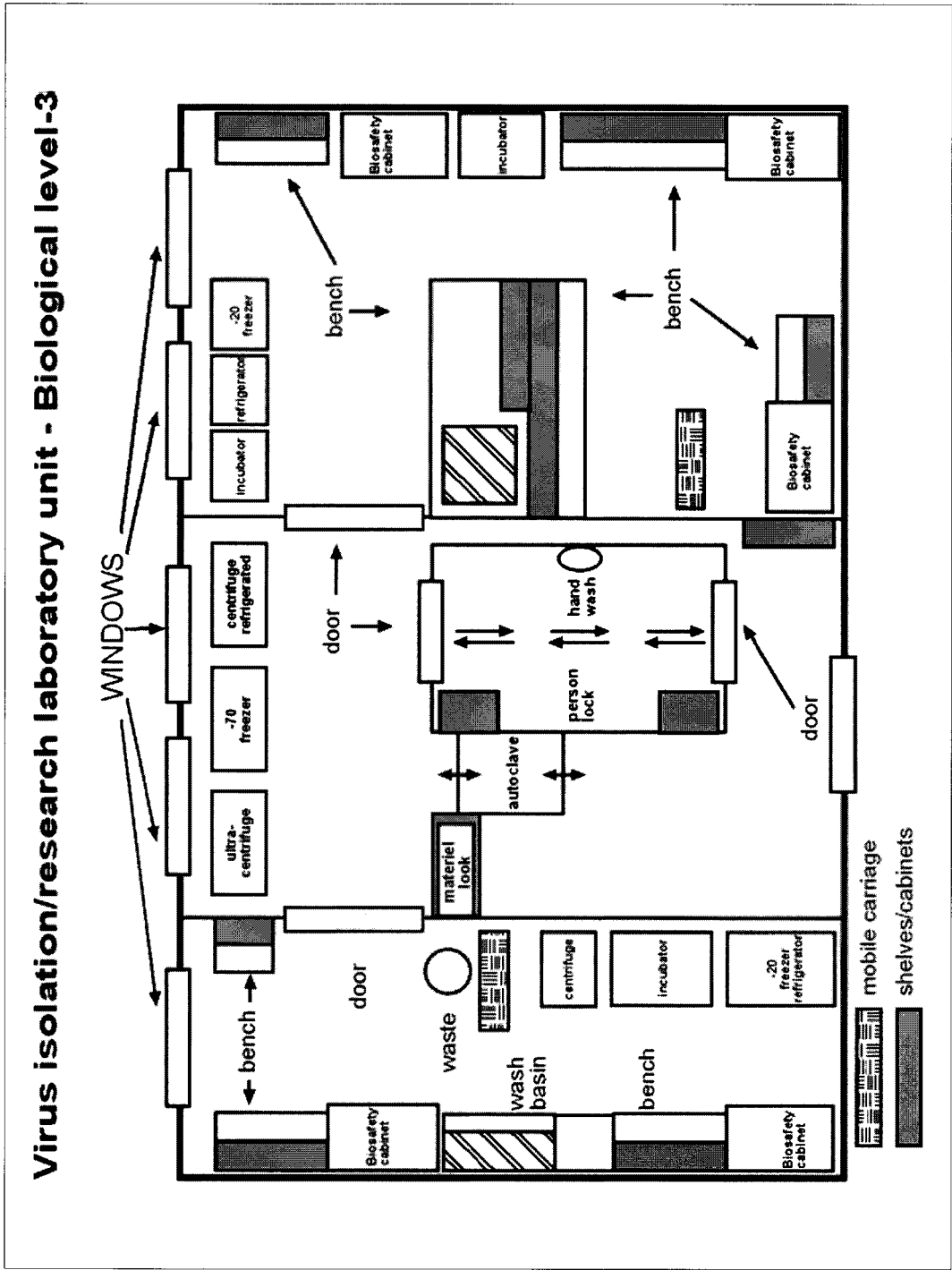
Electrophoresis equipment:

It is recommended to use front glass plates of size 19 cm h x 19.5 cm w, back plates of size 16 cm h x 19.5 cm w, and both plates 3 mm thick. Teflon spacers are 1.5 mm thick. If a V16 vertical gel apparatus is purchased from GIBCO/BRL, glass plates, spacers and a 20 well comb is provided with the apparatus. 15, 20 or 25 well combs can be purchased from BioRad.

Design of a virus isolation laboratory room BL-2



Design of a virus isolation laboratory unit BL-3



Annex 2:

Subject data form

WHO ID Number Collection Number	WHO-UNAIDS HVI Number Form Code
<div style="border: 1px solid black; display: inline-block; padding: 2px;">EPIDEMIOLOGICAL DATA</div>	<div style="border: 1px solid black; display: inline-block; padding: 2px;">EPIDEMIOLOGICAL DATA (cont'd)</div>
<p>1. (a) Group</p> <p>1 hospitalized (specify where)</p> <p>2 prenatal consultation (specify where)</p> <p>3 other consultation (specify where)</p> <p>4 blood donor (specify where donation made)</p> <p>5 cohort (specify)</p> <p>6 other (specify)</p> <p>(b) Specify</p> <p>(c) Group ID Number</p> <p>2. Sex</p> <p>1 male</p> <p>2 female</p> <p>3. Date of birth</p> <p>4. Age (years)</p> <p>5. Place of birth</p> <p>6. (a) Place of residence</p> <p>(b) Urban/rural residence</p> <p>1 urban</p> <p>2 semi-rural</p> <p>3 rural</p> <p>7 (a) Presumed transmission</p> <p>1 sexual (go to Q.7(b))</p> <p>2 parenteral sexual (go to Q.7(c))</p> <p>3 perinatal sexual (go to Q.7(d))</p> <p>4 unknown</p> <p>(b) If sexual transmission</p> <p>1 heterosexual</p> <p>2 bisexual</p> <p>3 homosexual</p> <p>(c) If parenteral transmission</p> <p>1 haemophiliac</p> <p>2 blood transfusion</p> <p>3 IV drug user</p> <p>4 other</p>	<p>(d) If perinatal transmission, Mother's WHO ID number</p> <p>8. Date of last seronegative sample</p> <p>9. Date of first seropositive sample</p> <p>CLINICAL STATUS</p> <p>10. (a) Presumed active infection</p> <p>1 no</p> <p>2 yes</p> <p>(b) If yes, date</p> <p>11. Clinical conditions</p> <p>12. Stage according to WHO HIV infection staging system</p> <p>1 Stage 1 Asymptomatic</p> <p>2 Stage 1 PGL</p> <p>3 Stage 2 Symptomatic early</p> <p>4 Stage 3 Moderate disease</p> <p>5 Stage 4 AIDS</p> <p>13. Antiviral treatment</p> <p>1 no</p> <p>2 yes</p> <p>ELIGIBILITY</p> <p>PERSON WITH SYMPTOMATIC INFECTION OR TREATED WITH ANTIVIRALS ARE NOT ELIGIBLE</p> <p>14. Eligible</p> <p>1 no</p> <p>2 yes</p> <p>If no, do not collect blood</p> <p>15. Consented to donate blood for research purposes</p> <p>1 no</p> <p>2 yes</p>

WHO ID Number	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	Collection Number	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
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LABORATORY HISTORY			
HIV1	First / Only Test	Second Test	
16. ELISA	day month year	day month year	
(a) Date	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(b) Specify kit:	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(c) Result 1 negative 2 positive 9 not done	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
17. Western Blot	day month year	day month year	
(a) Date	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(b) Specify kit:	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(c) Result 1 negative 2 positive 3 indeterminate 9 not done	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(d) Bands 1 not present	gp160	gp160	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
2 present	gp120	gp120	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
9 not done	p55/p61	p55/p61	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
	gp41	gp41	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
	p24	p24	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
	p17	p17	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
HIV2			
18. ELISA	day month year	day month year	
(a) Date	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(b) Specify kit:	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(c) Result 1 negative 2 positive 9 not done	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
19. Western Blot	day month year	day month year	
(a) Date	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(b) Specify kit:	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(c) Result 1 negative 2 positive 3 indeterminate 9 not done	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(d) Bands 1 not present	gp140	gp140	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
2 present	gp105	gp105	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
9 not done	p56/p68	p56/p68	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
	gp36	gp36	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
	p26	p26	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
	p16	p16	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>
HIV1/2			
20. ELISA	day month year	day month year	
(a) Date	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(b) Specify kit:	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div> <div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	
(c) Result 1 negative 2 positive 9 not done	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	<div style="border: 1px solid black; width: 20px; height: 20px; display: inline-block;"></div>	

