PN-ABN-707 81651

HIV Testing & Quality Control

A Guide for Laboratory Personnel

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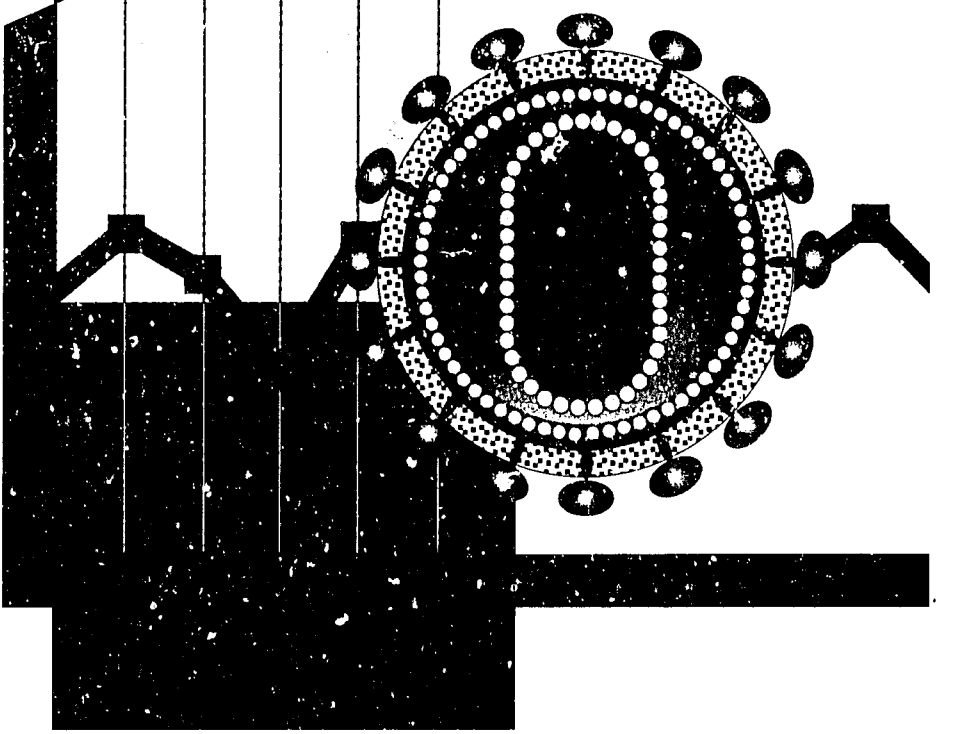

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A Guide for Laboratory Personnel

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AIDSTECH

1991
Family Health International
Durham, North Carolina, USA

The views expressed in this Guide are those of the authors and not necessarily those of the organizations with which they or the editors are affiliated.

Support for the publication of this Guide was provided by AIDSTECH/ Family Health International with funds from the United States Agency for International Development (USAID). Additional support was provided by the United States Naval Medical Research and Development Command, Bethesda, Maryland, Work Unit No. 3M463105.H29.AA335.

ISBN 0-939704-07-2
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ACKNOWLEDGMENTS

The authors, editors, and publisher wish to acknowledge the indispensable contributions of the following persons, who provided a thorough and thoughtful technical and editorial review of this publication: Dr. Guido Van der Groen of the Institute of Tropical Medicine, Antwerp, Belgium; Dr. Hiko Tamashiro of the Global Programme on AIDS, World Health Organization, Geneva, Switzerland; Dr. Debrework Zewdie, National Research Institute of Health, Addis Ababa, Ethiopia; and Debra Hanson, AIDSTECH/Family Health International, Durham, North Carolina, USA.

Text design and layout were provided at Family Health International by Chad Adams. The cover design was conceived and rendered by Dick Hill and Niel Constantine.

Crucial initial and ongoing professional and administrative support for this publication was provided by Dr. Peter Lamptey, Director of Family Health International's AIDSTECH division.

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PREFACE

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Laboratory services are an essential component in the diagnosis and treatment of persons infected with the human immunodeficiency Virus (HIV); in HIV screening of blood for transfusion; in surveillance for HIV infection; and in HIV/AIDS related research. Thus, accurate and cost-effective testing is of great importance in combating the spread of HIV. It is imperative that tests for diagnosis of HIV infection be as accurate as possible, given the serious ethical, legal and social issues that accompany HIV infection. Because of the lack of effective treatment and the necessity to prevent further spread of infection, counselling and confidentiality play an important role in HIV testing programmes used for diagnosis.

In this book, the authors describe concisely a series of issues related to HIV testing including a brief description of the virus and its immunology. Readers will also find guidance for overcoming economic and logistic problems associated with HIV testing without compromising diagnostic standards. This information is especially important for laboratories with limited resources.

The first commercially available HIV test was the enzyme-linked immunosorbent assay (ELISA), which was licensed for use in blood transfusion services in 1985. This test is still the most frequently used method for detecting HIV-specific antibody. The ELISA for HIV antibody was developed with unprecedented speed for any modern test: from the time the virus was isolated in cell culture, it took less than two years for the ELISA to be licensed.

Other forms of widely available assays such as agglutination and immunodot tests are simpler then ELISA, more rapid to perform and require no instrumentation. However, these tests, especially immunodot tests, are generally more expensive than the ELISA and are performed on individual serum rather than on groups of specimens. They are therefore more appropriate for use in peripheral laboratories which receive few specimens for testing, as compared to the ELISA system which is more appropriate for screening multiple specimens as required in blood banks or in HIV seroprevalence studies.

Although tests used for screening for HIV antibody are highly sensitive, specific and reproducible, none are free from false reactions. This poses a dilemma in interpreting test results, particularly in low HIV-prevalence areas: it is recommended that for patient diagnosis and research studies, sera that are reactive in a screening test be re-tested (or "confirmed") with a more specific verification (supplementary) assay. On the other hand, supplementary testing is usually not indicated for antibody screening for blood transfusion and surveillance. Alternative strategies for screening and supplementary testing are presented in this book. Their choice should be carefully considered especially in situations where there are limited resources.

Currently available HIV screening and supplementary tests have high sensitivity and specificity, but the quality of testing depends not only on their intrinsic performance characteristics, but also on the proficiency of laboratory technicians performing the test. Consistent standards assuring high quality of HIV testing remain to be established, especially in developing countries where laboratory infrastructure is not well developed. Many national HIV reference laboratories have enrolled in the WHO External Quality Assessment Programme, and results from this program suggest the performance of HIV tests at this level is good.

The proficiency of laboratories and technicians varies widely from laboratory to laboratory, and the need for quality assurance mechanisms at all levels within a country is critical. This book addresses in detail a series of important issues on quality assurance, offering practical advice on equipment maintenance, specimen collection and processing, and laboratory safety.

This *Guide for Laboratory Personnel* provides a useful resource not only to those specifically involved with HIV testing but also to many others concerned with prevention and control of AIDS/HIV infection, such as managers of national AIDS programs. As the HIV testing program itself in any country is expensive, program managers and laboratory personnel must carefully consider its sustainability during the years to come, keeping in mind that the pandemic of HIV infection has just begun. There is no doubt that laboratory personnel can play a significant role in curbing the spread of HIV. This book is an immense help to all those who wish to play such a role.

FORWARD

Human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) are of major concern to health care professionals in all parts of the World. The terms "HIV" and "AIDS" most likely have been used in every laboratory on every continent and at least one laboratory in each country probably tests for antibodies to HIV. In developing countries, adequate information on HIV and the testing for HIV infection may not be available. However, the World Health Organization (WHO) has been instrumental in the dissemination of information and providing guidelines for HIV testing in most countries.

Now that HIV testing has been initially established in these laboratories, it is time for education to proceed beyond the mechanics of performance of these tests. The next step is to ensure that the individuals who perform the tests have a thorough knowledge of the principles of these tests. This includes the proper use and choice of tests, the interpretation of results, and the necessary quality assurance measures needed to effectively and accurately provide a laboratory diagnosis. Laboratorians must possess the background knowledge needed to be confident that results are accurate within the limits of the tests.

Each individual performing tests for the laboratory diagnosis of HIV infection must conduct all aspects of the testing procedure with perfection. Laboratory supervisors must be able to teach and monitor the work of laboratory personnel who will provide a means to disseminate quality testing to all laboratories in each country. In order for laboratory personnel to contribute significantly to the assurance of quality results, they must have a basic understanding of HIV as an infectious agent, and the means by which it is able to cause mortality. Laboratory workers should pay particular attention to the biochemical composition of the virus, in order to understand the host's immune responses and the basic principles of the diagnostic tests. This understanding is important for the proper selection and usage of diagnostic tests.

All the authors of this guide have been trained in laboratory medicine with strong backgrounds in immunology. Niel Constantine has taught clinical immunology to medical technology students for over eight years, and has directed an HIV diagnostic and research laboratory in a developing country for almost three years. Douglas Watts currently heads a Virology Division and has

acted as a WHO technical representative in developing countries. Johnny Callahan has worked in a clinical laboratory for four years and has experience in an HIV testing laboratory in a developing country for nearly two years. Each author has trained technicians and technologists from many parts of the World, and each has acted as a WHO consultant or advisor in several countries for the laboratory diagnosis of HIV. In addition, all have taught HIV testing workshops in several developing countries over the past three years, and have developed insight into both the problems that laboratory personnel encounter, and areas in which a more thorough understanding is needed.

This guide was prepared with three objectives in mind: to provide basic information on HIV and HIV testing; to indicate the HIV testing technologies which are available, how these should be used, and how the results should be interpreted; and to describe the essential quality control and quality assurance measures that must be implemented to ensure results are as accurate as possible. The ultimate goal is to establish the capability for effective and accurate diagnosis of HIV infection. An incorrect HIV test result due to the lack of technical expertise or basic knowledge is inexcusable, and can lead to serious consequences for the individual being tested or the recipient of infected blood.

- Niel T. Constantine

PART 1
HIV : AN INTRODUCTION

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HIV: AN INTRODUCTION

The current number of reported acquired immunodeficiency syndrome (AIDS) cases worldwide exceeds one-third of a million. However, delays in reporting and a reluctance to accurately report numbers makes this number suspect. It has been estimated that the actual number of individuals with AIDS currently exceed one million. In addition, it is estimated that there are between 8 and 10 million persons presently infected with the human immunodeficiency virus (HIV). A recent report from The World Health Organization estimates that one-half of all the AIDS cases occur in Africa, and the largest rate of yearly increase in cases is occurring in some countries of Asia. In addition, many countries (including those in the Middle East) are now reporting indigenous transmission. The world-wide institution of accurate laboratory testing for HIV can have a major impact in helping to contain the epidemic.

