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GLOBAL PROGRAMME ON AIDS

WHO GUIDELINES FOR STANDARD HIV ISOLATION PROCEDURES

1994

WORLD HEALTH ORGANIZATION

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CONTENTS

Acknowledgements

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1. Introduction

These guidelines for human immunodeficiency virus (HIV) isolation and characterization are provided as part of the activities of the WHO Network for HIV Isolation and Characterization, established to coordinate epidemiological studies on HIV variation worldwide. The primary objective of the WHO Network is to acquire information relevant to the evaluation and/or design of antigenically appropriate HIV candidate vaccines for various geographic regions.

HIV evolves rapidly, and strains with widely divergent nucleotide sequences and biological properties have been isolated from a number of geographical locations. In addition, infected individuals can harbour mixed virus populations, the composition of which varies with time. Isolation procedures may greatly influence the biological, and perhaps antigenic, properties of virus isolates; therefore the use of a standard HIV isolation technique for comparative epidemiological studies is essential. As yet, it is not possible to fully correlate differences in nucleotide sequences with fundamental viral properties such as infectivity, transmissibility or pathogenicity. Detailed information on the number of distinguishable HIV serotypes circulating within different communities is lacking and the mechanism for inducing protective immunity is unknown. It is therefore an open question whether effective HIV candidate vaccine preparations will need to contain immunogenic material derived from viral variants geographically associated with a target population, or with phases of the epidemic. There is a need to establish a network designed to monitor genetic and antigenic variation of HIV in selected geographical locations. Such a network will make possible assessment of the impact of antigenic variation on vaccine efficacy, and potentially diagnosis, pathogenesis and improved antiviral therapy.

The WHO network comprises collaborating research laboratories for HIV isolation and characterization. This includes:

(a) "primary" collaborating laboratories for collection of biological samples and virus isolation at potential sites for HIV vaccine evaluation, in areas of high incidence of HIV infection, usually in developing countries;

(b) "secondary" laboratories for the molecular and immunological characterization of HIV isolates, especially in developed countries; and

(c) repositories for the storage of clinical specimens, including sera, viable cell samples and virus isolates.

These materials are being stored by, and distributed through, the existing WHO repositories at the National Institute for Biological Standards and Control (NIBSC) in the United Kingdom and the Basic Research and Development Programme, Division of AIDS of the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) in the USA.

The WHO/GPA Steering Committee on HIV Vaccine Development is coordinating implementation of the project. WHO/GPA designates appropriate primary and secondary collaborating laboratories, distributes the biological materials and reagents, collects and analyses the data and establishes links between vaccine development efforts worldwide. Guidelines for standard HIV isolation procedures are particularly important since establishing primary research laboratories in developing countries with a high incidence of HIV infection – and consequently potential sites for HIV vaccine testing – is among the primary goals of this programme.

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 of the world, are examples of the advances made in this area. The identification of a retrovirus as the etiological agent of acquired immunodeficiency syndrome (AIDS) has made the development of a vaccine to prevent this disease a possibility. A vaccine to prevent infection with HIV or the subsequent immunosuppression caused by the virus has to overcome 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, and
- the genetic and antigenic variability of HIV.

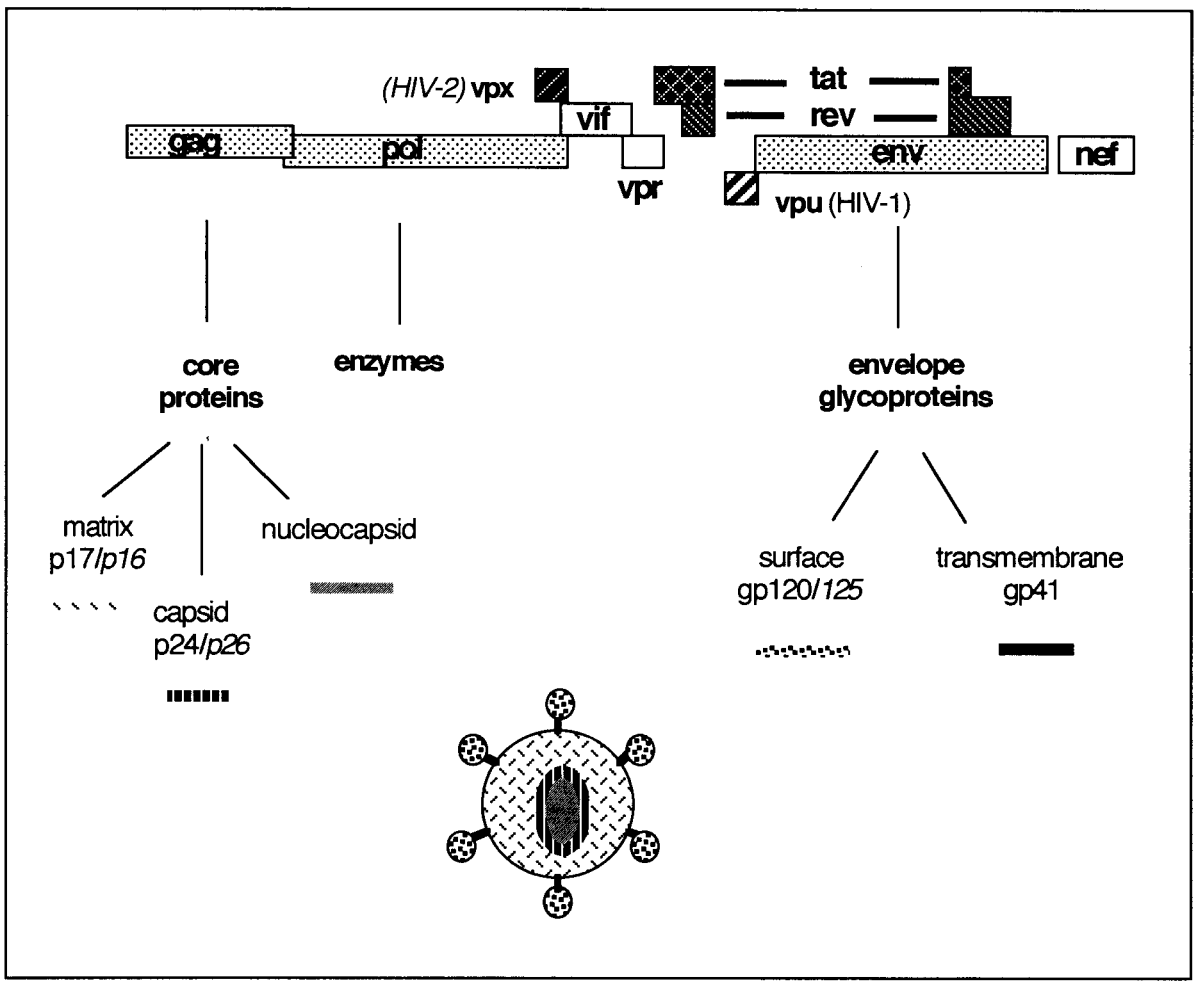
To fully understand these problems, a short description of the retroviral life cycle and the causes and consequences of variability will follow.

2.1 Genome organization and protein products

Based on electron microscopic morphology and studies of genomic organization and pathogenic features, HIV can be classified as a member of the lentivirus subfamily of retroviruses. Lentiviruses cause diseases with long incubation periods and protracted symptomatic phases. Like all replication-competent retroviruses, HIV has the genes, *gag* and *env*, encoding structural proteins and *pol* encoding enzymatic proteins. Three classes of messenger RNA (mRNA) size can be distinguished, namely genome length mRNA, singly-spliced mRNAs and multiply-spliced mRNAs. Translation of genome length mRNA yields a large polyprotein encompassing all *gag* and *pol* products. This precursor is cleaved in several steps by the viral protease to give 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 numerous regulatory functions. Two of these genes, *tat* and *rev*, are produced from overlapping reading frames by multiply-spliced mRNAs and encode small nuclear proteins that regulate transcription of the other HIV genes. *Tat* increases the steady state levels of all HIV mRNAs through binding to the *tat*-responsive-element (TAR) within a sequence termed the long terminal repeat (LTR), which comprises the two ends of the viral sequences. This mechanism is called transactivation. *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 single-spliced HIV mRNAs. Conceivably, the complex regulation of viral replication enables HIV to rapidly change from the latent to the productive phase, which is characterized by the output of large amounts of virus within a short time. This complexity also gives hope that intervention with virus replication will be an effective means by which disease progression and infectiousness of infected individuals could be controlled in the future.