<div style="border-bottom: 1px solid black; padding-bottom: 5px;"> WHO ID Number <div style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></div> </div> <div style="border-bottom: 1px solid black; padding-bottom: 5px; margin-top: 5px;"> LABORATORY HISTORY (cont'd) </div> <div style="margin-top: 5px;"> <div style="display: flex; justify-content: space-between;"> <div>21. HIV1 p24 antigen</div> <div>day month year</div> </div> <div style="margin-left: 20px;"> (a) Date <div style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></div> </div> <div style="margin-left: 20px;"> (b) Result <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div>1 negative</div> <div style="border-bottom: 1px solid black; width: 40px;"></div> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div>2 positive</div> <div style="border-bottom: 1px solid black; width: 40px;"></div> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div>9 not done</div> <div style="border-bottom: 1px solid black; width: 40px;"></div> </div> </div> </div> <div style="margin-top: 5px;"> <div style="display: flex; justify-content: space-between;"> <div>22. CD4 cell count</div> <div>day month year</div> </div> <div style="margin-left: 20px;"> (a) Date <div style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></div> </div> <div style="margin-left: 20px;"> (b) Absolute number / mm3 <div style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></div> </div> <div style="margin-left: 20px;"> (c) Percent <div style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></div> </div> </div> <div style="margin-top: 5px;"> <div style="display: flex; justify-content: space-between;"> <div>23. CD8 cell count</div> <div>day month year</div> </div> <div style="margin-left: 20px;"> (a) Date <div style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></div> </div> <div style="margin-left: 20px;"> (b) Absolute number / mm3 <div style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></div> </div> <div style="margin-left: 20px;"> (c) Percent <div style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></div> </div> </div> <div style="margin-top: 5px;"> <div style="display: flex; justify-content: space-between;"> <div>24. Total lymphocyte count</div> <div>day month year</div> </div> <div style="margin-left: 20px;"> (a) Date <div style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></div> </div> <div style="margin-left: 20px;"> (b) Absolute number / mm3 <div style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></div> </div> </div>

SPECIMEN COLLECTION

25. (a) Date specimen collected

day month year

(b) Time specimen collected

hour min

26. (a) Amount of whole blood taken

ml

vials

(b) Amount without anticoagulant

ml

vials

(c) Amount with anticoagulant

ml

vials

(d) Specify anticoagulant

27. Amount of serum

ml

vials

Collection Number

SEPARATION

28. Separation done at primary laboratory

1 no (go to Q.33)

2 yes

29. (a) Date of separation

day month year

(b) Time of separation

hour min

30. Serum

ml

vials

31. Plasma

ml

vials

32. PBMC

cells/ml

vials

SHIPPING

33. Specimen sent to:

34. Date of shipment

day month year

35. Whole blood

ml

vials

36. Serum

ml

vials

37. Plasma

ml

vials

38. PBMC

cells/ml

vials

REMARKS

Form-filling instructions

WHO-UNAIDS HIV ISOLATION AND CHARACTERIZATION NETWORK

Form-Filling Instructions for Primary Laboratories

NOTE: SHADED BOXES SHOULD NOT BE CODED

Page 1

WHO-UNAIDS ID Number

Use consecutive numbers, starting with 0001. A special logbook should be kept, showing the person's ID Number, Collection Number(s), name, address, and any other identifying information. This information may be required for follow-up, in case additional information is required, or if consecutive blood samples are needed.

Collection Number

This should be of the form CCYYNNN, where CC is the country code (already printed in the first two boxes), YY is the year (e.g. "02" for 2002), and NNN is the consecutive number (starting with 001 each year) given by the primary laboratory. This number should be marked, in its entirety, on all vials containing materials collected from a person each each time blood is collected.

Epidemiological Data

Q. 1 In part (a), enter the group to which the subject belongs, if any.

In part (b):

If the person was hospitalized when blood was collected
[code 1 in part (a)], specify where he/she was hospitalized.

If the blood was collected from a woman attending for a prenatal consultation
[code 2 in part (a)], specify where the consultation took place.

If blood was collected from a person attending for some other type of medical
consultation [code 3 in part (a)], specify where the consultation took place.

If the blood was collected from a person donating blood [code 4 in part (a)],
specify where the donation was made.

If the person is participating in a cohort study [code 5 in part (a)],
specify which cohort If the person from whom the blood was collected was
contacted in some other way [code 6 in part (a)], specify how, or to which
other group the person belongs.

In part (c):

Enter the person's ID number in the group to which he/she belongs,
for example, hospital number or the ID number given to him/her in the cohort
study.

-
- Q.3 If known, the person's date of birth should be recorded. If only incomplete information is available, such as month and year, or year alone, it should still be recorded, and the unknown part(s) of the date coded 99.
- Q.4 Enter the person's age in completed years.
- Q.5 Record the village/town, district and/or province.
- Q.6 In part (a), record the village/town, district and/or province.
In part (b), enter the appropriate code to indicate whether the place of residence is in an urban, semi-rural or rural area.
- Q.7 In part (a), code the most likely mode of transmission of HIV infection.
If the most likely mode of transmission is sexual [code 1 in part (a)], indicate in part (b) whether infection was most likely transmitted by heterosexual, bisexual or homosexual contact.

If the most likely mode of transmission is parenteral [code 2 in part (a)], indicate in part (c) whether infection was most likely transmitted by treatment for haemophilia, a blood transfusion, IV drug use, or some other route.

If the most likely mode of transmission is perinatal [code 3 in part (a)], and one or more Blood samples have been collected from the mother, enter the mother's WHO ID Number in part (d). If the most likely mode of transmission is perinatal, and the mother has not donated any blood samples to the HIV Isolation and Characterization study, code part (d) 9999.
- Q.8 Enter the date of the last blood sample which was proven to be negative for anti-HIV antibodies, if known. If unknown, code Q.8 999999.
- Q.9 Enter the date of the first blood sample which was proven to be positive for anti-HIV antibodies.

This information, together with that recorded in Q.8, will allow estimation of the date of onset and duration of infection. **Individuals who have been infected for more than 2 years should not be included in this study.**

Clinical Status

- Q.10 In part (a), indicate whether the person has or has had a presumed acute infection. If no, leave part (b) blank.
If yes, enter the date of onset of symptoms in part (b). If the date is unknown, code part (b) 999999.
- Q.11 As many as three clinical conditions can be coded in Q.11.
Codes to be used for these conditions are as follow:
- 01 Asymptomatic
 - 02 Persistent generalized lymphadenopathy (PGL)
 - 03 Weight loss < 10%
 - 04 Minor mucocutaneous symptoms/signs
(e.g. seborrheic dermatitis, folliculitis, pruritus, psoriasis, fungal nail infections, recurrent oral ulcerations/angular cheilitis)

-
- 05 Herpes zoster (including disseminated)
 - 06 Recurrent upper respiratory tract infection
 - 07 Progressive weight loss > 10%
 - 08 Unexplained diarrhoea > 1 month
 - 09 Fever > 1 month
 - 10 Oral candidiasis (thrush)
 - 11 Oral hairy leucoplakia
 - 12 Pulmonary tuberculosis
 - 13 Severe bacterial infection (e.g. pneumonia, pyomyositis)
 - 14 Cachexia
 - 15 Pneumocystis carinii pneumonia
 - 16 Toxoplasmosis of the brain
 - 17 Cryptosporidiosis with diarrhoea persisting > 1 month
 - 18 Cryptococcosis, extrapulmonary
 - 19 Cytomegalovirus disease of an organ other than liver, spleen or lymph nodes
 - 20 Herpes simplex infection (mucocutaneous > 1 month, or visceral any duration)
 - 21 Progressive multifocal leukoencephalopathy
 - 22 Any disseminated endemic mycosis (e.g. histoplasmosis, coccidioidomycosis)
 - 23 Oesophageal candidiasis
 - 24 Atypical mycobacteriosis (disseminated)
 - 25 Salmonella septicaemia (first episode or recurrent)
 - 26 Extrapulmonary tuberculosis
 - 27 Lymphoma
 - 28 Kaposi sarcoma (localized cutaneous < 10 lesions, or disseminated)
 - 29 HIV encephalopathy (according to CDC group IV B definition)

Q.12 Clinical conditions 01 and 02 correspond to Stage 1 infection. If the subject is asymptomatic (clinical condition 01), Q.12 should be coded 1. If the subject has POL (clinical condition 02) only, Q.12 should be coded 2.