AIDS has only been recognized for the past decade, and only within the last six years has a laboratory diagnostic test become available. Asymptomatic HIV infection presents considerable concern for physicians, laboratory workers, and the general public. Everyone must rely on the laboratory tests to reveal infection. The detection of antibody in serum is still the most widely used means for the laboratory diagnosis of infection, although some recent advances in technology offer alternatives. Until effective preventative and/or treatment strategies are developed, tested and universally implemented, the prevalence of HIV infection will continue to increase. For the foreseeable future, laboratory diagnosis will continue to play a significant role in screening blood for transfusion and in identifying infected individuals so that they can be counseled and educated to prevent the infection of others.

Those who are involved in the laboratory diagnosis of HIV infection must provide accurate results. To accomplish this, a thorough understanding of the tests is necessary, including test principles, the proper use of tests, and the measures that ensure that these tests are performing properly. In addition, a thorough background knowledge of the immune response and the composition of the virus will allow for effective teaching and technology transfer to other health personnel.

THE HIV VIRUSES

The HIV viruses (HIV-1 and HIV-2) are enveloped, RNA containing **retroviruses** that primarily infect human white blood cells (lymphocytes). They are called retroviruses because they have the ability to integrate their genetic material (RNA **genome**) into the host cell's DNA by first transcribing this genome into DNA. The host cell DNA (now containing the viral genome) harbors the HIV **provirus**.

This provirus is transcribed and translated along with the host cell's DNA (see Figure 1). This results in the synthesis of specific viral components. These components (the proteins and glycoproteins) eventually assemble to produce complete viral particles that are released from the cells as infectious virions. An enzyme called **reverse transcriptase (rt)** is required in order for the virus to transcribe RNA to DNA. This enzyme is important for diagnostic purposes since it can be detected in cell culture indicating the presence of virus. In addition, rt acts as a foreign substance when introduced into a human, and therefore it elicits an immune response (see Part 2).

The genome of the virus possesses two types of genes: the structural genes and the regulatory genes.

The structural genes are responsible for the direction of proteins and glycoproteins that give the virus its physical characteristics.

The regulatory genes stimulate production of proteins that can effect the activities of other components of the virus, or can specifically turn other genes on and off. These activities may therefore increase or decrease the replication and functioning of the virus.

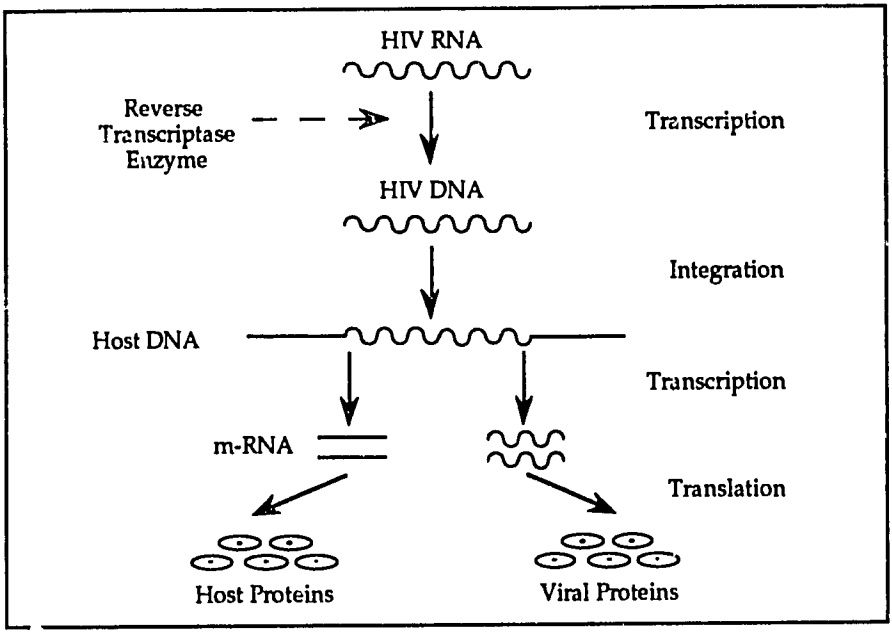

Figure 1. Transcription, Integration, and Translation of Virus Genome

HIV virus is composed of two major parts: an outer envelope and an inner core (see Figure 2).

The external envelope components are embedded in a lipid matrix and are involved in the binding of the virus to the host cells. The spikes or knobs extending from the lipid membrane are proteins with sugar molecules and are therefore called glycoproteins (gp).

The core components are internal and are bound by a protein coat encompassing two identical copies of the nucleic acid genome-RNA. Also within the core are three viral enzymes: reverse transcriptase, integrase, and protease.

The specific components of the envelope and core portions are described below under HIV antigens. HIV (as with most viruses) cannot survive long periods unless it is contained in a host cell (i.e. it must maintain an intracellular relationship.) Very importantly, the virus has the capability to modify some of its structural components (mainly the envelope components) in a way that may prevent the host's immune response from being effective in containing the infection. Therefore, small differences in the precise sequences within these components may occur between

the virus in different individuals, and even during the infection within the same individual. This is one of the reasons why an effective vaccine has been difficult to develop.

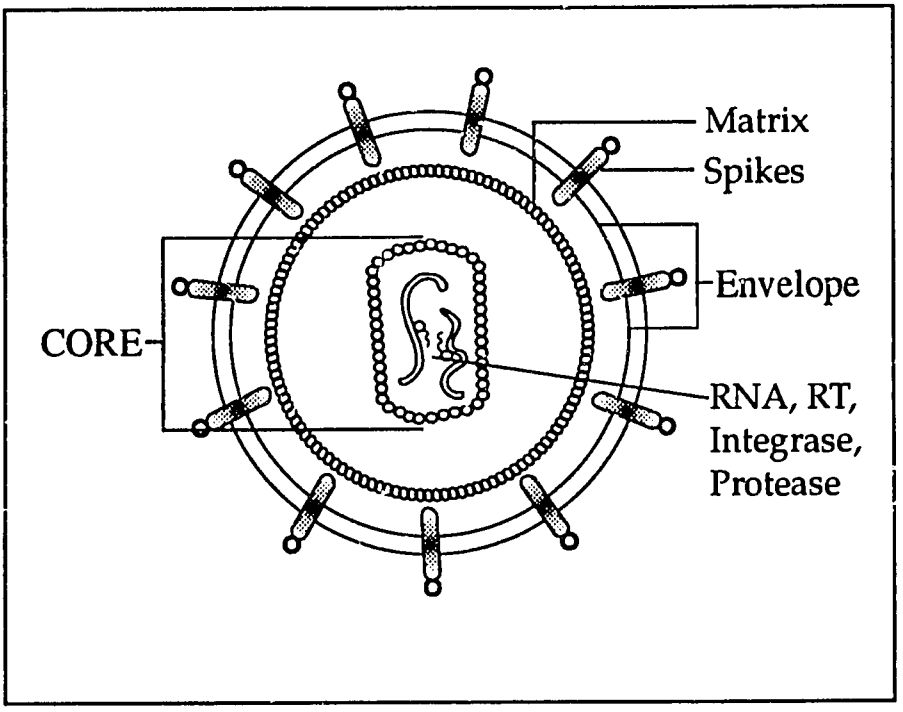

Figure 2. The Major Structural Components of HIV

THE BIOLOGY OF HIV INFECTION

The events leading to viral attachment, penetration, replication, and pathology are complex and need not be addressed here in detail. Basically, the virus attaches to the host cell (most often a **T-4 lymphocyte**) through a complicated mechanism involving an external envelope component of the virus and a protein on the host cell. Since the virus can combine with this protein, the protein can be considered as a **receptor** for the virus. This protein (receptor) is known as **CD-4** and is the molecule identifing these cells as a subpopulation of lymphocytes called **helper T lymphocytes**. Many other types of human cells (e.g. brain cells) may also contain this CD-4 molecule (or a similar one) and therefore also can be infected by the virus. Evidence is also accumulating to indicate that other body cells that do not have this CD-4 molecule can be infected, presumably by a different mechanism. In any event, the virus fuses with the cell, and the internal components of the virus are released into the host cell. At this stage, the person is **infected**.

After the viral genome integrates into the host DNA, the provirus directs the production of new viruses. During this infectious process, some host cells are destroyed, some fuse together (**syncytia**), and some simply exist--seemingly unaffected. Symptoms may or may not exist at this time, and may not appear for many years. Following some unknown event, the virus is activated and begins to replicate. It is at this time that symptoms usually appear and the individual may be diagnosed as having **AIDS related complex (ARC)** or, if a particular number of symptoms appear, full-blown AIDS.

Symptoms may vary because of the involvement of many organ systems. **The ultimate cause of death in persons suffering from AIDS is usually due to infectious diseases caused by the severe immunodeficiency associated with the involvement, compromise, and destruction of immune cells.** These infectious diseases are usually considered to be **opportunistic infections**, since they rarely cause disease in a person whose immune cells have not been severely compromised. Since the helper T lymphocytes orchestrate the activities and functions of most other immune cells, the effects of the virus on T lymphocytes can interfere with the functioning of other immune cells. When the necessary relationship between these other immune cells is lost, the entire immune system becomes ineffective.

IMMUNOLOGIC ABNORMALITIES DURING INFECTION

A thorough understanding of the immune system is necessary to fully appreciate the way in which the virus can establish infection and paralyze the protective effects of the system. The following brief description of the system will concentrate on the major immune cells and the basic effects following infection. **The major components of the immune system include:**

1. **the macrophages,** large cells that phagocytize, process antigens, and present these antigens to other immune cells;

2. **the lymphocytes,** which include T cells and B cells;

3. **the plasma cells,** small cells which result from the maturation of B cells and produce large quantities of antibodies;

4. **the natural killer cells (NK cells),** that are active against tumors;

5. **the polymorphonuclear leukocytes (PMN),** which are also phagocytic cells;

T lymphocytes are further classified as CD-4 (T-4) and CD-8 (T-8) cells:

- **the CD-4 lymphocyte** subpopulation contains cells that can either act as **helper cells** or **inducer cells**; and,

- **the CD-8 cells** can be **suppressor cells** or **cytotoxic cells.**

T-helper cells are responsible for providing assistance to other immune cells via direct contact, or by elaborating soluble substances **(lymphokines)**. This assistance to other cells can include events that bring about the other cells' maturation, activation, and proliferation.