Figure 1. The HIV genome and encoded protein products



2.2 The viral life cycle

Following attachment of a viral particle to a specific cellular receptor (in the case of HIV, this receptor is termed CD4) and penetration of the cell membrane, the viral enzyme transcribes the viral RNA into DNA, the RNA template is then degraded and a second DNA strand is synthesized. The double-stranded DNA copy of the viral sequences, also called the provirus, is transported to the nucleus and integrates into the host cell DNA. Integration of the provirus is assisted by another virus-specific enzyme, an endonuclease (also called integrase). Integration completes phase I of the retrovirus life cycle (Figure 2). The integrated provirus may be transcriptionally inactive and behave

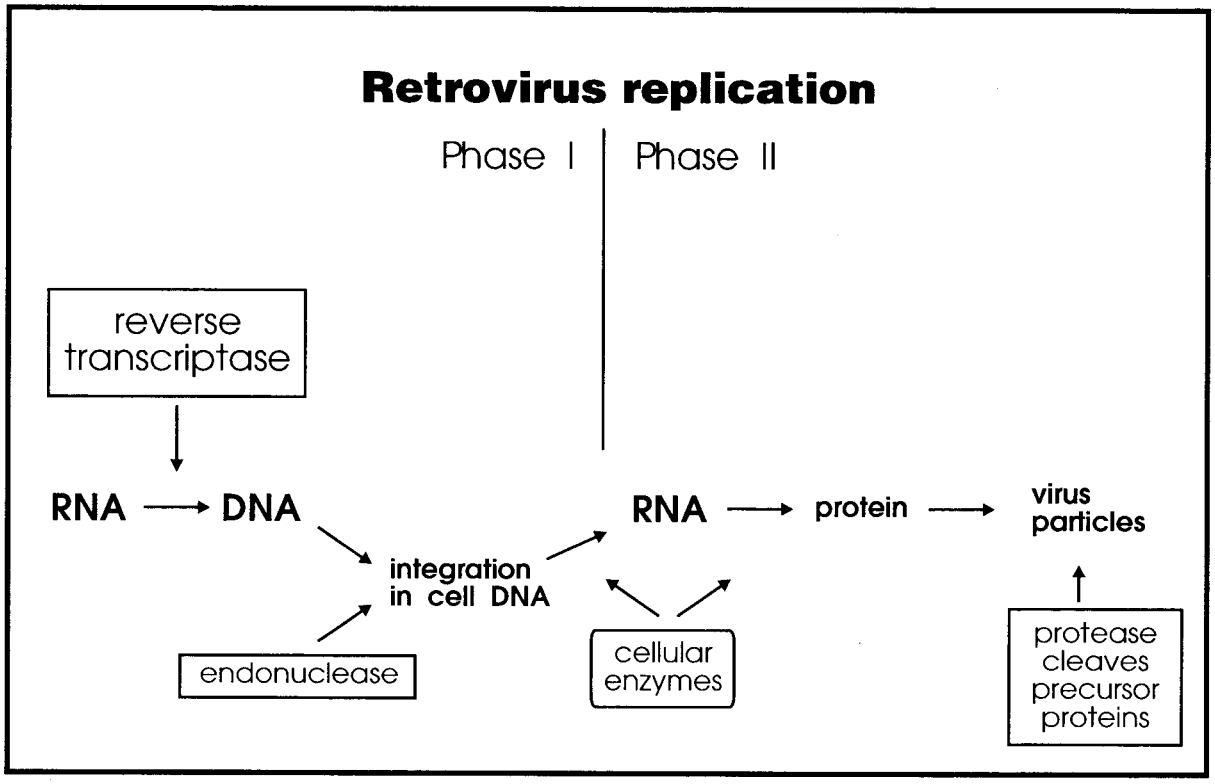
like any other silent cellular gene. When the host cell divides, the proviral gene is transmitted to daughter cells as part of the host cell chromosomes.

Retroviruses are unique in that they can exist in two forms, both as RNA containing virus particles and as host cellular genes. This latter feature enables them to persist in cells of an infected organism.

The integrated provirus may become transcriptionally active. In this phase of the retroviral life cycle, host cellular enzymes (RNA polymerases) carry out transcription of the integrated provirus DNA template, and cellular mechanisms also complete RNA processing and transport to ribosomes in the cell cytoplasm. Similarly, viral protein synthesis, further processing into viral glycoproteins, and transport to the cell membrane – where maturation of viral particles occurs through a process called budding – is carried out by the cell's machinery. A third virus-specific enzyme, a protease, is involved in cleavage of the large precursor proteins into functional capsid, nucleocapsid, matrix and enzymatic proteins. This cleavage occurs during the formation of the viral particle and is 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 asymptomatic HIV infection. For an effective virus infection to occur, HIV DNA has to 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. Retrovirus replication



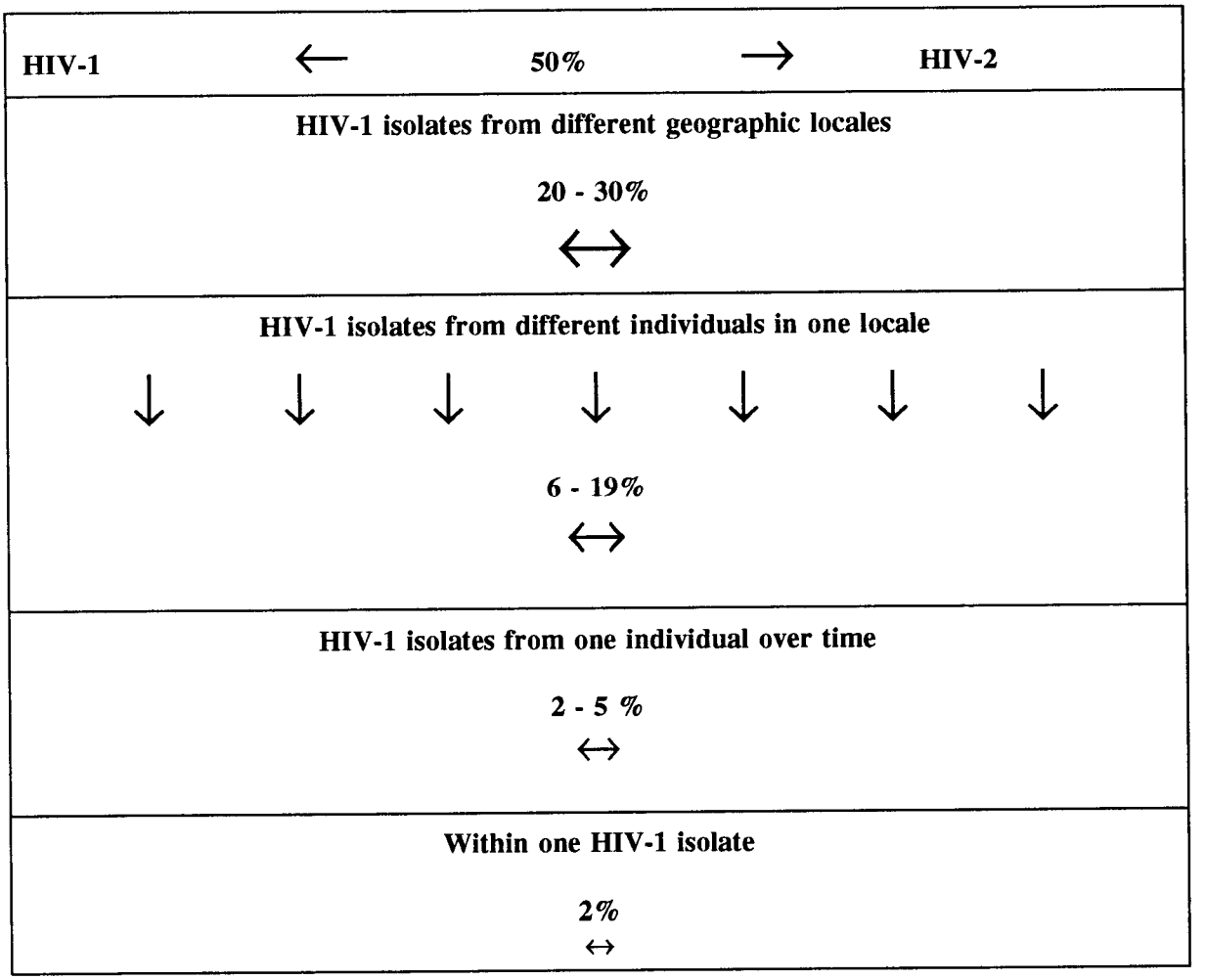
2.3 Genetic, antigenic and biological variability.