Clinical conditions 03-06 correspond to Stage 2 infection. If the subject has one or more of clinical conditions 03-06, but none with a code of 07 or higher, Q.12 should be coded 3.

Clinical conditions 07 -13 correspond to Stage 3 infection. If the subject has one or more of clinical conditions 07-13, but none with a code of 14 or higher, Q.12 should be coded 4.

Clinical conditions 14-29 correspond to Stage 4 infection. If the subject has one or more of clinical conditions 14-29, Q.12 should be coded 5.

Q.13 If the person has been receiving antiviral treatment, then he/she should not be included in the study.

Eligibility

Q.14 A person is ~eligible for inclusion in the study if the duration of infection is less than 2 years, if the infection is acute or Stage 1, and if he/she has not received any antiviral treatment.

Q. 15 In addition, the person must have consented to donate blood for research purposes. Indicate whether this consent has been obtained in Q.15.

Page 2

Ensure that the WHO-UNAIDS ID Number and Collection Number are entered at the top of Page 2 of the form.

Laboratory History

- Q.16- If available, enter the results of HIV testing on recent blood samples.
If results are available
- Q.20 for only one blood sample, enter the date of the test and the results in the boxes with the header "First/Only Test". If results are available for two blood samples, enter the date and results of the earliest test in the boxes with the header "First/Only Test", and the date and results of the more recent test in the boxes with the header "Second Test".

Page 3

Ensure that the WHO-UNAIDS ID Number and Collection Number are entered at the top of Page 3 of the form.

- Q.21 If available, enter the date and result of the most recent HIV p24 antigen test
- Q.22- If available, enter the dates and results of the most recent CD4 cell counts, CD8 cell counts
- Q.24 and total lymphocyte counts.

Specimen Collection

- Q.25 Enter the date and time the specimen was collected.
- Q.26 Vials of whole blood must be labelled with the Collection Number recorded at the top of Page 1.
- Q.27 Vials of sera must be labelled with the same Collection Number, with an "S" as a suffix.

Separation

- Q.28 Indicate whether separation was performed at the primary laboratory. If not, leave Q.29-Q.32 blank.
- Q.29 Enter the date and time of separation.
- Q.30- After separation record the amount of serum, plasma and cells saved.
- Q.32 Vials of serum must be labelled with the Collection Number followed by an "S".
Vials of plasma must be labelled with the Collection Number followed by a "P".
Vials of cells must be labelled with the Collection Number followed by a "C".

Shipping

- Q.33 Record the name of the HIV isolation laboratory or the repository to which the specimen is sent.
- Q.34 Record the date of shipment.
- Q.35- Record the amounts of whole blood, serum, plasma and cells shipped to the HIV isolation laboratory or repository.

When the form is complete, the original should be kept at the primary laboratory. **A copy of the form should be sent, with the specimen, to the HIV isolation laboratory or repository.**

Annex 3:

Reagents for virus detection in tissue culture

1. Solutions, tissue culture and other reagents

Solutions

Anticoagulants:

EDTA-stock solution: 40mM (pH 8.0)

Add 14.9 g of disodium ethylene-diamine-tetraacetate ($2\text{H}_2\text{O}$) to 800 ml of H_2O . Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~1.6 g of NaOH pellet). Adjust volume to 1 litre. Aliquot and sterilize by autoclaving.

Note: The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to 8.0 by the addition of NaOH.

A 100 mM solution is commercially available.

Sodium citrate-stock solution: 129mM

Dissolve 37.9 g of sodium citrate ($2\text{H}_2\text{O}$) in 1 litre H_2O . Aliquot and sterilize by autoclaving.

Phosphate-buffered saline (PBS):

	g/per litre
NaCl	8
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.24

Dissolve above amounts in 800 ml distilled H_2O . Adjust pH to 7.4 with HCl. Add H_2O to 1 litre. Aliquot and sterilize by autoclaving. Store at 18–22°C.

Phytohaemagglutinin (PHA):

Add 5 ml distilled H_2O to bottle containing 50 mg PHA. Add 15 ml RPMI 10% medium to obtain 2.5 mg/ml. Aliquot in 100 ml and store tubes frozen at -70°C.

Tissue culture media

RPMI-1640:

According to the formulation of Moore, G.E., Gerner, R.E., Franklin, H.A.
Journal of the American Medical Association, 1967, 199:519.

RPMI 10%:

	Per litre (ml)
RPMI-1640 (1x)	900
Fetal calf serum (FCS) (FDA approved)	100
Antibiotics in a maximum volume of (final concentration: penicillin 50 U/ml and streptomycin 50 µg/ml)	5

Stock solution for penicillin and streptomycin is commercially available.
Use a solution containing 10 000 U/ml of penicillin and 10 000 µg/ml of streptomycin in PBS.

RPMI 10% PB:

	Per litre (ml)
RPMI 10%	1000
Polybrene (PB) 3.2 mg/ml (final concentration: 2 µg/ml)	0.6

Polybrene stock solution: 32 mg Polybrene dissolved in 10ml deionized
(or distilled) H₂O and sterilized by filtration through a 0.22 micron filter.
Keep solution at 4°C.

IL-2 medium:

	Per 500ml
RPMI 10% PB	500 ml
Interleukin-2 (IL-2, recombinant) (final concentration: 5 U/ml)	2 500 U

The choice of IL-2 is of crucial importance for successful HIV isolation.
The use of recombinant IL-2 (rIL-2) is recommended. Crude IL-2 demonstrates batch variation, is labile if not stored at appropriate temperature (see manufacturer's recommendation) and quality control is time-consuming and in many instances not feasible. Each type of rIL-2 should be of consistent quality over extended periods of time.

Carefully select the rIL-2 to be used by testing at least two different concentrations (ranging from 5U to 20 U) for efficiency in HIV isolation and HIV titration on PBMC in a microtitre system.

Dulbecco's Modified Eagle Medium (DMEM)

According the formulation of Dulbecco, R., Freeman, G., *Virology*, 1959, 8:396. Smith, J.D., Freeman, G., Vogt, M. and Dulbecco, R., *Virology*, 1960, 12:185. Tissue Culture Standards Committee, *In Vitro*, 6:2, 93.

DMEM (High Glucose)

contains 4500 mg/l D-glucose
with L-glutamine (580 mg/l) or l-Alanyl-l-Glutamine (862 mg/l)

is commercially available. For preparing medium containing 10% FCS
see above RPMI 10%.

Reagents used in the reverse transcriptase assays

Use double-distilled (dd) or Milli-Q purified H₂O for preparation of all solutions.

VDB (virus disruption buffer)

Isotopic assay:

100 mM Tris	1.20 g
100 mM KCl	0.74 g
4 mM DTT (dithiotreitol)	0.06 g
1 mM EDTA	0.37 g
1.25 % Triton-X 100	

Dissolve Tris, KCl, DTT and EDTA in 80 ml ddH₂O and adjust pH to 7.6 with HCl. Add 12.5 ml 10% Triton-X 100 and adjust volume to 100 ml with ddH₂O. Dispense into 5-ml and 1-ml aliquots and store at -20°C.