In other words, T-helper cells can cause T-suppressor cells to suppress the activities of other cells, including: other T cells and B cells; T-cytotoxic cells to kill virus infected cells; B lymphocytes to mature to plasma cells producing antibody; NK cells to kill tumor cells; macrophages to become activated resulting in increased phagocytosis, killing, and processing of antigen.

The basic interactions of the components of the immune system are depicted in Figure 3.

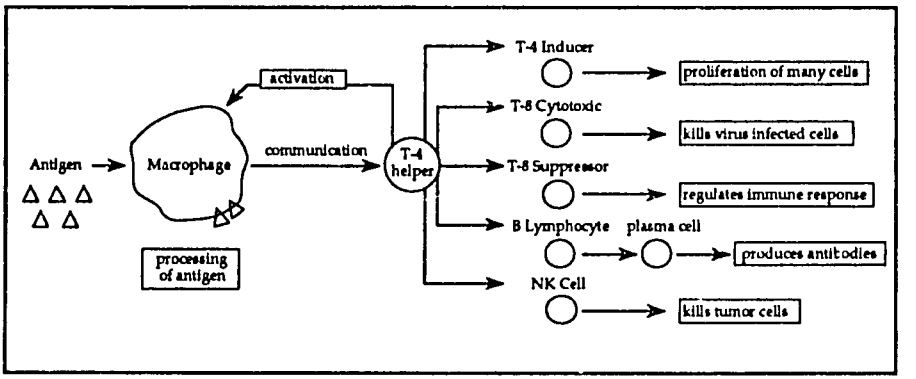

Figure 3. Immune System Interactions

As an infectious agent (such as HIV) enters the body, it is usually first detected by the scavenger cells -- the macrophages. The infectious agent (foreign substance) is phagocytized by the macrophage, and the antigens of the agent are degraded and processed. Subsequently, these antigens are presented to the T-4 helper cells in such a way that the T cells recognize the antigens and respond. Macrophages also produce soluble substances (e.g. interleukin 1) that activate the T-4 helper cells. The T-helper cells

later elaborate their own products (such as interleukin 2 and interferon) and these products effect the other immune cells as mentioned above.

From this simplified description of the events of the immune system, it is apparent that **when the T-4 helper cell is compromised by infection with HIV, the entire immune system is affected.** Eventually, T-8 cytotoxic cells and the macrophages become incompetent due to the lack of support by T-helper cells, and opportunistic infections are manifest; tumors may arise (possibly due to the decrease in NK activity), and antibodies to commonly encountered organisms (may not be produced). In addition, there is evidence that many immune cells may be destroyed as they become fused in masses (syncytia) composed of infected macrophages, T-4 cells, and other cells.

As the HIV infectious process evolves, there is a general lymphopenia. The primary effect is a decrease in the T-4 lymphocyte population. T-8 suppressor cells may initially increase and sometimes remain at high levels for long periods. The T-4 cell counts offer the most information for the clinical disease stages, and therefore are the most widely used parameter to assess the disease. The T-4 cell counts are reduced in about half of asymptomatic HIV infected individuals and in nearly all individuals with ARC and AIDS.

Normally, T lymphocytes (T 4 and T8) account for 70-90% of all lymphocytes. Their numbers are generally near 1000 cells/mm^3 and the decrease seen with HIV infection is gradual (50-100 cells/mm^3/year). Although there is no consensus on the exact value that indicates disease, most authorities agree that a T-4 count of less than 500 is highly supportive of a diagnosis of HIV infection, if other reasons for the decrease have been ruled out. Generally, the lower the T-4 value, the more severe the disease progression and the less favorable the prognosis. It is often helpful to determine the T-4/T-8 ratio. **Since T-4 cells account for about 60% of the T lymphocytes, and T-8 cells account for about 30% of the T lymphocytes, a normal ratio is always greater than 1.0 and usually greater than 1.5. Therefore, there is a significant decrease when the value of the ratio falls below 1.0 (a reversal of the ratio).** Methods to evaluate cells of the immune system are presented in Part 4.

HIV ANTIGENS

As with any infectious agent, HIV is composed of basic chemical components. The most important of these, for an understanding of the diagnostic tests are:

the **proteins** (chains of amino acids);

the **glycoproteins** (proteins coupled to carbohydrates or sugars).

Enzymes of the virus are also important and should be considered with the protein group. All these components are foreign to the human body and are termed **antigens** (to generate against). Many antigens are **immunogenic** (generating an immune response), and will result in an immune response when introduced into a human (see part 2). Antigens are produced as a result of instruction specified in the nucleic acids that make up the genome of the virus. These antigenic components may also be referred to as **gene products** (produced from the genes). As the virus replicates in the host, these antigens are being produced, and often they are released into the blood of the individual. Therefore, **at certain times during infection an antigenemia (antigens in the blood) occurs.**

The different antigens of HIV are encoded by three major structural genes and can be divided into three major groups:

1. **gag** proteins (encoded by the "gag" gene or group-associated antigen gene)

2. **env** (envelope) glycoproteins

3. **pol** (polymerase) proteins

The most important **gag** proteins (p) are those having molecular weights of 55,000 (**p55**), 24,000 (**p24**), 17,000 or 18,000 (**p17**), and 15,000 daltons (**p15**).

The p55 antigen is referred to as a precursor molecule because it is produced early during the infectious process and is eventually cleaved to produce the other core proteins.

Gag proteins are located inside the virus in the nucleocapsid.

The p17 protein lies between the protein core and the envelope (the matrix) and is embedded within the internal portion of the envelope.

The p24 and p15 proteins make up the core coat that surrounds the nucleic acids.

The **env** (envelope) antigens are glycoproteins (gp) and include molecular weights of 160,000 (**gp160**), 120,000 (**gp120**), and 41,000 daltons (**gp41**). **The gp160 is the precursor molecule** and is not a structural component; it is a component that is produced during infection, but is later degraded to form gp120 and gp41 (the structural envelope glycoproteins). These envelope glycoproteins contain:

- **conserved regions** (sequences constant from virus to virus),
- **variable regions** (sequences which may vary between viruses).

The presence of antibodies to the envelope antigens is extremely important for the laboratory diagnosis of HIV infection and at least one env component must be detected in order to confirm infection. The gp41 antigen is located between the inner and outer membrane, and is often referred to as the **transmembrane** glycoprotein. This particular antigen contains variable regions, and may be very specific for each type or strain of virus. The gp120 antigen is the major component of the external envelope and is responsible for the 72 knobs or spikes of the envelope. Together, gp41 and gp120 are involved with the attachment to the CD4 molecule on the host cells.

Pol (polymerase) antigens include the proteins **p66 (reverse transcriptase enzyme), p51,** and **p31 (integrase or endonuclease).** Polymerase components are responsible for the process of:

- conversion of RNA to DNA (reverse transcription);
- integration of viral DNA into cellular DNA;
- the proteases that cleave the precursor molecules into smaller active components.

Polymerase antigens are located within the core and are associated with the nucleic acids.

Figure 4 depicts the location of **gag, env,** and **pol** antigens within the virus. The precursor molecules gp160 and p55 are not structural components of the virus and are only included in the figure for

completeness. These precursor components are, however, true gene products and are produced during the transcription and translation processes. It is later (but before virus assembly) that they are cleaved to form the other structural components. **Therefore, during viral replication these precursor molecules may spill into the blood and elicit antibodies that can be detected by serologic techniques.**

Other components of the virus may be antigenic, and play a significant role in infection and the development of immune responses, but their exact roles are poorly understood. These virus components include the negative regulatory proteins **nef** (p27), and **vpr** and the positive regulatory proteins **vpu, tat** (p27), **rev**, and **vif**. Generally, these regulatory components are responsible for modifying the expression of viral proteins and the replication of the virus. They may also govern viral infectivity.

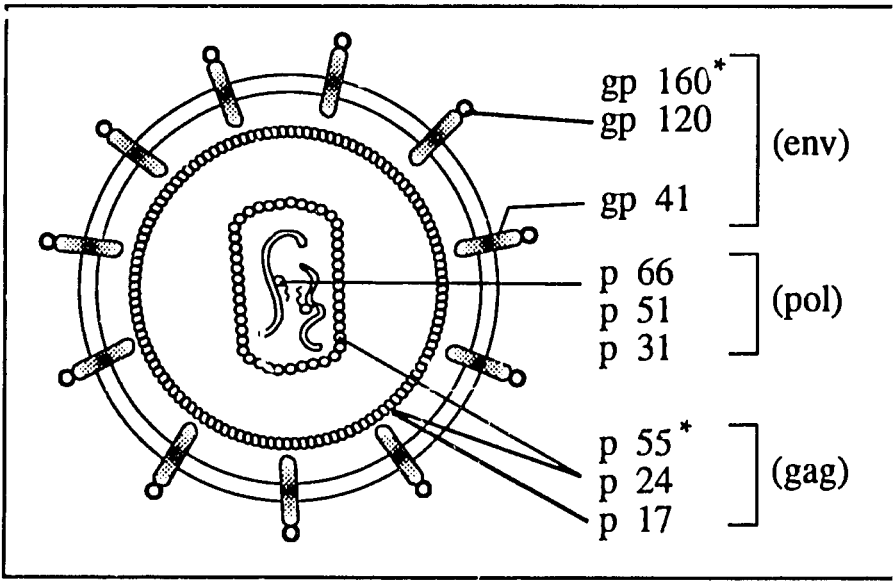

Figure 4. The Location of HIV Antigens
* Precursor molecules

HIV-1 vs HIV-2

Several years after the recognition of HIV, a similar virus was discovered. This led to the classification of HIV into HIV-1 (the initial virus) and HIV-2. **HIV-2 seemed to be endemic primarily in areas of West Africa, in contrast to the widespread prevalence of HIV-1.** However, HIV-2 infections have since been described in many other areas including Europe, South and North America, the

Persian Gulf Region, and in North and East Africa. Although most cases are linked to West Africa, it will probably not be long before cases of endemic transmission are described in many countries. Biologically, HIV-2 is very similar to HIV-1. The genome of HIV-2 shares 40 to 45% homology with HIV-1 and the virus causes the same pathological consequences. HIV-2 may have a longer latent period, although this has recently been challenged.