(a) Genetic variability

High rates of genetic variability are a characteristic feature of retroviruses. Reverse transcription of RNA to DNA, which occurs each time the virus infects a new cell, has an error rate several orders of magnitude higher than DNA polymerases of animal cells. In fact, the estimated error rate for HIV is one substitution in 10 000 nucleotides synthesized. In addition to substitutions, deletions and insertions can also occur, although the frequency of these errors is more difficult to estimate. The range of genetic variability encountered in HIV is illustrated in Figure 3. HIV type 1 (HIV-1), identified in viral isolates from North America, Europe and Central Africa, has only 50% nucleotide sequence homology with HIV type 2 (HIV-2), a related retrovirus common in West Africa. Variability in the *env* gene of HIV-1 isolates from different individuals in a single region is in the range of 6-19%, although differences higher than 30% have been noted. Isolates collected in geographically distinct locations may be more divergent than isolates collected from a population group within a defined area. Also, people infected with virus from a common source, like a group of haemophiliacs infected through the same HIV-1 contaminated batch of Factor VIII, harbour viruses that are more related to each other than to viruses in the general population. There

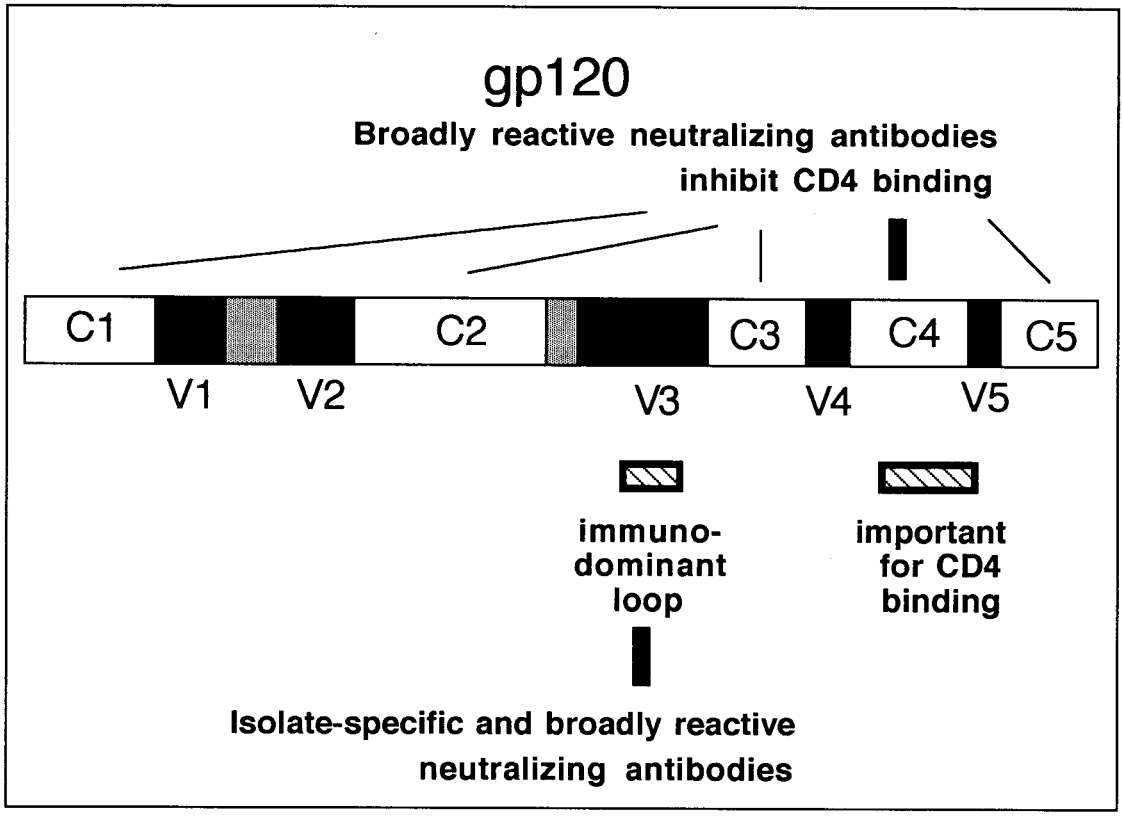
can also be intrapatient variation, observed as change over time and as coexisting closely related variants within one isolate. These variants may be separated by molecular techniques and analysed individually.

Figure 3. Differences in nucleic acid sequence composition



The various parts of the viral genome change at different rates. While the envelope seems to undergo rapid changes, alterations in the *gag* gene, encoding the viral capsid protein for example, are more constrained. 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 particularly attracted attention, since it appears to be an important epitope for eliciting neutralizing antibodies as well as for cell-mediated immune reactions thus far referred to as a principal neutralization domain (PND). Nucleotide changes in the V3 loop are normally substitutions, whereas in the V4 and V5 regions changes are typically deletions and insertions. Changes at the nucleotide level are followed by changes in the amino acid composition of the envelope proteins. Deletions in the V4 and V5 regions may change the distance between constant regions and thereby influence the tertiary structure of the whole 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.

(b) Biological variability

HIV isolates show distinct biological features according to 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 fail or result in only transient replication. In contrast, viruses from subjects with severe immunodeficiency and symptoms replicate rapidly and efficiently in PBMC as well as in cell lines. Hence the designation of slow/low and rapid/high isolates respectively. PBMC infected with slowly replicating viruses contain low levels of viral RNA and conversely cells infected with highly replicative viruses contain high levels of viral RNA. Slow/low and rapid/high

viruses can thus be distinguished at a single cell level. Restriction of viral replication may be imposed, at least in part, at the transcriptional level.

The two viral phenotypes can also be distinguished by their cytopathic effects on PBMC *in vitro*. Rapid/high viruses are characterized by extensive syncytia 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. In individual people, the shift from the slow/low to the rapid/high phenotype may be concomitant with or shortly prior to disease progression. Whether this shift signals the emergence of HIV variants with increased virulence or reflects damage to an immune system that no longer can control viral replication is not yet understood.

Recent attempts to map various biological properties of HIV genome suggest that the structure of the envelope plays an important role in determining replicative capacity as well as the cytopathic effects of the virus.

(c) Antigenic variability

Antibodies are the best tools to dissect relevant immunogenic portions of the viral envelope at present. The antigenic variability of HIV, as reflected by the neutralizing antibody response of the subject to his/her own virus, will be considered. The neutralizing antibody response has been chosen because in other viral infections the appearance of neutralizing antibodies is generally considered to be a sign of protective immunity. Moreover, the protective capacity of a vaccine is usually estimated by its ability to evoke neutralizing antibodies.

In the course of a primary HIV-1 infection, viraemia is followed by seroconversion to different viral antigens (seroconversion). As antibody titre increases and, presumably, cytotoxic T lymphocyte (CTL) activity appears, viraemia decreases, and for a variable length of time only antibodies, but not viral antigen, can be detected in serum of individuals with asymptomatic HIV-1 infection. Neutralizing antibodies to the subject's own virus isolated during the primary HIV-1 infection will appear concomitant with seroconversion. This early antibody is normally isolate-specific and directed to the V3 loop, and 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. Certain selected HIV-1 antibody-positive sera are able to neutralize virus from many HIV-1 infected individuals, i.e. contain broadly reactive neutralizing antibodies. Broadly reactive neutralizing antibodies may react with discontinuous (conformational) as well as continuous (linear) epitopes. The conformational epitope is composed of several constant regions along the gp120 molecule (Figure 4) and the neutralizing activity of antibodies is exerted mainly through inhibition of CD4 binding by viral particles. The linear epitope is the tetrapeptide GPGR that forms the tip of the V3 loop in many HIV-1 isolates.

In summary, an isolate-specific neutralizing antibody response develops early in HIV infection. Even if virus replication is suppressed, HIV will not be eliminated but persists in

the infected host. Low-level replication of variant viruses continues and, with time, may evoke a broadly reactive neutralizing antibody response.

2.4 Approaches to vaccination against HIV/AIDS

(a) Potential uses of HIV vaccines

There are three potential uses of HIV vaccines:

- as classic "preventive" vaccines, to prevent people from becoming infected
- as "therapeutic" vaccines, to be given to people who already have HIV infection, to delay progression of disease
- as "perinatal" vaccines, for vaccinating women with HIV infection who are pregnant or might become pregnant, to prevent perinatal transmission of HIV.