Salt mix

250 mM Tris	3.00 g
250 mM KCl	1.86 g
10 mM DTT	0.15 g

Dissolve in 90 ml dd H₂O. Adjust pH to 7.6 with HCl. Adjust volume to 100 ml with dd H₂O. Dispense into 2-ml aliquots and store at -20°C.

MgCl₂ solution 200 mM

Dissolve 0.8 g MgCl₂ in 10 ml dd H₂O. Dispense into 0.3 ml aliquots and store at -20°C.

Poly (rA) 40 mg/ml

Dissolve 400 mg PrA in 10 ml ddH₂O. Dispense into 0.3 ml aliquots and store at -20°C.

Oligo (dT₁₂₋₁₈)

1 unit/ml

Dissolve 5 units in 5 ml ddH₂O. Dispense into 0.3-ml aliquots and store at -20°C.

BSA (Bovine serum albumin)

mg/ml

Dissolve 50 mg BSA in 10 ml ddH₂O. Dispense into 0.3-ml aliquots and store at -20°C.

Yeast RNA

4 mg/ml

Dissolve 40 mg in 10 ml dd H₂O. Dispense into 0.3-ml aliquots and store at -20°C.

Non-isotopic assay:

Sample dilution buffer (SDB)

10mM Hepes buffer pH 7.6

4mM MgCl₂

50mg/l dextran sulfate

1% Triton X-100

2. Virus detection methods

HIV antigen (p24) assay

There are several commercially available ELISAs that can be used. The antigen detection test should be carried out according to the manufacturer's recommendations.

Practical considerations

1. Check the harvest-protocol against the samples.
2. If single samples are missing, leave corresponding wells empty and in place. Mark on the harvest-protocol that sample is missing.
3. In case of additional samples, enter those in the protocol before beginning testing.
4. Set the samples in the wells with a micro-pipette with disposable tips. Check location of each sample against the protocol.
5. Keep the remaining portion of each sample at -20°C until the test has given satisfactory results.
6. The antigen detection test should be carried out according to the manufacturer's recommendations.
7. Enter the results on the harvest-protocol (pages A2:6-7) and on the subject-list (page A2:5).
8. At least two antigen ELISA tests carried out on two sequential harvests should give positive results for a culture to be considered positive. Indeterminate cases should be re-tested with a different antigen ELISA. In addition, it is desirable to test cells for HIV-specific immunofluorescence or culture supernatant for reverse transcriptase activity.

Immunofluorescence assay

A) Sample preparation

1. Take 2-4 ml cell suspension in a 10-ml centrifuge tube (conical, translucent, with screw cap) and centrifuge at 1000 rpm (156 x g) for 5 minutes.
2. Wash cells twice in 10 ml PBS by centrifuging at 1000 rpm (156 x g), 5 minutes each.
3. Decant the supernatant (or remove with a pipette), resuspend cells in the remaining droplet.
4. Place one drop of the cells on glass slides, thoroughly cleaned with ethanol. Glass slides with 8 or 10 sample rings are to be preferred.
5. Air dry samples.
6. Fix in equal amounts of acetone-methanol at -20°C for 10 minutes.
7. Air dry. Keep slides refrigerated until tested. Slides may be stored at -20°C up to two months.

B) Fluorescence staining

Direct method

Reagent: fluorescein isothiocyanate (FITC)-conjugated IgG fraction of human serum from an HIV antibody-positive person (select a sample with high anti-HIV titre, >2500 in ELISA).

1. Add 15 ml of the above reagent to each ring of a multi-prep slide.
2. Incubate at 37°C for 30 min in a humidified chamber (box lined with wet paper).
3. Wash three times in PBS for 5 minutes with gentle agitation.
4. Wash once for 5 minutes in distilled H₂O with gentle agitation.
5. Counterstain in Evans' blue for 3 minutes.
6. Rinse slides in distilled H₂O. Remove excess water with a paper towel.
7. Mount cover slips with mounting fluid.

Indirect method

Reagents: polyclonal or monoclonal antibodies from human or animal source in the first step and appropriate FITC-labelled IgG (commercially available) in the second.

Incubation with the first antibody is performed at 4°C for 30 minutes and with the second antibody 37°C for 30 minutes, with three 5 minute PBS washes between these incubations. For all other steps, follow the direct method protocol.

Reverse transcriptase (RT) assay

Two alternative methods will be described. A: The classical assay using isotopic detection and B: an assay using monoclonal antibody to the incorporated BrdUMP substrate and a readout in ELISA format. The latter test is commercially available (RetroSys RT assay) from Cavid Tech AB, Uppsala Science Park, Staben, Uppsala, Sweden. Method A requires a multistep sample preparation, whereas culture supernatants can be directly used for the assay in method B.

Method A

Sample preparation.

1. Collect 1 ml supernatant culture fluid into 1.5-ml Eppendorf tubes. Since flasks are stored standing up in the incubator, the cells will sediment on the bottom of the flask. Handle flasks carefully; cell-free medium can then be conveniently harvested without centrifugation. Change pipette between each flask.
2. Pellet virus at 13 000 rpm (15 115 x g) for 90 minutes in an Eppendorf (or analogous) centrifuge.
3. Remove supernatant and add 100 µl virus disruption buffer (VDB). Dissolve pellet by tapping the tube. Samples may be kept at -70°C until tested.

The virus particles are now disrupted and the reverse transcriptase is accessible for assay. Disruption of virus particles inactivates virus infectivity; samples can now be removed from the safety laboratory.

When running the assay, all samples, reagents and test tubes/microplates **must be kept on ice**.

4. Thaw out samples and keep them on ice. Check the harvest protocol against the samples.
5. Thaw out reagents and keep them on ice. Prepare the RT-mix, the volume of which will depend on the number of samples to be tested.

RT mix	Volume in µl for each 10 samples
Salt mix	200
MgCl ₂	31
PrA*	50
OdT*	20
BSA	20
³ H-dTTP	25
dd H ₂ O	124
	500

MgCl₂: the HIV reverse transcriptase requires magnesium ions (Mg++) to function optimally; PrA: poly (rA), provides a synthetic template; OdT: oligo (dT₁₂₋₁₈) provides a synthetic primer to be elongated by incorporation of ³H-dTTP (tritiated deoxythymidine 5-triphosphate, specific activity 79.6 Ci/mmol, conc.: 1 mCi/ml).

The isotopic RT assay can be run in a “macro-” and “microassay” format. The “macroassay” uses tubes, 25-mm filter papers for collection of TCA (trichloroacetic acid) precipitates from individual samples and a standard scintillation, emission counter. The “microassay” uses 96-well microtitre plates, an automatic cell harvester apparatus for collection of TCA precipitates and a scintillation counter for 96-well-size filters.

RT assay in macro format

- Label 5-ml glass tubes with sample number and include two negative controls and two positive controls. It is recommended that two positive controls with different activity be used (one with high and one with relatively low reverse transcriptase activity) to facilitate comparison between assays carried out on different days.
- Tap the sample tubes to ensure mixing before removing 50µl. Add 50µl sample to tubes (50 µl VDB to the negative controls).
- Add 50 µl RT-mix to each tube.
- Cap tubes, shake rack to ensure mixing. Incubate at 37°C for 60 minutes.
- Place tubes on ice and immediately add ice-cold solutions of
 - 100 µl saturated sodium pyrophosphate (NaPP)
 - 10 µl yeast RNA
 - 200 µl 25% TCA.

* A PrA.OdT mixture is available commercially. It should be diluted with ddH₂O to 1 unit/ml and frozen in aliquots at -20°C. Use 100 ml from this solution for 10 samples.