Diagnostically, HIV-1 and HIV-2 can present problems. **Tests designed to detect infection by HIV-1 do not always detect infection by HIV-2 and vice-versa.** However, the two viruses may be differentiated serologically and by utilizing additional technologies; these issues are addressed in Parts 3 and 4.

Antibodies to HIV-1 will frequently cross-react with the antigens of HIV-2 and produce positive reactions in serological assays designed to detect antibodies to HIV-2. Conversely, antibodies to HIV-2 may react in HIV-1 assays. However, in many cases (perhaps 30-50%) HIV-1 serologic assays will not detect HIV-2 infections and HIV-2 tests will not detect HIV-1 infections (see Part 3). Most cross reactions represent antibody induced by the core antigens, since these are highly conserved between the two different viruses. The antigens of HIV-2 are similar to those of HIV-1, but the molecular weights may vary slightly. For example:

- some tests incorporate strains of HIV-2 that contain envelope antigens of molecular weights: gp140, gp105, and gp36;

- likewise, the pol and gag antigens of HIV-2 may also vary in molecule weights from those of HIV-1.

Therefore, it is not uncommon to see the transmembrane antigen of HIV-2 referred to as gp36 while others may call it gp41; likewise, the major core antigen may be referred to as p24 vs p26. The inconsistency in characterizing these antigens is unfortunate. Table 1 compares the antigens of HIV-1 and HIV-2.

OTHER RETROVIRUSES

At least two other retroviruses are known to cause human disease, but they do not cause AIDS:

HTLV-I (human T lymphotropic virus type I) is associated with adult T cell leukemia (ATL), HTLV-I associated myelopathy (HAM), and tropical spastic paraparesis (TSP). It was the first human retrovirus to be discovered and the first to be associated with human malignancy. There are generally no symptoms in early disease and in many individuals there is no apparent disease, even after many years. The highest prevalence of infection is in Japan, the Caribbean, and parts of Central America and Africa.

HTLV-II infection has been described in patients having "hairy-cell" leukemia, however the agent has not yet been conclusively linked to any specific disease.

Both viruses produce disease after long intervals of time and are transmitted by the same modes as the HIV viruses. Their effect is immuno-proliferative rather than causing an immunodeficiency, and therefore the HTLV's are referred to as transforming viruses.

Although these viruses are similar in many aspects to the HIV's, they contain distinct antigens. Therefore, **the HTLV's do not present a diagnostic problem when testing for antibodies to HIV.** Antibodies to HTLV antigens generally do not cross-react with the antigens of HIV. At most, there may be some minor cross reactivity between the p24/p26 core antigens. The major antigens of the HTLV's include the gag proteins p19 and p24, and the envelope glycoproteins gp61/68. Serologically, HTLV-I and HTLV-II antibodies cannot be differentiated. Therefore, it is not possible to identify the exact infection based on routine serologic tests.

Currently, the technology for confirmation of antibodies to the HTLV's includes the Western blot and the RIPA (see Part 4). Usually, both of these tests are required to confirm HTLV infection. Newer technologies, such as the synthetic peptide-based tests, may be beneficial in confirming and differentiating these infections.

HTLV infections and their transmission modes are similar to the HIV's; **therefore, all blood units for transfusion should be tested and discarded if positive.** Details on the HTLV's will not be presented in this laboratory guide.

Table 1
Recommended WB nomenclature of major gene products of HIV-1, HIV-2 and HTLV-I/HTLV-II

Gene	HIV-1		HIV-2		HTLV-1/HTLV-II	
	Gene products	Description	Gene products	Description	Gene products	Description
ENV	gp160	precurson gp	gp140	precursor gp	gp62/68	precursor gp
	gp120	external gp	gp125[a]	external gp	gp46	envelope gp
	gp41	transmembrane gp	gp80[b]	dimeric form of transmembrane gp		
			gp36/41	transmembrane gp		
GAG	p55 p40 p24 p17	precursor precursor core matrix	p56 p26 p16	precursor core matrix	p53 p24 p19	precursor core matrix
POL	p66 p51 p32	rt rt endonuclease	p68 p53 p34	rt rt (provisional) endonucleuse		
Other	p160	*gag/pol* precursor				

[a] Identified as gp105 by some WB manufacturers.
[b] Visible on radio-immunoprecipitation assay (RIPA), sometimes present in WB.
Note: In HIV-2 a gp300 band can be detected by RIPA, not by WB.
gp: glcoprotein rt: reverse transcriptase
Adapted from World Health Organization's Weekly Epidemiological Record, 14 September 1990, page 282.

PART 2
IMMUNE RESPONSES TO HIV

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ADULTS

As with most infectious agents, the human body responds to invasion by HIV with the production of antibodies. During HIV infection, these antibodies are usually produced between six and twelve weeks following infection, although in rare cases they may not be detected for months or years. **If an individual is tested soon after infection, he may not have antibodies, and will test negative.** The individual is truly infected at this time of testing, but since antibody will not be detected, a **biologic false negative** result will be obtained (a biologic false result occurs due to parameters related to the specimen or patient and not to the testing process.) Therefore, it is important to understand the kinetics of the immune response in order to realize when an individual should or should not be tested. Perhaps a test should be performed at the early stages in order to produce a baseline result for later comparisons; however, a negative result cannot be used as an indicator that the individual is absolutely not infected. As the concentration (titer) of antibodies gradually rises (as infection progresses), the individual's serum will change from negative to positive for antibodies (**seroconversion**) and infection will be verified. **Some antibodies to HIV will persist throughout the infection and the disease, while other antibodies will decrease to undetectable levels.** Specific antibodies are produced to all of the major antigens of the virus (p15, p17, p24, p31, gp41, p51, p55, p66, gp120, gp160), and perhaps to some of the regulatory proteins.

It is important to note that not all individuals will have the same immune response to the virus. Some persons may have stronger responses to p24, for example, while others may respond strongly to other antigens. Although it is not unusual to demonstrate different antibody profiles in the sera from different individuals, most individuals respond to all viral components during the course of infection. It should also be noted that the concentration of specific antibody can vary between persons, and vary during various times following infection.

Antibody titers are commonly as high as 50,000, but can also be very low. Low titers are present early in infection (during seroconversion) and later in disease when the individual becomes immunodeficient. Low titers may be responsible for weak reactions in serological assays and can result in biologic false negative results, or **test false negative** results if the test lacks sensitivity (see Part 5). In addition, the demonstration of specific antibodies may depend on the method used to detect these antibodies. For example, if a test

primarily detects antibodies to gp41, it may not produce a positive result if the individual only has antibodies to p24 and gp120. **Therefore, it is important to understand not only the kinetics of the response, but also the basis of the tests that are used.**

Figure 5 illustrates antibody response and occurrence of antigenemia following infection by HIV. As indicated, antibodies to the gag proteins (p55, p24) usually appear early during infection, while antibodies to the env and pol products are produced at the same time or somewhat later. However, it is important to remember that the detection of these particular antibodies depends on the assay used, (eg. tests designed to detect primarily gag components may yield reactive results before tests that are designed to detect env antibodies.)

Antibodies to p24 may decrease in time as the disease progresses, and as viral protein (p24 antigen) increases in the serum (see Figure 5). This reversal of antibody/antigen most likely reflects removal of antibody as it combines with an increasing amount of viral antigen produced as a result of virus replication. This viral replication usually occurs late in disease and is accompanied by symptoms in the patient (i.e. the syndrome of AIDS becomes evident).

The appearance of p24 antigen in the serum (antigenemia) very early after infection is also to be noted. Although this may occur, the quantities of antigen may be very low, and may not be detected by antigen assay methods (see Part 4). **The period after infection has occurred but before antibody can be demonstrated (generally 0-6 weeks), is called the "window period."**

The antibody responses indicated in Figure 5 signify IgG antibody. Although IgM responses occur in most viral infections, there does not seem to be a consistent IgM response during HIV infection. Alternatively, it may be that IgM tests need improvement. IgM assays, if effective in identifying infected individuals, would be beneficial for use during early infection (the window period) before IgG antibodies are produced. Currently, tests to detect IgM and IgA specific antibodies are being evaluated to determine the immune status of newborns suspected of being infected.

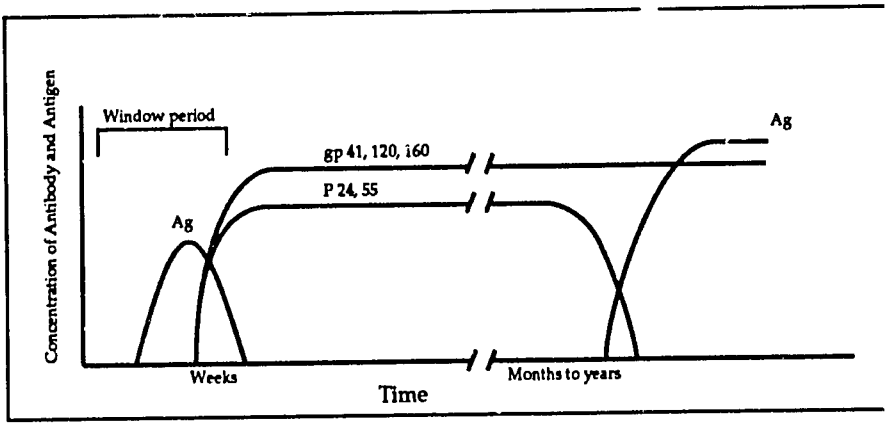

Figure 5. The Immune Response to HIV Infection

NEWBORNS

HIV infection in newborns presents diagnostic problems because of the presence of maternally acquired antibodies in the newborn's serum. IgG antibodies from an infected mother cross the placenta and will be detected when assaying the newborn's blood for antibodies. The detection of IgM antibodies which do not cross the placenta would be of value in diagnosing newborn infection, but assays for IgM have not been shown to produce adequate results, and therefore are not used routinely. Recently, promising results have been obtained using IgA specific assays for newborns. These tests seem to be more valuable when testing a newborn after several months of age. Newer, more specific (but technically difficult) assays such as the polymerase chain reaction to detect viral nucleic acids are currently helpful in detecting the presence of the virus in the newborn's lymphocytes. Viral culture and antigen assays may also be of value for identifying HIV infection in newborns (see Part 4). These assays are of minimum use in the neonate, but their sensitivity increases as the age increases beyond six months.