The ultimate goal of HIV vaccine development, and a high priority for WHO/GPA, is to develop safe and effective preventive vaccines. Such vaccines would have the highest public health impact, not only by protecting individuals from HIV infection but also by interrupting different chains of HIV transmission. Development of therapeutic and perinatal vaccines is important, not only because of their own merits but also because of their potential value for the development or improvement of preventive vaccines. A perinatal vaccine could possibly act dually as a therapeutic vaccine in the mother and as a preventive vaccine in the fetus.

(b) Approaches to vaccine production

HIV vaccines could be produced by at least four different approaches, using:

- live attenuated virus
- whole inactivated virus
- isolated viral proteins (or protein subunits)
- live recombinant viral or bacterial vectors expressing immunogenic HIV proteins, or injected "naked" DNA.

Although most viral vaccines used today in humans are based on 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 they are being tested as therapeutic vaccines in volunteers with HIV infection. Whole inactivated and live attenuated vaccines are producing encouraging results in experimental animals, and should not be excluded as potential preventive vaccines for human use. Most of the candidate HIV vaccines being developed are based on immunogenic subunits of HIV produced by recombinant DNA techniques, or on the use of recombinant bacterial or viral vectors expressing HIV proteins. If safety concerns are addressed, live vectors 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 the further potential benefit of inducing mucosal immunity, which may increase immune protection against sexual transmission of the virus.

(c) Phases of HIV vaccine development

Vaccine development proceeds through a well-defined series of preclinical and clinical phases. In the preclinical phase, experimental vaccines are tested in animal models to assess safety, ability to induce immune responses (immunogenicity) and, in some cases, ability to protect against infection with the virus (protective efficacy). Many experimental HIV vaccines are presently being characterized in animals, including whole inactivated virus, attenuated strains, and a number of recombinant vaccines.

Some of these candidate vaccines have already entered phase I trials in humans; phase I trials are designed to evaluate safety and immunogenicity in humans, and include a small number of volunteers. Phase II trials are designed to obtain additional information on safety and immunogenicity, usually including a larger number of volunteers, and to evaluate a number of additional variables such as different doses, routes of administration, adjuvants and duration of immunity – with HIV vaccines phases I and II are usually carried out as a single phase, to assess safety and immunogenicity in a broader sense. Finally, phase III trials are large-scale field trials designed to assess the efficacy of the candidate vaccine in protecting against HIV infection or disease.

(d) Experiments with animal models

Current optimism regarding the development of a safe and effective HIV vaccine has arisen from the demonstration of protection induced in immunized animals by experimental vaccines. Two animal model systems have been extensively used for HIV vaccine development purposes:

- simian immunodeficiency virus (SIV) infection of macaque monkeys
- HIV infection of chimpanzees – the only nonhuman primate which can be consistently infected experimentally with HIV-1.

Macaques have also been used to evaluate HIV-2 vaccine approaches.

In the SIV/macaque model, protective immunity has been achieved by using vaccine preparations containing complete virus particles rendered inactive by chemical or physical treatments. Macaques have also been protected by vaccines based primarily on the envelope glycoprotein gp160. Protection of macaques has also been achieved with live attenuated viruses. On the other hand, chimpanzees could not be protected by whole inactivated HIV, although protective immunity has been induced using vaccines based on the envelope glycoproteins gp120 and gp160, sometimes using boosting immunizations with synthetic peptides representing the V3 loop.

Interestingly, immunization has sometimes failed to protect against SIV infection in the macaque model, but was effective in slowing disease development, a concept that cannot

be tested in the HIV/chimpanzee model since HIV-infected chimpanzees do not appear to develop AIDS. Such observations could have important implications in the development and evaluation of HIV vaccines in humans.

An important requirement for an effective HIV vaccine is that it should induce protection against infection by different strains (heterotypic protection). It is encouraging that experimental immunization of macaques has protected them against strains other than those used in vaccine preparation.

Another important requirement for an effective HIV vaccine is that it should protect against sexual transmission of the virus, perhaps by inducing mucosal immunity. Protection against mucosal virus exposure in the SIV/macaques model has been reported, but remains to be confirmed and extended to the HIV/chimpanzee model. Reports of protection against a cell-associated challenge (using virus-infected cells rather than free virus, which may more accurately represent the way HIV is transmitted), in both SIV and HIV models, are also encouraging.

The importance of neutralizing antibodies in protective immunity has been confirmed by the fact that passively-transferred antibodies have protected macaques (with polyclonal antibodies) and chimpanzees (with anti-V3 monoclonal antibodies) against challenges with cell-free SIV and HIV, respectively. No clearcut correlation has been found between protection and the level of neutralizing antibodies in macaques vaccinated with SIV vaccines. However, such a correlation seems to exist in the HIV/chimpanzee model, with antibodies against the principal neutralization domain (V3 loop). The observation of a correlation between protection and the presence of antibodies against contaminant human host cell components present in whole inactivated virus preparations is disturbing, suggesting that at least some of the protection observed in the SIV/macaque experiments was probably not due to virus-specific immune responses.

(e) Preparation for phase III efficacy trials of preventive candidate vaccines

Although a number of candidate vaccines tested in phase I/II trials have been found to be safe and immunogenic in those without HIV infection, there is no general agreement on the requisite criteria to enter preventive candidate vaccines into large-scale efficacy trials. Presumably, these requirements might include one or more of the following criteria:

- demonstration of protective immunity in animal models
- induction of long-lasting, high-titred, humoral and/or cellular immune responses
- cross-immunity against heterotypic strains of HIV
- induction of mucosal immunity, protecting against sexual transmission of HIV.

New vaccine designs and antigen delivery systems may both help to address the above requirements and to develop vaccines which would be appropriate for use in developing countries (e.g. thermostable and easy to administer, perhaps by the oral route, thus stimulating mucosal immunity).

Criteria for commencing efficacy trials should not only encompass characteristics of the vaccine product itself, but also the implementation of study designs that are appropriate to the population in which the vaccine is to be evaluated. A major international effort is being made by WHO and other international HIV vaccine research programmes to identify, and when possible, prepare appropriate study populations in both developed and developing countries, where efficacy trials could be conducted with high scientific and ethical standards.

(f) The WHO Strategy for HIV Vaccine Development

All of the above considerations are addressed by the WHO Strategy for HIV Vaccine Development, which includes three major components:

- support for the development of appropriate vaccines, especially for use in developing countries

- evaluation of candidate vaccines, emphasizing the strengthening of field sites in developing countries

- ensuring the worldwide availability of safe and effective vaccines for public health use.

WHO is presently working with the national authorities and scientists from Brazil, Rwanda, Thailand and Uganda to implement WHO-sponsored comprehensive national plans for HIV vaccine research in these four countries. The national plans have four major components:

- virus isolation and characterization (part of the WHO Network for HIV Isolation and Characterization)

- clinical trials, initially to repeat phase I/II trials of selected candidate vaccines

- epidemiological research, especially the establishment or adaptation of specific population cohorts for efficacy trials

- research on the social and behavioural aspects of participation in HIV vaccine trials.

The national plans have been developed with the ultimate goal of testing the efficacy of preventive vaccines, although the evaluation of therapeutic and perinatal vaccines is also included as part of the WHO strategy.

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 the result of penetrating injuries caused by sharp objects and 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 and must be continually strengthened. 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 your hands with soap and water after removing gloves.
- Never cross your 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.

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[1] *Biosafety guidelines for diagnostic and research laboratories working with HIV.* Geneva, World Health Organization, 1991 (WHO AIDS Series, No. 9).

(b) **Serological laboratories**

Laboratory facilities

- It is desirable to have a laboratory (or laboratory room) devoted exclusively to work 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 have 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 your 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 use mouth pipetting.**

- 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 apply to virus isolation laboratories, but they should be strengthened. **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 work with HIV-contaminated material.

- Biological safety cabinets (class I or II) are the equipment of choice for work with HIV isolation. 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
- 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% 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% 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 waste[1]

- 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.

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

- 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.

4. 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 identified groups 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 be able to carry out serological tests for diagnosis of HIV infection. Enzyme-linked immunosorbent assay (ELISA) and Western blot tests 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 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 with the Western blot test. 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.

5. Specimen Collection

Experience accumulated over the last eight years indicates that blood is the best source for HIV isolation. 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.

5.1 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). Heparin should be avoided since it may have an antiviral effect and may inhibit the Taq polymerase used in subsequent sample analysis.