-
11. Shake tube rack and leave on ice for at least 15 minutes.
 12. Filter precipitate on glassfibre filters (Whatman GF/A) (for example in a Millipore manifold apparatus).
 13. Rinse tubes four times, pass washings through filter and then wash the filter itself with ice-cold 10% TCA. Rinse the filters once with ice-cold 99.5% ethanol to shorten drying time for filters (optional).
 14. Place filters in scintillation vials and allow to dry in an oven at 70-80°C for at least 60 minutes.
 15. Add scintillation fluid (commercially available, for example Quicksafe A, Zinsser) and cool vials for 30 min and count in scintillation counter for emission.

RT assay in micro format

6. Transfer the 50-µl samples to wells of a microtitre plate. The samples must be set in horizontal order (A1® A12). A1, A2 are the positive controls and A3, A4 are the negative controls.
7. Add 50µl RT-mix to each well. Use a multichannel pipette or a repeating pipette. Tap the plate gently to ensure mixing.
8. Incubate the plate with the lid on at 37°C for one hour.
9. Terminate the reaction by adding 50 µl 10% ice cold TCA with 0.02 M NaPP. Let the plate stand on ice for at least 15 minutes, during which time a precipitate will form.
10. Wash the plate in a cell harvester apparatus according to the manufacturer's recommendations. Wash solution: 10% TCA with 0.02 M NaPP.
11. Place the filter in a bath of 99.5% ethanol for 5 minutes.
12. Dry the filter (as above or in a microwave oven for 10 minutes, power level 6).
13. Place the filter into a plastic bag, add 10 ml scintillation fluid and seal the top of the bag.
14. Count in a scintillation counter for 96-well filters.

Method B

Corrigan et al. *AIDS Research and Human Retroviruses*, 1998, 14:347.

1. Dilute cell culture supernatants with sample dilution buffer (SDB) in three or four five-fold dilution steps beginning with 1:5. See template for virus dilution in section 9.3. Since the SDB contains Triton-X 100, the virus particles will be disrupted and the reverse transcriptase becomes accessible for assay. Disruption of virus particles inactivates virus infectivity; samples can now be removed from the safety laboratory.
2. Add 50-µl aliquots of serially diluted samples to poly(rA) coated wells of a 96-well microtitre tray. Use mock-infected culture supernatants as controls.
3. Add 150µl of the reaction mixture containing oligo(dT)₂₂ as primer and bromodeoxyuridine 5'-triphosphate (BrdUTP) as substrate. The reaction mixture is provided with the kit and should be stored and used according to the manufacturer's recommendation.
4. Incubate 3 hours - overnight at 37°C, as necessary. RT catalyzes along the poly(rA) template the polymerization of a new DNA strand consisting of incorporated BrdUMP.
5. Wash plates twice to remove substrate.
6. Add 100 µl of alkaline phosphatase (AP)-conjugated anti-BrdU antibody to each well and incubate at 33°C for 90 min.
7. Wash plate four times and add substrate, p-nitrophenyl phosphate. The intensity of the color reaction can be read in a standard plate reader (405 nm) at defined times, for example at intervals of 0.5, 1, 2, 4 and 24 hr.
8. Calculate the amount of RT present in each sample dilution as the concentration (in ng/ml) relative to a serially diluted reference enzyme of known concentration present on all plates. The concentration in each sample dilution can then be used to calculate the mean concentration in the undiluted sample. All samples with RT activities corresponding to =0.003 ng/ml and having absorbance greater than two times the background are deemed positive.

Extended biological characterization

Cell lines

MT-2 cells were derived from cord blood cells by infection with human T-cell leukemia virus type I (HTLV-I) (Harada et al. 1985). The cell line carries HTLV-I and is sensitive to infection with HIV-1 that employ CXCR4. Productive infection with such viruses results in syncytium induction. Due to this property, MT-2 cells provide a test system which can easily be used to characterize HIV-1 isolates into syncytium inducing (SI) and non-syncytium inducing (NSI) (Koot et al. 1992). MT-2 cells grow in suspension (RPMI 10%) and can be carried by dividing cultures 1:10 twice a week. The recommended number of passages is 20, after which new cultures should be thawed from frozen stock. It is advisable to produce a large frozen stock of cells, because sensitivity to HIV-1 infection decreases with time.

U87.CD4 and GHOST(3) cell lines have been engineered to stably express CD4, co-receptors for HIV and, in the case of GHOST(3) cells, also the green fluorescent protein (GFP) [34, 35]. The cell lines are available from the repositories in the USA and UK [18, 27]. Stability of the different markers is, however, highly variable. It is, therefore, mandatory that upon receipt of the indicator cell lines, each laboratory freezes a large stock of cells (at least 10 tubes). Continuous passage of any of the U87.CD4 cell series should not exceed two months. After this time the cultures must be discarded and a new tube thawed out from frozen stock. GHOST(3) cells grow faster than U87.CD4 cells and may be even more prone to loss of markers. It is, therefore, advisable to thaw out the cells shortly before use for experiments (2-3 days before). The cell lines may then be used for a maximum of three weeks.

Note: It is important to include control viruses with defined co-receptor usage (and defined TCID₅₀ titre on PBMC, optimally 1000 TCID₅₀[PBMC]) in each experiment. Using the same virus stock, co-receptor usage patterns (also time to syncytium induction in U87.CD4 cells and the proportion of cells showing fluorescence in GHOST(3) cells) should be highly reproducible between experiments. Another way of testing receptor expression is by flow cytometry using monoclonal antibodies to CD4 or the chemokine receptors (anti-CCR3, -CCR5, and -CXCR4 are available). All reagents are commercially available.

Should the proportion of receptor-positive cells decrease with time, the cell lines may be re selected by culturing in selective medium for two weeks. Expression of the different markers can be selected according to the following scheme:

- CD4 - 300 µg/ml of G418 for U87.CD4 and 500 µg/ml for GHOST(3)
- CCR1, CCR2, CCR3, CCR5, CXCR4, BOB, Bonzo - 1 µg/ml puromycin
- GFP - 100µg/ml hygromycin for GHOST(3) (reduce to 50µg/ml if cells appear too sensitive)

Following selection, passage cells at least once in DMEM 10% (or DMEM 7.5% for GHOST(3) cells), then freeze stocks and use for experiments.

Culturing of U87.CD4 cells

U87 is a human glioma cell line into which the CD4 receptor has been introduced and, subsequently, the chemokine receptors CCR1, CCR2B, CCR3, CCR5 or CXCR4. The cell lines grow as monolayer cultures.

1. Thaw cryotube rapidly in lukewarm water.
2. Transfer cells to a 10-ml centrifuge tube. Add 8 ml DMEM 10% (high glucose), prewarmed to 18-22°C.
3. Centrifuge at 1000 rpm (156 x g) for 10 minutes.
4. Resuspend cells in 10 ml DMEM 10% and seed cells in a 25-cm² flask. Place flasks horizontally in a humidified 5% CO₂ atmosphere at 37°C.
5. Observe cultures, change medium or subculture as necessary (approximately twice weekly). If cultures grow very slowly, use medium containing L-alanyl-L-glutamine (Glutamax) rather than L-glutamine (less stable).

6. Subculture:

- remove medium and rinse the monolayer with 5 ml sterile PBS.
- add 0.5 ml EDTA (5 mM, pH 8) and place flasks horizontally (preferably at 37°C) for 5 min.
- when cells detach, add 5 ml DMEM 10%, resuspend and split cultures 1:3 (1:5).
- add 10 ml DMEM 10% and return flask to the incubator (humidified 5% CO₂ atmosphere at 37°C).

Culturing of GHOST(3) cells

HOS is a human osteosarcoma cell line into which the CD4 receptor has been introduced and, subsequently, the chemokine receptors CCR3, CCR5, CXCR4 or the orphan receptors Bonzo or BOB. The cell line also carries GFP driven by the HIV-2 LTR. HIV infection activates GFP and, consequently, infected cells fluoresce green. GHOST(3) cells grow as monolayer cultures.