Presently, a newborn must be monitored using standard IgG antibody tests for at least 16 months before the absence of infection can be determined. Once maternal antibody has disappeared from the newborn's serum (approximately 8 to 14 months), the newborn may **sero-revert** (become negative). However, it may be several more months before the newborn's immune system is competent enough to produce antibodies, and the newborn may still subsequently seroconvert to positive. Therefore, a newborn must be monitored for nearly 18 months before the absence of infection is verified. **Approximately 20 to 30% of babies born to infected mothers will be truly infected with HIV.**

PART 3
TESTS TO SCREEN FOR HIV INFECTION

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INTRODUCTION

There are no available tests to diagnose AIDS, only HIV infection. These tests should never be referred to as AIDS tests; this is misleading and incorrect. Diagnostic screening tests to detect HIV antibodies are many in number and variety.

The initial tests developed were ELISAs (enzyme-linked immunosorbent assays) because these types of tests had been used successfully for a variety of other infectious agents. Also, ELISA tests are generally easy to perform, adaptable for testing many samples, do not require the use of radioactive substances, and are sensitive and specific (see Part 5). When the ELISA for HIV became widely used, there were already a number of different companies producing these tests in a variety of different formats.

Not long after the ELISAs were marketed, other techniques became available such as latex, red cell, and gelatin particle agglutination tests. Subsequently, dot-blot assays were introduced.

Most of the "early" tests and many of the ones still used are based on viral lysates (ie. the antigens used in the test are prepared from whole, disrupted HIV virions.) These lysates may contain various contaminants derived from the host cells in which the virions are propagated. These contaminants can introduce unwanted components in the assay system and can sometimes lead to biologic false positive results.

Newer tests incorporate the use of recombinant or synthetic peptide antigens (see below).

As technology evolved, so did the newer tests, both in performance and in the ease of operation. At present, a range of assays are available:

- some can be performed in 22 hours and others in 5 minutes;
- some require sophisticated instrumentation while others require none;
- some require volumes of 800 ul and some need only 1 ul;
- some produce color, and others produce no color as an indicator system;

- some are read visually, some with a fluorescence detector, and others with a spectrophotometer;

- some tests require a great deal of expertise to perform while others are so simple that a laboratorian with only the most fundamental skills could perform them accurately.

The cost of these tests also varies, with the range being from less than 1 US dollar per test, to more than 12 dollars per test.

Screening assays are designed to detect all positive sera (i.e. to be sensitive), even if some false positives do occur. It must be noted that screening assays are tests that possess a high degree of sensitivity in order to identify all individuals who may be infected. They are not to be used as the final analysis, since some individuals will inevitably produce reactive results, even though they are not infected. This concept is further discussed under predictive values in Part 5.

Mistakes in the testing procedure may occur due to technical error; repeat testing is mandatory. A result must be repeatedly reactive at least 2 of 3 times before the sample is considered as truly reactive by the screening assay. Sera that produce a positive result by screening assays are termed **reactive**. If sera are found to be further reactive by confirmatory tests, they are usually considered **positive**.

PRINCIPLES OF THE TESTS

ELISAs

A variety of different ELISA tests are available, but most of the ones used for detection of HIV antibodies are classified as **indirect, competitive, or antigen capture assays.** Most of these types use HIV antigen attached on a solid phase (support), together with a conjugate, and a substrate. Solid supports are supplied by the kit manufacturer with the viral antigen (viral lysate, recombinant or synthetic peptide) already attached.

These **solid supports** can be "wells" of a microtiter plate, plastic beads, or a type of paper--usually nitrocellulose. Recently, microbeads with an increased surface area have been used as the solid support in order to absorb larger quantities of antigen.

If antibody to HIV is present in the sample, the antibody will bind to the HIV antigen on the solid phase. Following a wash step to remove unbound serum constituents, conjugate is added and incubated. Conjugate will bind to the patient's antibody (if present) with the excess removed during another wash step.

Conjugates are antibodies directed against either a human antibody (in the indirect assays) or the HIV antigen on the solid support (in the competitive assays). In either case, these antibodies are coupled (conjugated) to an enzyme.

Enzymes are either alkaline phosphatase or horseradish peroxidase. These enzymes are capable of modifying a **substrate** in the presence of a chromogen (color producing compound) to produce a colored product that can be detected either visually or by an instrument (**spectrophotometer**). Therefore, in an indirect assay, the addition of substrate will produce a color reaction if the conjugate has been bound to the patient's antibody (which is bound to the antigen on the solid support). A typical enzyme-substrate reaction is as follows:

$$\underset{\text{(substrate)}}{H_2O_2} + \underset{\text{(chromogen)}}{OPD} \text{ — } \underset{\text{(enzyme)}}{\textbf{Horseradish peroxidase}} \text{ —> } \underset{\text{(water)}}{H_2O} + \underset{\text{(radical)}}{O^-}$$

$$\underset{\text{(radical)}}{O^-} + \underset{\text{(oxidizes chromogen)}}{OPD} \text{ ——————————> colored product}$$

The colored product detected by a spectrophotometer must be detected at a particular wavelength of light where the color absorbs the incident light. This results in a signal usually converted by the instrument into optical density (O.D.) units. These O.D. readings refer to the light transmitted through the colored product, and are a measure of the intensity of the colored product. Therefore, the amount of color produced by the enzyme-substrate reaction is determined by the readings in O.D. units.

All ELISAs are easy to perform, but require careful adherence to procedures. Any deviation in incubation times, temperatures, or volumes can result in dramatic changes in test results. Wash procedures must be thorough, and reagents must be prepared exactly as directed. Conjugates must be thoroughly, but gently mixed in order to not denature the enzyme. Substrates should be prepared immediately before use and should be kept in the dark prior to addition to the test system.

1. Indirect ELISAs

In the **indirect ELISA**, the patient's serum is added to the solid phase containing the antigen and is incubated for a specified time period and at a particular temperature. The indirect ELISAs produce more color as the unknown antibody concentration in the sample increases (Figure 6). Conversely, with small amounts of antibody, smaller quantities of conjugate will bind, resulting in less substrate cleaved, less color produced, and lower O.D. readings. Each component is added separately, with wash steps before the addition of conjugate and before the addition of substrate. These wash steps remove unbound material.

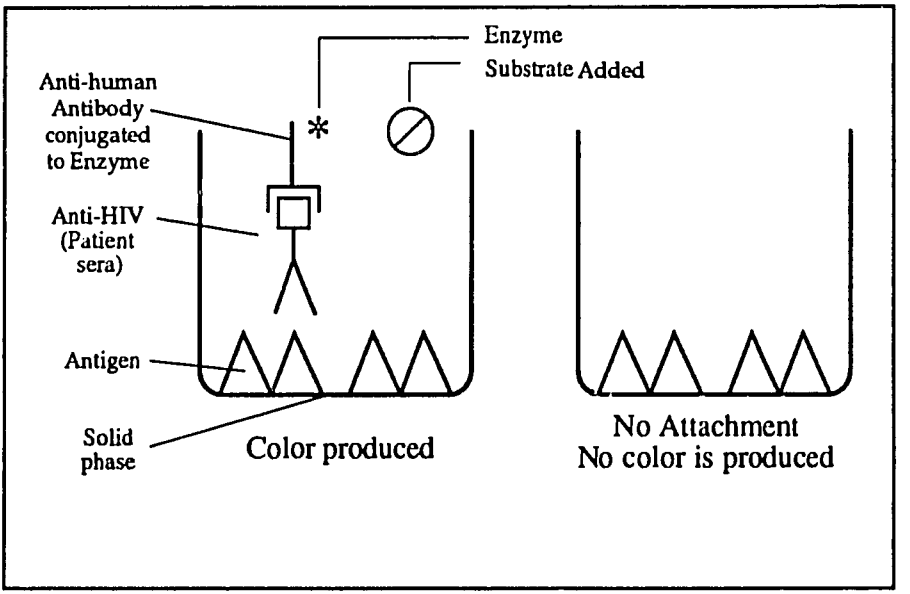

Figure 6. Principle of the Indirect ELISA

2. Competitive ELISAs

Competitive ELISAs differ in that the antibody to HIV in the sample competes with the conjugate (which is an antibody also directed against the HIV antigen) for reactive sites on the bound antigen (Figure 7). Therefore, in Competitive ELISAs, both the sample containing the antibody to HIV and the conjugate are added to the solid phase (containing the HIV antigen) at the same time. If the concentration of antibody in the sample is high, very little conjugate can bind to the immobilized antigen because the two compete for sites on the antigen. There will be little color development since there will not be much enzyme attached to cleave the

substrate. Conversely, with samples that contain little or no HIV antibody, more conjugate will bind to the antigen on the solid phase and the subsequent addition of substrate will cause more color development. Hence, **the amount of unknown antibody in the sample is inversely proportional to the amount of color produced.**

Competitive ELISAs require less total assay time since there are fewer steps (conjugates and sample are added together). Many laboratorians consider these types of tests to be more specific (but perhaps less sensitive) than the indirect ELISAs; however, this point of view has been recently challenged.

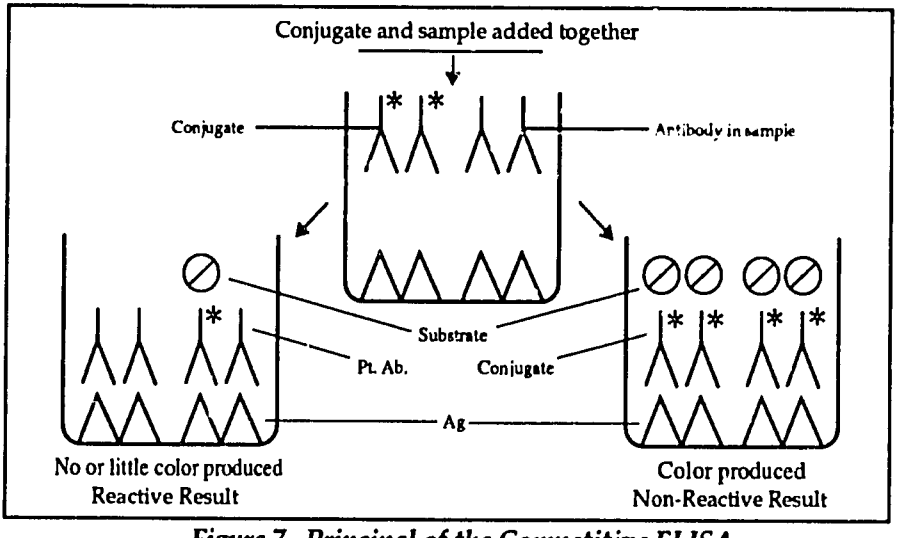

Figure 7. Principal of the Competitive ELISA

3. Antigen Capture ELISAs

Antigen capture ELISAs can be of the indirect or competitive type, and differ only in the initial step of attaching antigen to the solid phase. A monoclonal antibody (very specific antibody directed toward only one antigenic determinant) is bound to the solid support to "capture" the specific HIV antigen. This tends to decrease the amount of contaminating substances bound to the solid support resulting in less non-specific binding. These assays are also considered to be more specific than the indirect assays, which incorporate total viral lysates. Figure 8 illustrates the principle of this type of assay.