For venepuncture the following equipment can be used:

(a) Safety-Monovette used according to the vacuum principle. Ten millilitre 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.

5.2 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):[1]

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.

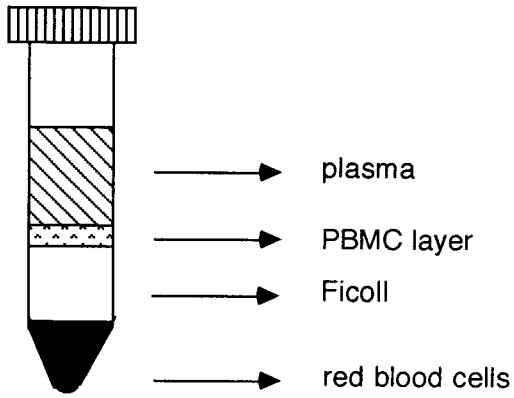
Ficoll and phosphate-buffered saline (PBS) should be prewarmed to 18-22°C before use.

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

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

[1] 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 has to be 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. Centrifugation of 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 µl). Count at least 5 small grids within different quadrants of the counting grid and calculate sample cell number (average count/grid x 90 = cells/µl sample). See Annex 3 for further details.

7. Distribute cells into 15-ml tubes, 5 x 10^6 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 10^6 cells for this purpose (see section 7 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.

8. Resuspend cells in 1 ml fetal calf serum (approved by the Food and Drug Administration, USA) containing 10% dimethyl sulfoxide (DMSO).[1]

9. Label the 1-ml cryotubes with date, name of site and subject identification number.

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 6, 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 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.)

6. Shipment of Specimens[2]

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.

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

[2] For further details, see Madeley, C.R. *Guide to the collection and transport of virological specimens (including chlamydial and rickettsial specimens).* Geneva, World Health Organization, 1977.

6.1 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.

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 (Annex 4)

   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.

6.2 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 for shipment of blood samples above.

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

CO_2 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 for blood samples above.

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.

7. HIV Isolation

The recommended HIV isolation procedure involves coculture of subject PBMC with phytohaemagglutinin-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.

7.1 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 5.

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.

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 x 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-cm^2 tissue culture flasks (sterile, translucent, screw cap). Add 40-100 ml RPMI medium to adjust cell concentration to 1 x 10^6 cells/ml. Add 100 µl PHA to each flask (to a final concentration of 2.5 µg PHA/ml).[1] 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% CO_2), 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 5. 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 you will need.

2. Transfer cells to a 50-ml centrifuge tube. Add 10 ml 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.

[1] The final concentration may be increased up to 10 µg PHA/ml. The optimal concentration of PHA used may be determined in each laboratory.

7.2 Virus isolation

Day 1: Mix 2 x 10^6 subject PBMC with 10 x 10^6 PHA-stimulated blood donor PBMC (preferably from two donors) in a 25-cm^2 tissue culture flask and add up to 10 ml 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 x 10^6 PHA-stimulated donor PBMC (preferably from two donors) is cultured in IL-2 medium and culture fluids regularly harvested and tested for p24 antigen.

Day 4: Half of the volume of culture medium is exchanged for fresh medium.

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 10^6 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 10^6 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-X100. Samples may be stored at -20°C prior to testing.

Following the harvest of medium, cultures can be observed microscopically. Note: Keep flasks 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 p24 antigen ELISA, reverse transcriptase assay and immunofluorescence on fixed cells see Annex 3.

8. Preliminary Biological Characterization of HIV Isolates

Knowledge about the biological characteristics, replicative capacity and cytopathogenicity of the virus isolates facilitates antigenic characterization by virus neutralization. Two kinds 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 adequate 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 with 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, remove the mycoplasma from cell lines.

8.1 Primary characterization

The virus isolation procedure as outlined in section 7 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 regularly observed 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 people infected with HIV-1 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*. Virus production is not only 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, 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. The slow *in vitro* replication of the majority of HIV isolates makes it important that virus-negative cultures be continued for four weeks; they may be exceptionally extended to 5 weeks.

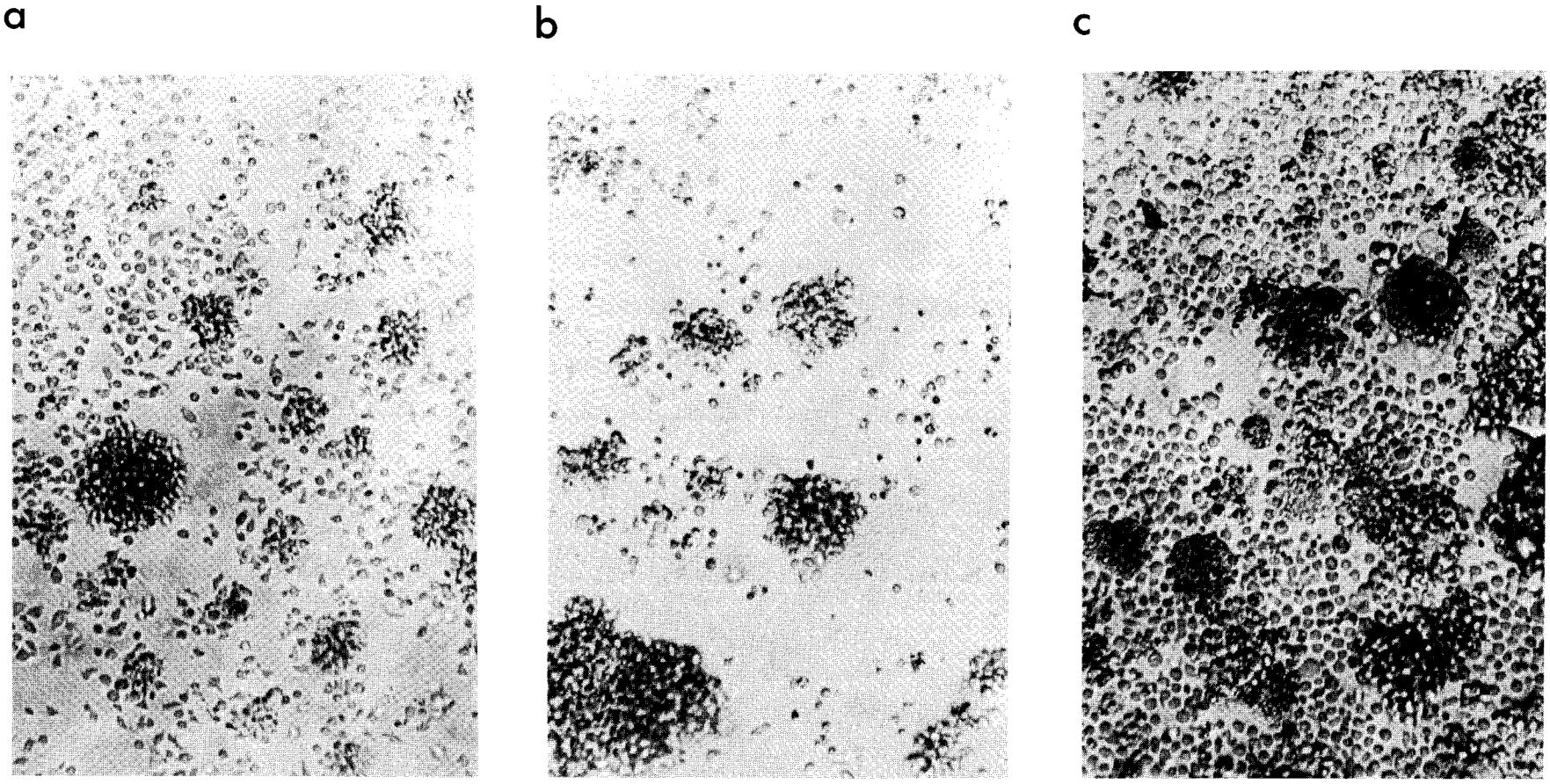
Viruses replicating in PBMC cultures display distinct cytopathic characteristics. Accordingly, slowly replicating viruses may not display any cytopathic effect at all. Some slow/low isolates form small syncytia sparsely present in PBMC cultures (Figure 6a), other isolates kill single cells (Figure 6b). Syncytia formation and single cell killing may occur in the same cultures. Extensive syncytia formation accompanies replication of rapid/high viruses (Figure 6c). 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, slow/low and rapid/high HIV isolates have also been named non-syncytia-inducing (NSI) and syncytia-inducing (SI), respectively. Since the

cytopathic effects may be slight and in some cases transient, cultures have to be observed twice a week for the entire isolation period.