1. Thaw cryotube rapidly in lukewarm water.
2. Transfer cells to a 10-ml centrifuge tube. Add 8 ml DMEM 7.5%, prewarmed to 18-22°C.
3. Centrifuge at 1000 rpm (156 x g) for 10 minutes.
4. Resuspend cells in 8 ml DMEM 7.5% and seed cells in a 25-cm² flask. Place flasks horizontally in a humidified 5% CO₂ atmosphere at 37°C.
5. Observe cultures, change medium and subculture twice weekly.
6. Subculture:
 - remove medium and rinse the monolayer with 5 ml sterile PBS.
 - add 0.5 ml EDTA (5 mM, pH 8) and place flasks horizontally (preferably at 37°C) for 2-3 min.
 - when cells become detached add 5 ml DMEM 7.5%, resuspend and split cultures 1:10-1:20, as necessary.
 - add 8 ml DMEM 10% and return flask to the incubator (humidified 5% CO₂ atmosphere at 37°C).

Preparation of GHOST(3) cells for flow cytometry

Infection of GHOST(3) cells see section 9.3.2.

- Day 4: Check cultures under a fluorescence microscope. If fluorescence intensity has increased from the previous day, cultures are ready for flow cytometric analysis (FACS).
1. Wash wells with 350 µl PBS.
 2. Add 200 µl 5mM EDTA, place trays at 37°C and wait a few minutes until cells detach.
 3. Add 200 µl 4% paraformaldehyde to the FACS tube (5 ml polystyrene tubes, sterile).
 4. Resuspend cells and transfer into the FACS tube. Mix well. The final concentration of paraformaldehyde is 2%.
 5. Keep samples in the dark at 4°C for at least 1h before FACS analysis. Samples can be kept up to 2-3 days before reading.

Mycoplasma testing

Cell lines kept in continuous passage (like MT-2 cells) should regularly, preferably monthly, be tested for contamination with mycoplasma. For this purpose, a mycoplasma test detecting mycoplasma-specific nucleic acid is appropriate. Such a test does not require separate laboratory rooms for lengthy cultivation of mycoplasma test cultures, but can be performed on any laboratory bench and yields results within a few hours. Tests of this kind are commercially available. As a rule, 1.5-2.0 ml supernatant fluid from 3-4 day cultures is tested according to a procedure described by the supplier. Indicator cell lines changed frequently (every 1-2 months) may be tested once or twice a year.

Mycoplasma-contaminated cultures must be treated with antibiotics. Antibiotic combinations for this purpose are commercially available. Treated cultures should be re-tested for mycoplasma to confirm the success of treatment. If negative, freeze several aliquots in liquid nitrogen to have a supply of mycoplasma-free cells. Thaw a new aliquot every two months. It is recommended that the U87.CD4 and GHOST(3) series of cell lines be re-selected following treatment for mycoplasma infection.

p24 Antibody Interference Assay

The potential for residual serum p24 antibodies to interfere with p24 antigen detection by ELISA, giving a false indication of virus neutralization, is of concern. Approximately 10% of sera from HIV-1 infected individuals contains high levels of anti-p24 antibodies, and these could potentially interfere with the readout ELISA. The assay detailed below gives a semi-quantitative assessment of the level of serum p24 antibodies in test serum and their potential for p24 antigen complexing.

1. Serially dilute (2-4 fold dilutions) HIV-1 positive test serum in a suitable assay buffer covering a wide range (e.g. 1/25 to 1/102,400). The buffer is ultimately dependent on the p24 antigen ELISA used, but most isotonic buffers will suffice (e.g. PBS, Tris-buffered saline or culture medium). The buffer should also contain 5-15% animal serum (calf serum or sheep/lamb serum) and detergent (e.g. 0.5% Empigen or 0.5% Triton X100). In addition to the HIV-1 positive sera to be tested, a p24 antibody control serum should be used. This can be either a pool of p24 antigen monoclonal antibodies diluted in human HIV-1 seronegative serum or an animal p24 antigen immune serum. Care should be exercised to avoid strain-specific antibody interference with the p24 antigen ELISA used.
2. Add 75 µl of each serum dilution to 75 µl of assay buffer, containing recombinant p24 antigen. The amount of p24 antigen used should be in the range expected from cultured virus and give a high (1,500 to 2,000) optical density. Two additional assay controls are required; (i) at least six replicate wells containing 75 µl of the lowest dilution of HIV-1 seronegative serum (e.g. 1/25) plus 75 µl of p24 antigen; and (ii) 4-6 replicate wells containing 75 µl of seronegative serum (1/25) plus assay buffer.
3. Incubate together in a 96-well round bottomed tray for 2 hours at room temperature.
4. Perform p24 antigen ELISA on all samples. The volume used (in this case 100 µl) may need to be adjusted or diluted depending on the antigen ELISA used. The last dilution of test serum to reduce the level of p24 antigen to 50% of that detected in the positive control wells, incubated with seronegative serum, is scored. Most test serum will have no discernable interference beyond a dilution of 1/3,200. For the neutralization assays described above, the level of p24 antibodies is reduced to 1/21,870 and 1/43,740 for the *Endpoint Neutralization Assay* and *Infectivity Reduction Assay*, respectively. This is based on an input serum dilution of 1/10 or 1/20, a 3-fold dilution for the final 225 µl volume, and three 9-fold washes.

Determination of CCR5 genotype of PBMC by PCR

A portion of the *ccr5* gene can be amplified by PCR from genomic DNA using the following primers:

Forward - CTCGGATCCGGTGAACAAGATGGATTAT

Reverse - CTCGTGACATGTGCACAACTCTGACTG

These primers flank the 32 base pair deletion in the *ccr5* receptor gene, which has been associated with the inability of R5 strains of HIV-1 to infect PBMC carrying this deletion, and are used to generate wild-type and mutant fragments of 182 bp and 150 bp, respectively. For most applications, only those PBMC with a *ccr5* wild-type genotype - and expressing CCR5 on the cell surface - should be used.

The method described in the following is a variant of the one described in section 11.1.2, the difference being that the cells are first lysed to separate a nucleic fraction to enrich for genomic DNA, followed by lysis of nuclei and PCR amplification of resulting DNA.

1. A crude genomic DNA lysate can be prepared by lysing PBMC in 500µl of TST buffer (10mM Tris-HCL [pH 7.5], 5mM MgCl₂, 0.32M sucrose, 1% TritonX100). Sterile microfuge tubes should be used throughout. The nuclear pellet obtained following a 1 minute centrifugation step (15000 x g, or maximum setting of any benchtop microcentrifuge) is resuspended at the equivalent of 5-10x10⁶ cells per 10 µl of TENT buffer (10mM Tris-HCL [pH 8.5], 1mM EDTA, 0.5% NP40, 0.5% Tween-20) containing 200 µg/ml of proteinase K. The lysate is heat-treated overnight at 56°C, followed by a 10 minute enzyme inactivation step at 85°C. Samples should be stored at -70°C until use.
2. Each 50 µl PCR reaction consists of 5 µl of crude DNA lysate, 1X PCR buffer (see Annex 4), 1.5mM MgCl₂, 0.25mM dNTPs (dATP, dGTP, dTTP, dCTP at equimolar proportions), 20 pmoles of each primer, 1.5U AmpliTaq DNA Polymerase, nuclease-free H₂O, and overlaid with DNase-free light mineral oil. Each PCR amplification consists of 42 cycles, with five initial cycles of 94°C for 30 seconds, 50°C for 45 seconds and 72°C for 1 minute, followed by 37 cycles of 94°C for 30 seconds, 60°C for 45 seconds and 72°C for 1 minute.
3. Amplified PCR fragments are then visualized on a 3% agarose gel. The gel is prepared by dissolving 3g agarose in 100ml 1X TE buffer [1M Tris-hydroxymethyl-aminoethane, 0.1M EDTA, pH 8], microwave for 3-5 minutes, allow to cool slightly and add ethidium bromide (final concentration 0.2µg/ml). Ethidium bromide is a potent carcinogen and should be handled with extreme caution. Five microlitres of amplified PCR product is mixed with 1 µl of 6X loading buffer (containing 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol). These dyes migrate with DNA fragments of 2000 bp and 200 bp, respectively. Suitable molecular weight markers (e.g. a Hae III digest of plasmid f174 contains DNA fragments ranging from 72-1353 bp) should be used to confirm the size of the amplified PCR products. Samples should be carefully placed into adjacent wells of the gel and allowed to run for ~45 minutes at 100V. The gel can be visualized using a UV transilluminator and photographed.