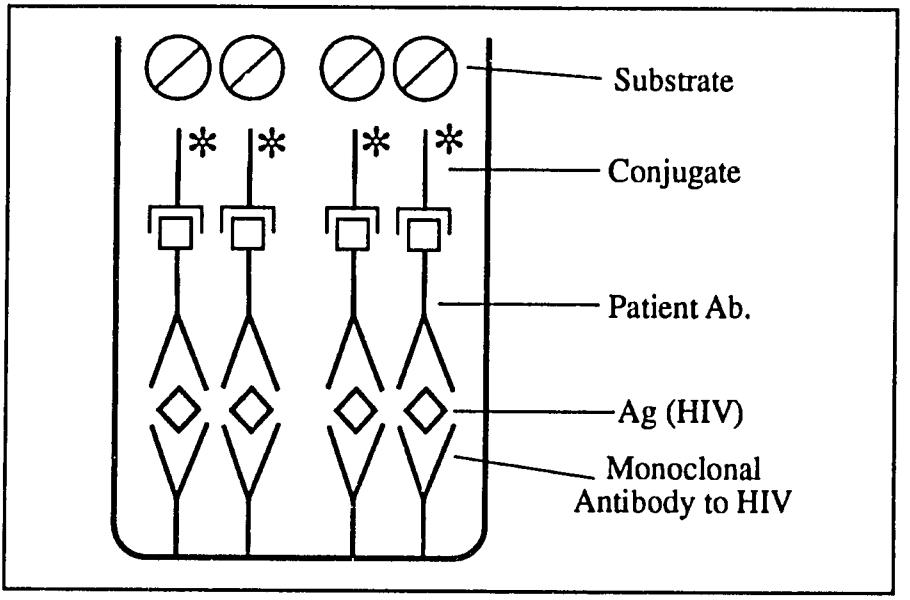

Figure 8. Principle of the Antigen Capture ELISA

Agglutination Tests

Tests employing agglutination as the indicator system have been used for diagnosing various infectious diseases for many years because they generally have good sensitivity for detecting antibody. However, specificity is sometimes compromised. Agglutination tests can incorporate a variety of antigen coated carrier . **Carriers are particles used to "carry" or support the antigen.**

- In HIV tests, red blood cells, latex particles, gelatin particles, and microbeads are commonly used as carriers.

- HIV antigens (viral lysate or specific components) are absorbed onto the carrier, and these antigen-coated reagents come ready to use.

Antigens are bound without specific attachment (i.e. they are passively absorbed onto the carriers) and therefore the technique is referred to as **passive agglutination.** If RBC's are used as the carrier system, the assay is called a **passive hemagglutination assay (PHA).**

A lattice network is formed between the antigen coated particles and the antibody as antibody in the sample reacts in the system. This reaction brings about the clumping (agglutination) of particles (see Figure 9).

One potential and serious problem with agglutination assays is the phenomenon of prozone reactions.

Prozone refers to the inhibition of agglutination when excess antibody is present, thereby preventing the proper combination (or lattice network) from forming. In other words, the concentrations of antigen and antibody are not optimal to allowing clumping to occur (see Figure 10). This may result in a falsely negative reaction (no agglutination) when higher quantities of antibody are present.

Antibody in the sample can only be detected if the sample is diluted and re-tested (see Part 7 for preparation of dilutions). Dilution of the serum decreases the concentration of antibody to a point where the optimal concentration of antigen and antibody occurs, and agglutination proceeds. It is important that laboratorians be aware of this phenomenon. Sometimes an equivocal reaction (+/-) may occur with the undiluted sample, suggesting that prozone may be occurring. **Although prozone reactions do occur, initial dilutions of the test sample as recommended by the manufacturer usually prevent prozone from presenting a significant problem in HIV test performance.**

Agglutination assays are very easy to perform and require no wash procedures. All that is needed is an initial dilution of serum, addition of coated particles, mixing, and incubation (usually at room temperature). Reactions are read macroscopically, or with the aid of a magnifying mirror. A control (non-coated or unsensitized particles) should be used to detect agglutination due to reactions against the particles themselves. If agglutination is detected in both the control and test, the serum must be retested following adsorption of the serum with uncoated particles to remove these non-specific agglutinations.

Weak agglutinating patterns are sometimes difficult to observe. Therefore, **all tests must be read very carefully, usually under a high intensity lamp.** Typical patterns produced in an agglutination assay are shown in Figure 11.

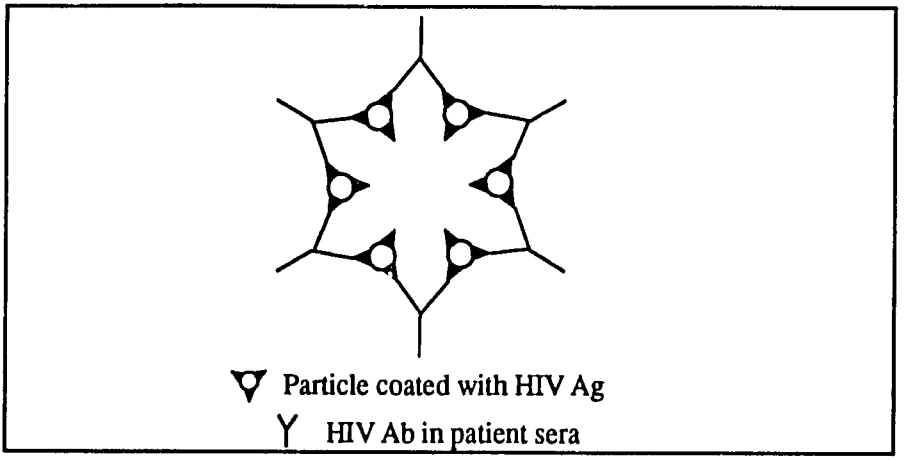

Figure 9. Formation of the Lattice Network and Agglutination

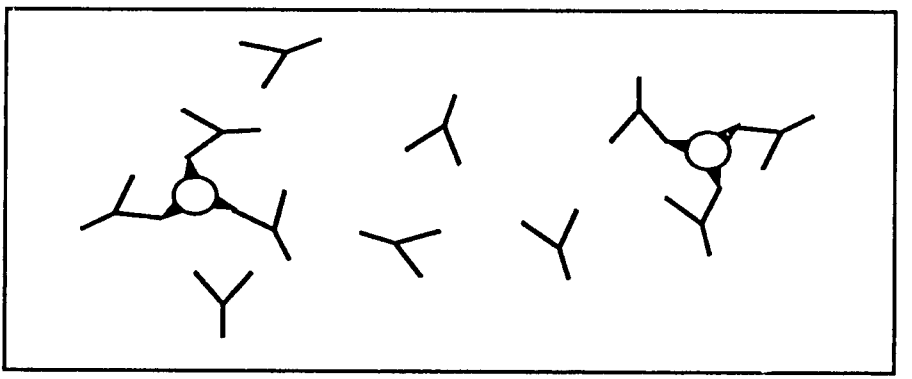

Figure 10. Absence of Agglutination Due to Prozone (Antibody Excess)

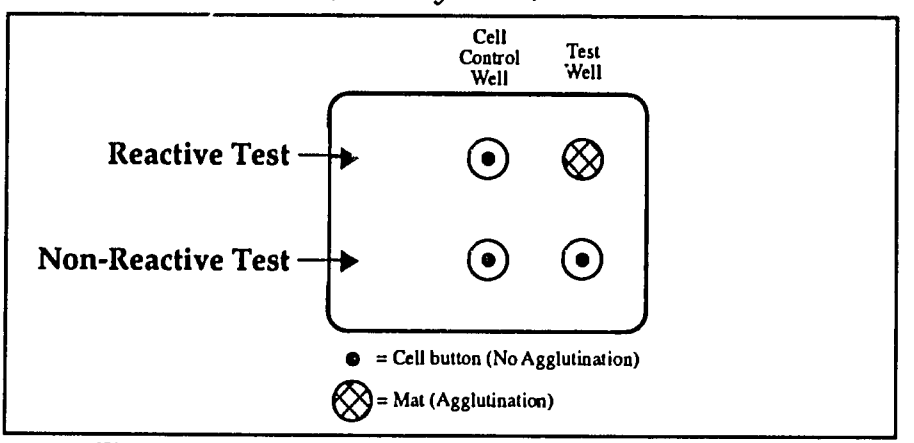

Figure 11. Schematic Illustrating Results of a PHA Test

Dot Blot Assays

HIV assays that incorporate paper or nitrocellulose as the solid support are usually referred to as dot-blot assays. This is because the antigen is passively blotted (absorbed) onto the support. Most often, the antigen is blotted as a small circle (dot), and is usually a recombinant or synthetic peptide antigen. A plastic device often holds the solid support and contains absorbent pads under the paper to collect the serum and reagents after addition.

Recently, a similar assay has become available that incorporates antigen coated microparticles that are trapped within the membrane. These microparticles are microscopic and are very efficient in carrying large quantities of the antigen due to their large surface area. In this assay, the reaction occurs on the microparticles that are subsequently collected on the paper so the resultant color reaction can be visualized.

Dot blot assays are rapid and easy to perform, but they are also expensive. Many yield results within five or ten minutes, some in as little as three minutes. Anti-human immunoglobulin conjugates attached to enzymes are commonly used to bind to the patient's antibody. The addition of a suitable substrate produces a color on the paper. One manufacturer uses a dye such as colloidal gold instead of a substrate, and conjugates the dye to a substance called protein A. Protein A has the ability to bind to human immunoglobulin, and therefore will attach to the patient's antibody. Because the dye is conjugated to this substance, a colored dot will appear on the paper if antibody is present in the sample.