8.2 Extended biological characterization

This involves cell-free transfer of viruses to donor PBMC and cocultivation of infected PBMC with cell lines. The virus dose can be standardized on the basis of p24 antigen content, 1 ng/10^6 PBMC or on the basis of reverse transcriptase activity, 20 000 cpm/10^6 PBMC. In both cases, a productively infected culture will result 6-10 days after infection with most isolates. PBMC 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 has been performed in donor PBMC following cell-free transmission of virus.

The first peak of virus production is also the time when cocultivation with cell lines should be performed. This procedure – cocultivation of virus producer PBMC cultures with cell lines – will to some extent circumvent the difficulties in quantifying infectious viruses and will allow classification of HIV isolates into rapid/high (= replication in cell lines) and slow/low (= no or transient replication in cell lines). For details see Annex 3.



Figures 6a, 6b and 6c

Cytopathic effects of HIV-1 isolates in PBMC cultures. a and b: slow/low virus; c: rapid/high virus. Photograph taken at peak of virus replication. Phase contrast, magnification x 250. a: shiny living cells, single or in clumps, appearance as in virus negative cultures. Two small syncytia can be observed. b: pyknotic single cells as well as dead cells in clumps can be observed. Dead cells and cell fragments have a dark appearance. Tendency for "ballooning" at the surface of clumps. c: extensive syncytia formation, with marked "ballooning" at the edge of clumps.

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 allows 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
CO_2 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

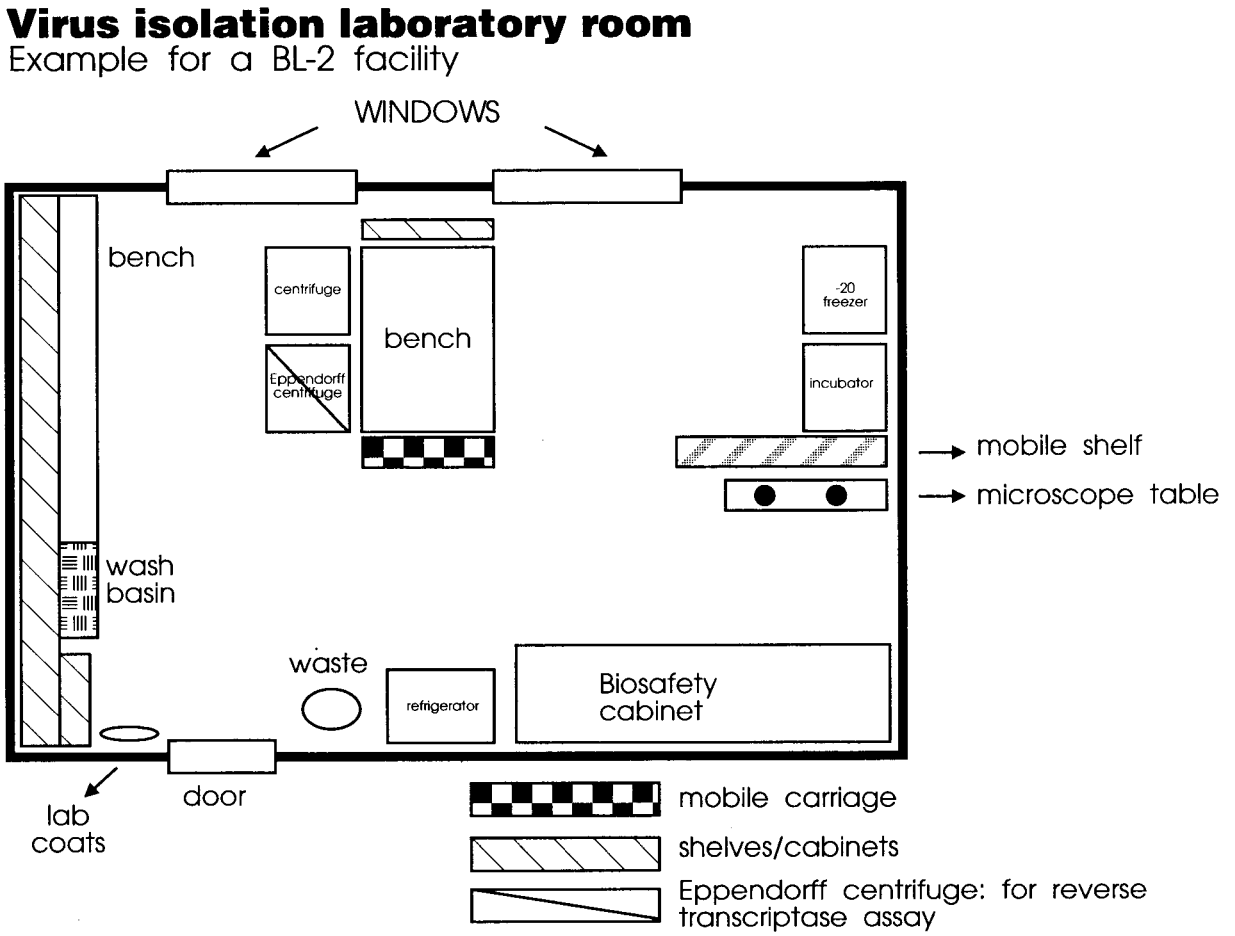
Equipment available in the same building:

Autoclave
Freezer, -70°C
Freezer, -140°C or liquid nitrogen
ELISA reading apparatus
Immunofluorescence microscope

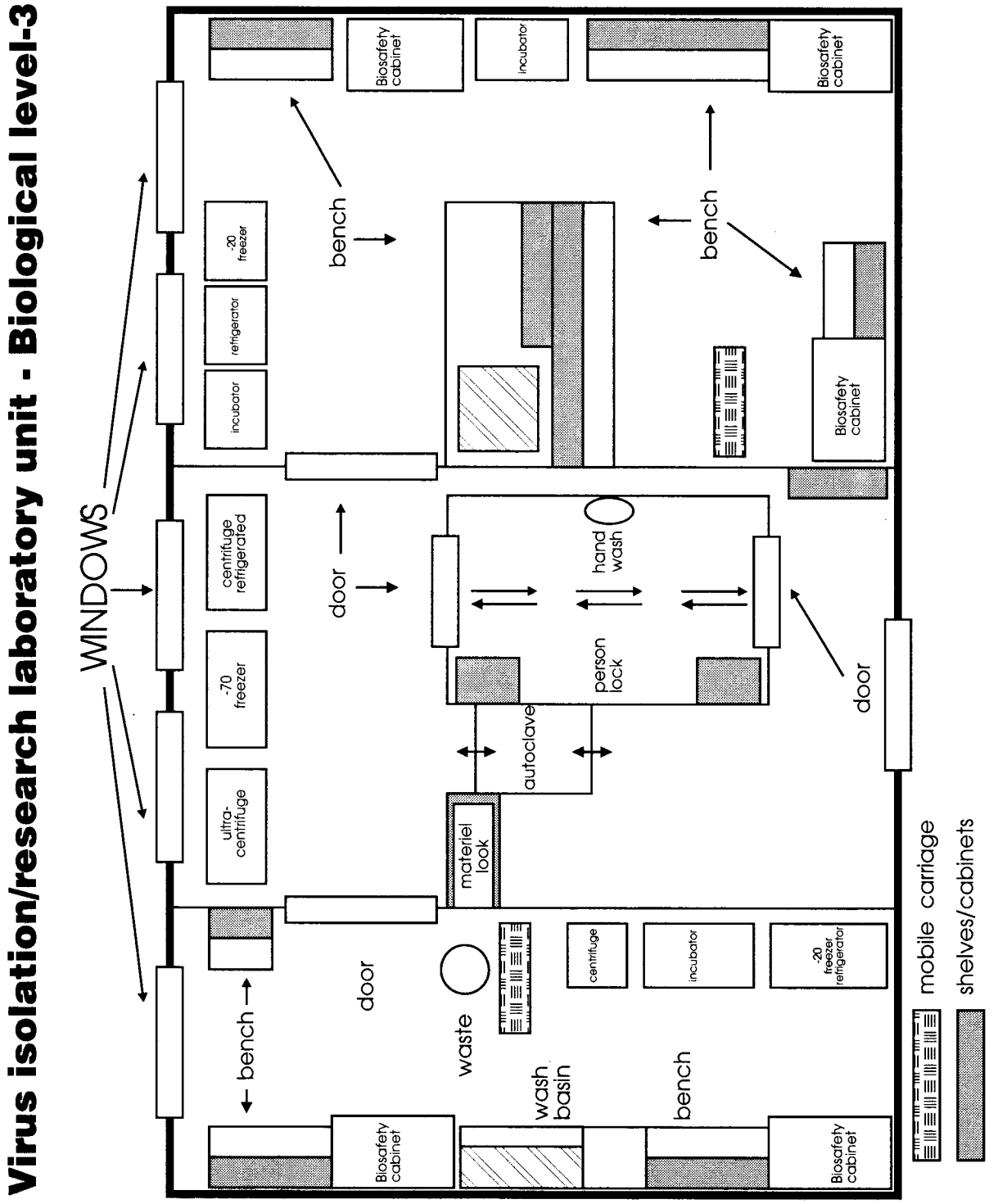
Supplementary equipment for research laboratories:

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

Design of a virus isolation laboratory room BL-2



Design of a virus isolation laboratory unit BL-3



Annex 2

Subject data form

WORLD HEALTH ORGANIZATION
GLOBAL PROGRAMME ON AIDS
VACCINE DEVELOPMENT UNIT / OFFICE OF RESEARCH
HIV ISOLATION AND CHARACTERIZATION NETWORK

PLB
Page 1

WHO ID Number
Collection Number

GPA/RES Number
Form Code: V 0 1 / P L B

EPIDEMIOLOGICAL DATA

1. (a) Group
   1 hospitalized (specify where)
   2 prenatal consultation (specify where)
   3 other consultation (specify where)
   4 blood donor (specify where donation made)
   5 cohort (specify)
   6 other (specify)

   (b) Specify _____

   (c) Group ID Number

2. Sex
   1 male
   2 female

3. Date of birth (day month year)

4. Age (years)

5. Place of birth

6. (a) Place of residence

   (b) Urban/rural residence
   1 urban
   2 semi-rural
   3 rural

7. (a) Presumed transmission
   1 sexual (go to Q.7(b))
   2 parenteral (go to Q.7(c))
   3 perinatal (go to Q.7(d))
   4 unknown

   (b) If sexual transmission
   1 heterosexual
   2 bisexual
   3 homosexual

   (c) If parenteral transmission
   1 haemophiliac
   2 blood transfusion
   3 IV drug user
   4 other

EPIDEMIOLOGICAL DATA (cont'd)

(d) If perinatal transmission, Mother's WHO ID Number

8. Date of last seronegative sample (day month year)

9. Date of first seropositive sample (day month year)

CLINICAL STATUS

10. (a) Presumed acute infection
    1 no
    2 yes

    (b) If yes, date (day month year)

11. Clinical conditions

12. Stage according to WHO HIV infection staging system
    1 Stage 1 Asymptomatic
    2 Stage 1 PGL
    3 Stage 2 Symptomatic early
    4 Stage 3 Moderate disease
    5 Stage 4 AIDS

13. Antiviral treatment
    1 no
    2 yes

ELIGIBILITY

PERSONS WITH SYMPTOMATIC INFECTION OR TREATED WITH ANTIVIRALS ARE NOT ELIGIBLE

14. Eligible
    1 no
    2 yes
    If no, do not collect blood

15. Consented to donate blood for research purposes
    1 no
    2 yes

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PLB
Page 2

WHO ID Number ☐☐☐☐ Collection Number ☐☐☐☐☐☐☐

LABORATORY HISTORY

HIV1

	First / Only Test	Second Test
16. ELISA (a) Date	day ☐☐ month ☐☐ year ☐☐	day ☐☐ month ☐☐ year ☐☐
(b) Specify kit:	☐☐	☐☐
(c) Result 1 negative 2 positive 9 not done	☐	☐
17. Western Blot (a) Date	day ☐☐ month ☐☐ year ☐☐	day ☐☐ month ☐☐ year ☐☐
(b) Specify kit:	☐☐	☐☐
(c) Result 1 negative 2 positive 3 indeterminate 9 not done	☐	☐
(d) Bands 1 not present 2 present 9 not done	gp160 ☐ gp120 ☐ p55/p61 ☐ gp41 ☐ p24 ☐ p17 ☐	gp160 ☐ gp120 ☐ p55/p61 ☐ gp41 ☐ p24 ☐ p17 ☐

HIV2

18. ELISA (a) Date	day ☐☐ month ☐☐ year ☐☐	day ☐☐ month ☐☐ year ☐☐
(b) Specify kit:	☐☐	☐☐
(c) Result 1 negative 2 positive 9 not done	☐	☐
19. Western Blot (a) Date	day ☐☐ month ☐☐ year ☐☐	day ☐☐ month ☐☐ year ☐☐
(b) Specify kit:	☐☐	☐☐
(c) Result 1 negative 2 positive 3 indeterminate 9 not done	☐	☐
(d) Bands 1 not present 2 present 9 not done	gp140 ☐ gp105 ☐ p56/p68 ☐ gp36 ☐ p26 ☐ p16 ☐	gp140 ☐ gp105 ☐ p56/p68 ☐ gp36 ☐ p26 ☐ p16 ☐

HIV1/2

20. ELISA (a) Date	day ☐☐ month ☐☐ year ☐☐	day ☐☐ month ☐☐ year ☐☐
(b) Specify kit:	☐☐	☐☐
(c) Result 1 negative 2 positive 9 not done	☐	☐

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PLB
Page 3

WHO ID Number

Collection Number

LABORATORY HISTORY (cont'd)

21. HIV1 p24 antigen
    (a) Date — day month year
    (b) Result
        1 negative
        2 positive
        9 not done

22. CD4 cell count
    (a) Date — day month year
    (b) Absolute number / mm3
    (c) Percent

23. CD8 cell count
    (a) Date — day month year
    (b) Absolute number / mm3
    (c) Percent

24. Total lymphocyte count
    (a) Date — day month year
    (b) Absolute number / mm3

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

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 — ml / 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 — ml / vials

REMARKS

Form-filling instructions

WHO HIV ISOLATION AND CHARACTERIZATION NETWORK

Form-Filling Instructions for Primary Laboratories

NOTE: SHADED BOXES SHOULD NOT BE CODED

Page 1

WHO ID Number

Use consecutive numbers, starting with 0001. A special "WHO HIV Isolation and Characterization" logbook should be kept, showing the person's WHO 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. 92), 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 Asymptoniatic
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 leucoencephalopathy
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 PGL (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 only 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 ID Number and Collection Number are entered at the top of Page 2 of the form.

Laboratory History

Q.16-Q.20 If available, enter the results of HIV testing on recent blood samples. If results are available 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 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-Q.24 If available, enter the dates and results of the most recent CD4 cell counts, CD8 cell counts 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-Q.32 After separation record the amount of serum, plasma and cells saved.
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 Q.38 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. A second copy of the form should be sent to Vaccine Development Unit at the address below.

Addresses

1. World Health Organization:

Vaccine Development Unit
Research and Intervention Development
Global Programme on AIDS
World Health Organization
1211 Geneva 27
Switzerland

Fax : (41) (22) 7910746
Telephone: (41) (22) 7914392

2. HIV Isolation Laboratory/Repository

2.1 Prof. Helga Rubsamen-Waugmann
Chemotherapeutisches Forschungsinstitut Georg-Speyer-Haus
Paul Ehrlichstrasse 42-44
D-6000 Frankfurt 70
Germany.

2.2 Dr Harvey Holmes
National Institute for Biological Standards and Control
Blanche Lane
South Mimms
Potters Bar
Hertfordshire EN6 3QG
U.K.

Annex 3

Solutions

Anticoagulants:

EDTA-stock solution: 40mM (pH 8.0)

Add 14.9 g of disodium ethylendiaminetetraacetate ($2H_2O$) 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. Dispense into aliquots 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 ($2H_2O$) in 1 litre H_2O. Dispense into aliquots and sterilize by autoclaving.

Phosphate-buffered saline (PBS):

	g/per litre
NaCI	8
KCI	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 HCI. Add H_2O to 1 litre. Dispense the solution into aliquots 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 µl 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 10 ml deionized (or distilled) H_2O and sterilized by filtration through a 0.22 mikron 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 have consistent quality over extended periods. 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.

Reagents used in the reverse transcriptase assay

Use double-distilled (dd) or Milli-Q purified H_2O for preparation of all solutions.