Annex 4:

Reagents and associated methods for HIV genetic typing

Lysis buffer

To prepare cell lysates for PCR amplification:

10 mM	Tris HCl, pH 8.3
50 mM	KCl
2.5 mM	MgCl ₂
0.45 %	NP-40
0.45 %	Tween-20
100 µg/ml	Proteinase K

Reagents for first round PCR:

10X PCR Reaction Buffer (no MgCl₂)

1X =	50 mM	KCl
	10 mM	Tris, pH 8.3
	1 %	DMSO
	1 %	Glycerol
	10 mM	MgCl ₂

10X dNTP mixture

1X =	200 µM	of each dATP, dCTP, dGTP, TTP (Pharmacia).
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Positive control DNA

pNL4-3	10 copies/µl
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Reagents for agarose gel electrophoresis:

Stock solution of TAE 50X (1 litre)

2M Tris base	(242 g)
1.66M NaAc	(136 g)
0.1M EDTA	(37.2 g)

Stir until dissolved and add acetic acid to a pH of 7.8. Add water to 1 litre.

Work solution of TAE 1X:

Dilute the TAE (50X) stock solution 1/50 with water to a final concentration of:

40mM	Tris
33mM	NaAc
2mM	EDTA

Loading dye:

25 %	Ficoll
1 %	Orange G

10X Heteroduplex annealing buffer

1X =	100 mM	NaCl
	10 mM	Tris, pH 7.8
	2 mM	EDTA

Polyacrylamide gel electrophoresis

Preparation of gel matrix: 5% polyacrylamide (using a stock of 30% acrylamide, 0.8% bis-acrylamide) in 1X TBE. A 50-ml mixture is prepared per gel using 8.3 ml acrylamide stock, 5 ml 10X TBE and 36.7 ml H₂O. Degassing by exposure to a vacuum is recommended. Polymerization is initiated with 50 mg of ammonium persulphate and 33 µl of TEMED.

Gel Electrophoresis buffer (TBE):

1X =	88 mM	Tris-borate
	89 mM	Boric Acid
	2 mM	EDTA

One litre of 10X stock is prepared by dissolving in deionized H₂O:

108 g of Tris base, 55 g of boric acid and 40 ml of 0.5 M EDTA.

5X Ficoll/Loading dye

25%	Ficoll
1%	Orange G

Reagents for Phenol extraction of DNA

Phenol/CHCl₃

BEFORE PREPARING READ section 4.4 about safe handling of phenol/CHCl₃

Recrystallized phenol is stored at -20°C in 250ml plastic bottles. When needed, a bottle is placed in a 56°C H₂O bath long enough to melt the phenol. Phenol is **carefully** poured into a glass bottle containing an equal volume of chloroform. Add 0.02 g hydroxyquinoline then add an equal volume of STE. Mix well then add sufficient NaOH (1-3 ml 10 N) to achieve pH 7.

STE - 10X STE (Sodium chloride/Tris/EDTA; filter sterilize)

for 1 litre:

1 M	NaCl	58.44 g
0.02M	Na ₂ EDTA	7.44 g
0.1M	Tris (pH 7.4)	13.22 g Tris-HCl [33.47 ml 2.5M stock] 1.94 g Tris-Base [5.34 ml 3M]

Buffer A - 10X Buffer A (filter sterilize, store at 4°C)

for 1 litre:

1.5 M	NaCl	87.66 g
0.05 M	KCl	3.73 g
0.055 M	glucose	9.91 g
0.25 M	Tris (pH 7.3)	33.05 g Tris-HCl [83.67 ml 2.5M] 4.85 g Tris-Base [13.36 ml 3M]

RNase – 20 mg/ml in H₂O: boil 20 minutes, aliquot and store at -20°C.

Proteinase K – 20 mg/ml in 1XSTE, stored at -20°C.

SDS - 10% Sodium dodecyl sulfate stored at room temperature.

SEVAG - 24:1 (v/v) mix of CHCl₃ and isoamyl alcohol.

TLE - 0.01M Tris (pH 7.8), 0.1mM EDTA

Alcohols:

100% isopropanol, 95% EtOH, 70% EtOH, each stored at -20°C.

5M NaCl. Filter sterilized.

10mM EDTA pH 8, 1% SDS.

AmAc - 7.5M Ammonium acetate, pH 7.5. Filter sterilized.

Method of Phenol extraction:

1. Begin with 2×10^6 Ficoll purified and washed PBMC (see chapter 8). Use of more cells requires scale up of the following volumes.
2. Pellet cells by centrifugation at 1500 rpm for 5 min or in a microcentrifuge for 1min.
3. Add 1/2 volume (relative to initial media volume, assuming 2×10^6 cells/ml) cold Buffer A, and vortex or pipette to resuspend.
4. Pellet cells and repeat wash.
5. Thoroughly resuspend cells in 160 μ l STE.
6. Add 20 μ l Proteinase K, mix.
7. Add 180 μ l 10% SDS, mix well, make sure clumps disappear during subsequent incubation.
8. Incubate at 56°C for 1-2 h. to overnight.
9. Dilute sample by adding 140 μ l with STE, mix, then add 500 μ l of Phenol/ CHCl_3 and mix very well. Invert several times to generate a homogenous milky emulsion, let sit for 2-5 min at room temperature. Mix again.
10. Spin in a microcentrifuge for 1 min.
11. Carefully remove aqueous phase (upper layer, colourless), trying to avoid collecting the white protein precipitate at the interface. However, it is better at this point to take the interface than to leave DNA behind that may be trapped within the protein precipitate (ppt.).
12. Add 250 μ l 7.5M AmAc; mix by inversion until homogenous.
13. Add 750 μ l cold isopropanol; mix by inversion until ppt. is completely fibrous (no gelatinous material left).
14. If only high MW DNA is required and a large fibrous ppt. forms, wind out DNA precipitate using a flame sealed glass rod. Otherwise, chill tube at -20°C (duration depends on expected yield, approximately 0-2 h), and spin in a microcentrifuge for 1-5 min.
15. Redissolve ppt. in 250 μ l 10mM Tris, 10mM EDTA (pH 8) containing 100 μ g/ml boiled RNase A. Incubate at 37°C, 30 min or until ppt. is dissolved.
16. Add 5 μ l NaCl and 28 μ l SDS, mix, add 15 μ l Proteinase K, mix, incubate at 56°C for ≥ 1 hr.

17. Phenol/CHCl₃ extract as needed until the ppt. at interface is gone (usually 1-3 times). When removing final aqueous phase be sure not to carry over any precipitate.
18. SEVAG extract aqueous layer once to remove traces of phenol.
19. Add 10 µl NaCl, mix. Add 750 µl cold 95% EtOH and mix by inversion until ppt. is completely fibrous (no gelatinous material left).
20. Spin out DNA precipitate using a flame sealed glass rod. Otherwise, chill tube at -20°C (length depends on expected yield, approximately 0-2 h), and spin in a microcentrifuge for 1-5 min.
21. Rinse ppt. with ice cold 75% EtOH, air dry briefly, and redissolve in 0.1 ml TLE at 4°C. Ensure DNA is fully redissolved: carefully examine solution while pipetting up and down.
22. To determine the DNA concentration and purity, pipette 1 µl of DNA into 0.5 ml TLE and read the absorbance in a UV spectrophotometer at 260 nm using a TLE blank. The purity of the sample can be estimated by the ratios of the absorbance at 230, 260 and 280 nm.

A_{260}/A_{280} should be 2.0-2.5.

A_{260}/A_{230} should be 1.7-1.9.