Some of these dot blot assays contain a control dot indicating that the test is working properly, that all reagents are good, and all have been added in the proper order. This control is a human immunoglobulin on the solid support that should bind the conjugate (or protein A) to produce a color reaction. If this control does not result in a reactive result, the test is not performing as required. Figure 12 illustrates typical reactions occurring in the dot-blot assays. Recently, dot-blot assays have become available that have separate dots for HIV-1 and HIV-2.

Dot blot assays are convenient to use and can be a valuable asset in certain testing situations such as emergency rooms, blood banks, or autopsy rooms; however, if large numbers of sera are to be tested, their advantage as rapid assays is lost. Some of these tests can be stored at room temperature, and none require instrumentation.

Rapid assays are good tests, but because of their expense, their use may be limited.

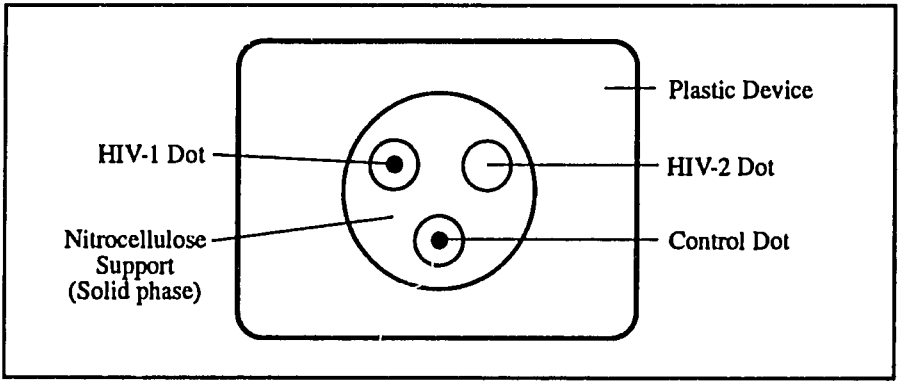

Figure 12. Typical Reaction by Dot Blot Tests of a Serum Positive for HIV-1

Recombinant and Synthetic Peptide Antigen-Based Tests

Assays based on viral lysate derived antigens are good tests but generally lack specificity (i.e. produce some false-positive reactions). This is due to the presence of contaminating components derived from the cells in which the virus was propagated. If an unknown sample contains antibodies which react with these contaminating components (e.g. antibodies against HLA antigens), a false positive reaction may occur. In addition, viral lysate tests are usually not 100% sensitive. This is most likely because cellular contaminating substances will also occupy sites on the solid phase, thereby decreasing the space that is needed for optimal quantities of the HIV antigen to attach. With less antigen attached to the solid support, less antibody can be detected and the indicator signal (color) will be low.

1. Recombinant based tests

Recombinant antigens are produced when a portion of the HIV genome is inserted into a biological vehicle (e.g. bacterial cells) resulting in the production of the gene product (antigen) by the modified vehicle. Since these vehicles can be grown easily in cell culture, they produce large quantities of the product.

The advantage of this technique is that the antigen of interest is the only antigen that is produced, since it was encoded by a particular gene. For example:

If the gene coding for gp120 were inserted into the vehicle, the antigen gp120 would be produced without any p24, p55, etc.

This results in the production of specific antigens that can subsequently be used to develop serologic tests with a high specificity (i.e. few false positives will be produced).

In most cases, antigens used in these recombinant antigen-based tests consist of mixtures of two or more of these recombinant antigens, or portions of antigens, in order to detect antibodies to several specific components. In addition, these recombinant antigens can be produced in large quantities and without the hazards associated with growing live HIV virus.

One disadvantage of this technique is that there are also some contaminates from the vehicle's components (i.e. the vehicle produces its own proteins.) The presence of these contaminating proteins may lead to some false positive reactions; however, efforts are made to partially purify the recombinant antigens. In most instances, these recombinant antigen-based tests are excellent.

2. Synthetic peptide antigens

Synthetic peptide antigens are the most recent addition for attempting to improve HIV testing technology. Tests based on this technology are gaining popularity. Once the amino acid sequence of an antigen (its basic building blocks) is determined, the antigen can be made in the laboratory by constructing it from a pool of free amino acids. These amino acids are combined in the same order as that of the original antigen. Furthermore, portions of antigens (epitopes) can be synthesized to yield antigens that are only reactive with specific antibodies.

Synthetic peptides often consist of a chain of only 10-30 amino acids. Serologic assays can therefore be developed using these highly specific peptides or mixtures of 2 or 3 of these peptides. The assays will only detect antibodies to the antigens (or epitopes) of interest. These synthetic peptides are synthesized using an instrument called a protein synthesizer.

Synthetic peptide antigens can be made in large quantities (grams), similar to recombinant antigens. They have the added advantage of being highly reproducible in a lot to lot manner. Since they are in a relatively pure form, there is a high signal to noise ratio (low background) when tested, and they are essentially devoid of

contaminating components (no host cell contaminants). **These qualities mean that synthetic peptide-based tests are extremely sensitive and specific, and probably the best choice for a high quality assay. Their specificity is adequate enough to differentiate HIV-1 and HIV-2.**

HIV-2 TESTS

Screening tests for the detection of antibodies to HIV-2 are identical to those for HIV-1, with one exception: the antigens that are incorporated. **To adequately detect antibodies to HIV-2, antigens from HIV-2 must be used.** Although many antigens are shared between HIV-1 and HIV-2, each virus contains specific antigens. However, these specific antigens may have the same molecular weights. For example: antibodies to gp120 of HIV-1 may not react with gp120 (or gp105) of HIV-2. This is evident when some HIV-1 positive sera do not react in HIV-2 screening assays and vice versa. In fact, only about 50-70% of positive sera will react with the antigens of the heterologous virus. Antibodies to the gag and pol components generally cross react to a much greater extent than antibodies to the env components:

If a test primarily incorporates env antigens from a particular virus, it will be more specific for detecting antibodies to that virus. Conversely, tests using primarily gag antigens will detect both infections more readily, but not always. This may or may not be advantageous.

Recently, tests have been developed using antigens from both viruses in order to detect either infection (see discussion of combination tests below). The differentiation of HIV-1 and HIV-2 infections can usually be accomplished using highly specific ELISAs (e.g. synthetic peptide-based), Western blots, radio immunoprecipitation assay (RIPA) or polymerase chain reaction (PCR) see Part 4.

HIV-1/HIV-2 COMBINATION ASSAYS

Antibodies to either HIV-1 or HIV-2 can be detected using assays designed to detect both. These combination assays usually incorporate env antigens from both viruses because it is the env antigens that are highly variable between the two viruses. Sometimes, the cross reacting gag proteins are also incorporated, but not exclusively since many non-specific reactions may occur. Some individuals have antibodies to p24 and are not infected with either virus (see Part 4).

Our laboratory has used combination assays with much success and on two occasions we have detected HIV-2 positive sera not identified using HIV-1 screening tests. There was no loss in sensitivity of these assays for detecting HIV-1 antibodies when compared to tests designed to detect antibodies to HIV-1 only.

At least five commercial companies are marketing HIV-1/2 combination assays. As with HIV-1 and HIV-2 tests, a variety of formats are available, including beads, microtiter wells, and dot blots. There are similarities and differences between the combination assays:

- all incorporate recombinant and/or synthetic peptide antigens in order to obtain adequate specificity;
- some use a combination of 2 synthetic peptide and others as many as 4;
- some use both recombinant and synthetic peptides simultaneously.

Most combination tests do not identify which antibodies are present (HIV-1 or HIV-2); a positive reaction only indicates that antibodies to one or the other are present. Some dot-blot combination assays are available that have separate dots containing antigens for each virus. With these tests, differentiation of HIV-1 and HIV-2 infections can be accomplished with one test. This saves time and money. Combination tests are more expensive than single detection tests, but are less expenesive than using separate tests for HIV-1 and HIV-2. Tests are under evaluation to detect HIV and hepatitis B simutaneously.

URINE AND SALIVA TESTS

Within the last 2 years, assays have been developed that use body fluids other than serum for detecting antibodies to HIV. Saliva and urine have been chosen since they are easily collected and do not require an invasive technique. These assays could be valuable in certain countries where needles are still reused for blood drawing (placing the individual at risk for infectious diseases eg. hepatitis, HIV). In addition, the collection of saliva and urine is much less expensive than the collection of blood. There have been some problems associated with developing these assays, however.

Although it has long been known that antibodies are present in saliva, the testing of saliva for antibodies has not been routinely adopted most likely because serological assays have previously lacked adequate sensitivity to consistently detect these antibodies. With the development of the newer assays (eg. ELISAs), sensitivities have dramatically increased.

The development of tests to detect antibodies in urine have not been previously attempted, probably because antibodies are considered too large (molecular weight) to pass through the kidney glomerulus, unless significant kidney damage has occurred. In addition, most researchers have considered that the pH of the urine would interfere with pH dependent immunological assays.

Recently, both urine and saliva have been used successfully for the detection of antibodies to HIV. However, the sensitivities of the tests are less than optimal. In general, the tests can only detect antibodies in about 92% of true infections. Many companies are actively pursuing the development of these assays and it may not be long before they are used routinely in laboratories. Some recent publications have indicated that the performance of tests using urine is approaching those of tests using serum. They have also indicated that the antibodies detected in urine are intact IgG molecules, not just fragments.

In conclusion, HIV antibody tests using urine and saliva are producing encouraging results. As technology proceeds, these fluids may become the choice for the detection of HIV infection.

SELECTION OF A SCREENING TEST

One of the most commonly asked questions is: "Which screening test is the best?" **At present there is not one single test that stands above all others. In addition, there is no combination of tests that can be most appropriate for all testing situations.** Tests offer different advantages and characteristics which must be considered in any particular testing situation. Some tests are very specific, while others offer great sensitivity; some are rapid but expensive; some require less expertise and equipment but are cumbersome to use if large numbers of sera are to be tested. Each laboratory must decide which test to adopt depending on their needs. As stated before, no test is perfect, but most offer equivalent sensitivities and specificities.

The choice of a test should not be determined on the basis of the manufacturer's claims, or even based on the studies of others. Claims and studies should be used as a guide in selection but adoption should be made on the basis of test performance in one's own laboratory setting if feasible (see Part 8). Test performance is known to vary between laboratories, probably due to differences in such variables as the quality of distilled water, temperatures, pressures, instrumentation, and laboratory technique.