VDB (virus disruption buffer)

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, KCI, DTT and EDTA in 80 ml dd H_2O and adjust pH to 7.6 with HCI. Add 12.5 ml 10% Triton-X 100 and adjust volume to 100 ml with dd H_2O. Dispense into 5-ml and 1-ml aliquots and store at -20°C.

Salt mix

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

Dissolve in 90 ml dd H_2O. Adjust pH to 7.6 with HCI. Adjust volume to 100 ml with dd H_2O. Dispense into 2-ml aliquots and store at -20°C.

$MgCl_2$ solution 200 mM

Dissolve 0.8 g $MgCl_2$ in 10 ml dd H_2O. Dispense into 0.3 ml aliquots and store at -20°C.

Poly (rA) 40 µg/ml

Dissolve 400 µg PrA in 10 ml dd H_2O. Dispense into 0.3 ml aliquots and store at -20°C.

Oligo (dT_{12-18}) 1 unit/ml

Dissolve 5 units in 5 ml dd H_2O. Dispense into 0.3-ml aliquots and store at -20°C.

BSA (Bovine serum albumin) 5 mg/ml

Dissolve 50 mg BSA in 10 ml dd H_2O. Dispense into 0.3-ml aliquots and store at -20°C.

Yeast RNA 4 mg/ml

Dissolve 40 mg in 10 ml dd H_2O. Dispense into 0.3-ml aliquots and store at -20°C.

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 or 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 µl 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 H_2O with gentle agitation.

5. Counterstain in Evans' blue for 3 minutes.

6. Rinse slides in distilled H_2O. Take away 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 "macroassay" uses tubes, 25-mm filter papers for collection of TCA (trichloroacetic acid) precipitates from individual samples and a standard scintillation ß emission counter. (b) 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.

Sample preparation.

1. Collect 1 ml supernatant culture fluids 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 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_2O	124
	500

* A PrA·OdT mixture is available commercially. It should be diluted with dd H_2O to 1 unit/ml and frozen in aliquots at -20°C. Use 100 µl from this solution for 10 samples.

$MgCl_2$: the HIV reverse transcriptase requires magnesium ions (Mg^{++}) to function optimally; PrA: poly (rA), provides a synthetic template; OdT: oligo (dT_{12-18}) provides a synthetic primer to be elongated by incorporation of ^{3}H-dTTP (tritiated deoxythymidine 5-triphosphate, specific activity 79.6 Ci/mmol, conc.: 1 mCi/ml).

Method A

6. 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.

7. Tap the sample tubes to ensure mixing before removing 50 µl. Add 50 µl sample to tubes (50 µl VDB to the negative controls).

8. Add 50 µl RT-mix to each tube.

9. Cap tubes, shake rack to ensure mixing. Incubate at 37°C for 60 minutes.

10. 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.

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, pan 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 ß emission.

Method B

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 50 µl RT-mix to each well. Use a multichannel pipette or a serial dispenser 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.

Extended biological characterization

Infection of donor PBMC

1. Virus stocks (the supernatant culture fluids from primary PBMC cultures) are stored at -70°C. 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 and calculate how many cells you need in total. 3 x 10^6 PBMC is optimal for a starting culture in 10 ml IL-2 medium using a 25-cm^2 flask. (If necessary, cell number per culture may be increased to 5 x 10^6 cells; increase also the volume of medium to 15 ml).

3. Transfer 3 x 10^6 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 10^6 cells in a volume of 0.5-1 ml will give optimal results. (Amount of virus added may, however, vary between 5 000 and 50 000 cpm per 10^6 PBMC.) Mix cells and virus thoroughly by gently tapping the tube.

5. Tubes should then be incubated for a minimum time of one hour in a humidified 5% $C0_2$ atmosphere at 37°C. Incubation may be prolonged overnight, but in such a case increase volume by addition of 1 or 2 ml (depending on cell number) Il-2 medium to each tube.

6. Following incubation, pellet the cells by centrifugation at 1200 rpm (225 x *g*), remove the virus containing supernatant 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-cm^2 tissue culture flask. Place flasks upright in a humidified 5% $C0_2$ atmosphere at 37°C.

8. Culture medium should be harvested twice a week as follows:

   - 3.5 ml for storage at -70°C (extended virus stock)
   - 1.2 ml for reverse transcriptase assay
   - 0.9 ml for p24 antigen ELISA.

9. Depending on cell number and strength of cytopathic effect, cultures may be split 1:2 or 1:3. If necessary, add 3 x 10^6 fresh blood donor PBMC once a week. Restore culture volume to approximately 10 ml with IL-2 medium.

Cocultivation of PBMC with cell lines

If infected in the standard way, PBMC cultures become productively infected with most HIV isolates 7-10 days post infection. Cocultivation with cell lines can then be performed. It is recommended that three cell lines be used for each virus: a T-lymphoid cell (HuT-78, H9, CEM, Jurkat etc.); a monocytoid 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 the transactivator protein of HIV-1) supports replication of all HIV isolates that also replicate in PBMC. Jurkat-tat is therefore an essential control for slow/low viruses that otherwise do not replicate in cell lines.

For cocultivation, use 1 x 10^6 cells of a cell line.

1. Harvest medium from PBMC cultures, as usual. In the remaining culture volume the approximate cell concentration will be 10^6 cells/0.5 ml.

2. Count cultures of cell lines to be used. Dispense 3 x 10^6 cells into labelled new 25-cm^2 flasks.

3. Transfer 0.5 ml of 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% $C0_2$ atmosphere at 37°C.

6. Harvest culture fluids twice a week and test for presence of virus by reverse transcriptase assay or p24 antigen ELISA. Cultures can be split 1:2-1:10, depending on the cell line and the strength of cytopathic effect. Restore culture volume to 10-12 ml each time.

7. Cultures which are virus-positive on three consecutive testings can be discontinued. Virus-negative cultures should be regularly tested for 28 days and then discarded (see "practical considerations" in section 7).

All cell lines used 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. Each cell line used should be tested monthly.

Mycoplasma-contaminated cultures must be treated with antibiotics. Antibiotic combinations for this purpose are commercially available. Treated cultures should be retested for mycoplasma to confirm the success of treatment. If negative, freeze several ampoules in liquid nitrogen to have a supply of mycoplasma-free cells. Do not keep cell lines in continuous passage. Thaw a new ampoule every two months.

Subject list

Name ..... Clinical Symptoms ..... Birth Date/Code No										Serum Antigen Test ..... Absorbance .... Cut-off .....
Sample no ..... Type ..... Volume ..... Anti coagulant..... ..... Date	Date									Cell number..... Total ..... In culture ..... Frozen ..... Comments..... .....
	Antigen ELISA									
	RT									
Sample no..... Type ..... Volume..... Anti coagulant .... Date	Antigen ELISA									Cell number ..... Total ..... In culture ..... Frozen ..... Comments ....
	RT									
Sample no ..... Type..... Volume ..... Anti coagulant .... Date	Antigen ELISA									Cell number ..... Total..... In culture ..... Frozen ..... Comments..... .....
	RT									

Protocol for harvesting of virus isolation cultures

Date of harvest: ..... HIV-antigen test: ..... Plate no. .....

Well no.	Sample no.	CPE	PBMC	FREEZE	SPLIT	Results of HIV-antigen test	RT	Comments on culture conditions
1A-B	Blank							
1C-D	*							
1E-F	*							
1G-H	*							
2A-B	*							
2C-D	*							
2E-F	*							
2G-H	Negative							
3A-B								
3C-D								
3E-F								
3G-H								
4A-B								
4C-D								
3E-F								
3G-H								
4A-B								
4C-D								
4E-F								
4G-H								
5A-B								
5C-D								
5E-F								
5G-H								
6A-B								
6C-D								
6E-F								
6G-H								

* Antigen dilution in 6 2-fold dilution steps

Protocol for harvesting of virus isolation cultures

Date of harvest: ..... HIV-antigen test: ..... Plate no. .....

Well no.	Sample no.	CPE	PBMC	FREEZE	SPLIT	Results of HIV-antigen test	RT	Comments on culture conditions
7A-B								
7C-D								
7E-F								
7G-H								
8A-B								
8C-D								
8E-F								
8G-H								
9A-B								
9C-D								
9E-F								
9G-H								
10A-B								
10C-D								
10E-F								
10G-H								
11A-B								
11C-D								
11E-F								
11G-H								
12A-B								
12C-D								
12E-F								
12GH								

Annex 4

Warning label proposed by WHO for consignments of infectious substances, including virological material

White background **black symbol and lettering**

Dimensions: 10 cm x 10 cm

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