All A_{260} readings should be 0.2-0.8 for maximum precision, therefore you should preload the cuvette with an amount of DNA (uninfected placental DNA may be used for this purpose) to reach a baseline OD of 0.2. To use as little DNA as possible, we add 1 µl samples sequentially to the cuvette and measure the change in OD for each sample. The DNA concentration in µg/ml can be calculated by multiplying the A_{260} reading by the dilution and 50:

$$\text{e.g. } \frac{500 \text{ } \mu\text{l tot} \times A_{260} - 0.2 \text{ (i.e., change in OD)}}{1 \text{ } \mu\text{l DNA}} \times 50 \text{ } \mu\text{g} = \frac{\text{XXX } \mu\text{g/ml}}{A_{260}}$$

23. Quantitate and store at -20°C.

“Sterilization” of PCR reaction

There are two times in a PCR experiment that sterilization can be implemented:

1. **Before** amplification – the goal is for carryover molecules present in the reaction mix to be rendered inactive, i.e., non-amplifiable.
2. **After** amplification – the goal is for all nucleic acids, including PCR products to be inactivated.

“Sterilization” Protocols:

The sensitivity of PCR performed using of the protocols discussed below are comparable, eliminating up to about 3×10^9 copies of the product. Each of the systems, however, presents certain drawbacks.

1. **Isopsoralen.** Isopsoralen is a furocoumarin, which, when excited by UV light, reacts with pyrimidines to form cyclobutane rings. Taq DNA polymerase will halt synthesis when it encounters such a photochemically modified base in a template strand. Psoralen or isopsoralen is added to a PCR mixture prior to amplification and will not be significantly degraded by thermal cycling. It is then photoactivated after amplification, by placing the closed PCR tube near an UV light source where it reacts with the newly synthesized DNA. If the damaged DNA that is produced is carried over into a new reaction vessel, it cannot serve as a template for the PCR process. All nucleic acids, including PCR products, are thus “sterilized” before the reaction tube is opened and exposed to the environment. However, the DNA remains detectable when analyzed by gel electrophoresis and ethidium bromide staining, and can be probed by hybridization without significant loss of sensitivity. Isopsoralen compounds are toxic and potentially carcinogenic and must be handled with care in the laboratory.
2. **Uracylglycosylase or UNG.** This enzymatic procedure is included in the commercially available DNA detection kit from Roche. It is based on the use of dUTP instead of dTTP in PCR reactions. All subsequent pre-PCR reactions are treated with the enzyme dUDG uracyl DNA glycosylase, followed by inactivation of this enzyme with heat. UDG cleaves the uracyl base from the N-glycosidic bond between the base and the sugar phosphate backbone. The resulting apyrimidinic site blocks DNA replication. The dUTP protocol is very attractive because the enzyme attacks many sites within contaminating PCR products, while being nonreactive on both the reaction reagents and on any natural DNA whose amplification is desired. The UNG protocol is expensive because of the higher cost of dUTP compared to dTTP. Furthermore, care must be taken to prevent residual UNG activity from destroying the amplification product prior to analysis. Note that this method does not eliminate contamination from DNA fragments that were not synthesized using dUTP.
3. **Primer hydrolysis.** This is a chemical procedure employing modified primers. The primers are modified near their 3' ends to contain one or two ribose residues. After amplification NaOH is added and the ribose linkages are susceptible to base hydrolysis and cleavage that greatly reduces the efficiency of reamplification. Primer hydrolysis is inexpensive, but tubes must be opened after amplification to allow addition of NaOH. This will provide opportunities for aerosolization of amplification products.

Despite the best measures taken to avoid sample contamination, it still does occur on occasion and thus additional measures are required to detect contamination after it has occurred. As mentioned above, all PCR experiments should include negative control reactions. If negative controls produce PCR product, contamination has definitely occurred in the experiment, although it is not clear which, if any, of the unknown samples have been contaminated. To avoid this uncertainty, it is customary to discard all of the results of the experiment and re-evaluate procedures and reagents. Even if negative control samples are not positive, contamination may have occurred in one or more of the unknown specimens. Thus, procedures to detect contamination after it has occurred, usually after the genetic screening test, need to be implemented. Please refer to Learn et al. [43] for further discussion.

Annex 5:

Sequence analysis tools at the HIV Database

HIV-MAP: This tool allows you to obtain all sequences (or a subset of sequences) which contain a selected region. Entries can be downloaded as Genbank records, or as nucleotide alignments in FASTA or IG/Stanford format, or as protein alignments. The tool allows searches on a variety of information: subtype, country, sequence name, accession number

Dbsearch: The regular sequence search interface, which allows searches on virtually all information present in the HIV database, such as country of sampling, patient characteristics (disease stage, risk group, country and year of infection, etc), sequence name, publication information, coreceptor usage, and gene or region

Motif Scan: Search HXB2 or your own amino acid sequence(s) for an HLA peptide binding motif.

Primalign: Automatically align your primer or sequence fragment to the complete genome alignment. The interface returns the coordinates (HXB2 numbering) and an alignment of the fragment to all sequences in the whole genome alignment.

Epilign: Automatically align your amino acid epitope or short functional domain against the protein alignments we have up on the web.

HIV-BLAST: Finds sequences similar to yours in the HIV database. Sequences are sorted by % similarity rather than by BLAST score.

HXB2 Numbering Engine: A quick way to find the correct position numbers in an HIV sequence relative to HXB2, which was selected as the standard; for numbering primers, epitopes, and sequence regions.

SeqPublish: Formats your alignment for publication: identical columns are replaced by dashes, and the sequences are printed in blocks of user-determined length.

Recombinant Identification Program (RIP): a program to identify mosaic sequences by comparing your query sequence to a set of background sequences. We provide background alignments that consist of one consensus sequence for each HIV subtype. Most sequences can easily be aligned to these sets. You can also use your own background alignment, so you can use RIP to analyze your own sequences of interest, including non-HIV sequences.

SNAP (Synonymous/Non-synonymous Analysis Program) calculates synonymous and non-synonymous substitution rates based on a set of codon-aligned nucleotide sequences, based on the method of Nei and Gojobori, incorporating a statistic developed in Ota and Nei. It produces a plot of cumulative syn and nonsyn changes, providing a rapid summary of the results.

HYPERMUT: This interface takes a nucleotide alignment and documents the nature and context of nucleotide substitutions in a sequence population relative to a reference sequence. Identifying hypermutants in a patient's viral population can be critical when reconstructing viral phylogenies (to assess the effects of drug therapy, immune surveillance, etc.). The apparent rate of viral evolution can be dramatically exaggerated by hypermutant sequences, when in reality these viruses are evolutionary dead ends; their profound divergence is an artefact of a single aberrant round of replication.

Neighbor TreeMaker takes a sequence alignment, converts it to PHYLIP format, runs it through the PHYLIP programs Dnadist (Distance Matrix program), and Neighbor (treefile generator), then displays a tree. An additional advantage is that Treemaker allows sequence names longer than 10 characters.

PCoord (Principal Coordinate Analysis) is a procedure to find meaningful patterns in sequence data. The method was developed by the mathematician J.C. Gower in 1966. This web interface uses the PCOORD program suite, written by Des Higgins. The procedure takes an alignment as input, and summarizes the variation in the sequences in ten 'axes' or 'dimensions'. Each sequence gets a score on each of the dimensions, and these scores can be plotted pairwise.

HMA gel analysis: An interface to HDent and HDdist, programs for quantitating diversity from heteroduplex mobility and tracking assays.

Vespa: Signature Pattern Analysis: A program for identifying sites which are shared by one group of sequences, and are rare in another group, and for quickly summarizing amino acid or nucleotide frequencies across all positions in an alignment.

Tutorials:

- The Sequence quality control tutorial gives examples of what contamination in a sequence set can look like, and lists some tips on how to detect and guard against contamination.
- The Tree making tutorial gives a basic introduction to phylogenetics, and lists some frequently made mistakes and how to avoid or remedy them

Annex 6:

Publications obtained within the framework of the WHO Network:

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