There are a wide variety of factors to be considered in test selection:

1. Some testing situations may dictate that a test be used requiring no instrumentation, while other situations may need a fully automated test.

2. The shelf life of reagents and the storage conditions must also be considered.

3. In certain situations (such as blood donor screening) a test possessing high sensitivity (detects all positives) must be selected to ensure that the blood supply is safe.

4. In other situations (such as private physician offices where therapy may be instituted for a patient) a test must have a high positive predictive value (see Part 5).

5. Some laboratories may be able to afford the presumably better, more expensive tests (recombinant and synthetic peptide), while others cannot.

6. If HIV-2 infection is suspected in a geographical locale, perhaps the use of a combination test should be considered.

7. The characteristics of the population to be tested must be considered in the adoption of a test.

The choice of a test depends on the situation and the need. With such a large number and variety of tests available, each laboratory should be able to find an appropriate test to produce reliable results.

It is fortunate that so many commercial companies produce such a variety of HIV tests using different technologies. As these companies compete, technology is driven forward, and better tests become available. Perhaps we will eventually have tests that are essentially perfect for every situation.

AVAILABLE SCREENING TESTS

Because there are such a large number of HIV screening tests on the market, it is not possible to list all of them. Some tests are not available for use in all countries. Table 2 offers a partial list of some of the available tests that can be used to screen for antibodies to HIV. This list provides some basic characteristics of each test. Organizations such as WHO may be able to provide more extensive information on the characteritics of available screening assays.

Table 2
Conventional Screening Assays for HIV

Test Manufacturer	Recombinent HIV-1/HIV-2 Abbott	TESTPAK HIV-1/HIV-2 Abbott	Enzygnost Behring	Recombinent HIV-LA Cambridge BioScience	serodia-HIV Fujirebio	LAV-EIA Genetic Systems	Vironostika HIV-1 Organon Teknika	HIVCHEK 1+2 Ortho-Diagnostics	Immunocomb Bispot PBS Organics	Wellcozyme recombinent Wellcome Diagnostics
Principle	Indirect EIA	immunobinding "Dot blot"	Indirect EIA	latex agglutination	particle agglutination	Indirect EIA	Indirect EIA	immunobinding "Dot blot"	immunobinding "Dot blot"	competitive EIA
Format	beads	individual test cartridge +/- readout	microtiter plate	four tests per card	microtiter plate	microtiter plate	microtiter plate	individual test cartridge	12 test comb	microtiter plate
Antigen	recombinent proteins HIV-2/HIV-2	recombinent proteins HIV-1/HIV-2	synthetic peptides HIV-1/HIV-2	recombinent protein HIV-1 GP120/41	H9 infected cell lysate HIV1	purified inactivated HIV-1	viral lysate	recombinent proteins HIV-1/HIV-2	synthetic peptides HIV-1/HIV-2	recombinent proteins HIV-1
Additional supplies or equipment required	Abbott leader, washer, incubator micropipettors	none	microtiter reader, washer, water bath micropipetors	high intensity light	micropipettor	microtiter reader, washer incubator micropipettors	microtiter reader, washer incubator micropipettors	[illegible]	pipettors	microtiter reader, washer incubator micropipettors
Time required	2.5 hours	10 min.	2.1 hours	5 minutes	2.5 hours	3.5 hours	2.3 hours	10 minutes	40 minutes	1.5 hours
Relative* cost US$	1.75	4.00	2.30	3.00	1.20	.90	1.80	3.65	4.0	1.50

* Cost based on purchase of 10,000 tests in US$
This table is reflective of different types of technology available and is not meant to be all inclusive nor endorse these specific tests

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TESTS TO CONFIRM HIV INFECTION

Although HIV screening tests are adequate for identifying most HIV infections, they generally lack a sufficiently high degree of specificity. This means these screening tests are responsible for producing some false positive results (biologic false positives). **It should be remembered that screening tests were designed to protect the blood supply, and their sensitivity is adequate enough to accomplish that purpose. However, since their specificity may be less than optimal, results must be verified to substantiate HIV infection.**

As discussed previously, most widely-used screening tests are based on viral lysate technology, and consequently contain host cell contaminates. Because of the occurrence of false positives, **the results of the screening assays cannot, and must not, be considered as conclusive.** This is particularly evident when testing certain low risk populations, where the screening tests exhibit a poor positive predictive value (see Part 5).

Confirmatory (or supplemental) testing is highly recommended for samples that are repeatedly reactive for antibodies to HIV by screening assays. In some countries, confirmatory testing consists of retesting the repeatedly reactive sample using a second screening assay. The principle of the second screening assay should be different from that of the first. The objective in determining the best combination of assays is to detect all false positive sera as well as all true positives. For example, if an indirect ELISA were repeatedly reactive initially, then a repeatedly reactive result by a competitive ELISA is sometimes considered a final result of positive for HIV.

Most protocols, however, require the use of very specific assays such as the Western blot, indirect fluorescent antibody assay (IFA), or the radioimmunoprecipitation assay (RIPA). These tests are extremely specific, and if performed and interpreted correctly, should not produce biologic false positive results. They are, however, more laborious and more expensive than screening assays.

As discussed below, confirmatory assays may not always produce definitive results (i.e. do not always result in a positive or negative), and therefore, additional testing may be required before a conclusion can be made. **The primary purpose of confirmatory tests is to ensure that individuals who test reactive by screening assays are not incorrectly identified as being HIV infected.**

PRINCIPLES OF TESTS

Western Blots

The Western blot (WB) is probably the most widely accepted HIV confirmatory assay. The technique had been developed many years ago and was recognized as a very specific technique for detecting antibody to many infectious agents. Many authorities consider it the "gold standard" for validation of HIV results. It is at least as sensitive as the screening assays. The WB is very expensive and labor- intensive to perform, and therefore cannot effectively be used as a screening tool.

The WB technique owes its specificity to two factors: component separation and component concentration. As the mixture of viral components is separated into specific "bands", each component becomes relatively pure. In addition, the separation of antigens allows for the identification of specific antibodies to each of these antigens. In principle, it is conducted in three parts (see Figure 13):

1. separation of viral lysate antigens by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE);

2. transfer (blotting) of the separated antigens onto nitrocellulose paper; and

3. testing of the unknown serum sample directly on the blotted membrane using a technique similar to the ELISA (enzyme-substrate reaction).

Most WB presently used for HIV are supplied by commercial companies in kit form and only part 3 (above) is performed by the laboratory. The tests are easy to perform, but require strict adherence to procedure, and most importantly, the proper interpretation of results (see below).

Laboratories that develop their own WB may save money, but require facilities to propagate the virus, expensive equipment for electrophoresis and blotting, and must contend with the hazards associated with handling live virus. In addition, the use of preblotted strips from manufacturers has greatly reduced the variability associated with WB preparations. **In order to develop WB, large quantities of virus must be:**

- grown in culture,

- chemically treated for disruption and inactivation,
- separated in an electric field in the presence of SDS*,
- blotted electrophoretically onto nitrocellulose.

*SDS is an anionic detergent that denatures the viral components and yields proteins with net negative charges. These negatively charged proteins migrate in polyacrylamide gel according to their molecular weights.

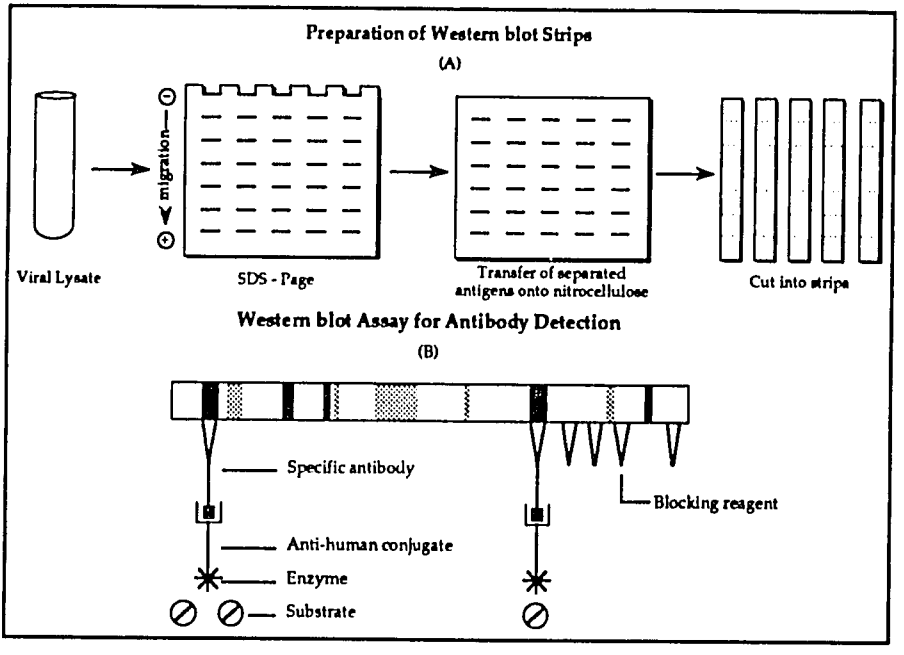


Figure13. Western Blot Principle
(A) Performed by manufacturer;
(B) Performed routinely in laboratory

The low molecular weight components migrate the fastest, and hence are found furthest from the point of origin (in most preparations, toward the bottom of the strip at the anode). Conversely, the higher molecular weight components migrate slowly, and stay near the origin (toward the top at the cathode).

After blotting on nitrocellulose, the viral antigens are separated as follows (from top to bottom): gp160, gp120, p66, p55, p51, gp41, p31, p24, p17, and p15. It is important to remember that other non-

viral components derived from the host cells in which the virus was grown are also present on the nitrocellulose. They can occur in many places, but often are near the middle molecular weight region (40,000 -60,000).

As mentioned before, commercial Western blot kits are supplied with individual strips of nitrocellulose that already contain the separated antigens. Each strip (all are identical) is used as a solid phase and an ELISA procedure (indirect) is performed directly on the strip (see Figure 13).

Test strips are placed in individual troughs of a plastic tray. Care must be taken to avoid splashing or contamination between wells. **In the initial step, each strip is wetted and incubated in the presence of a blocking solution** (usually albumin or a milk powder protein). The protein in this solution binds to the strip in areas where there are no components (i.e. no antigens or host cell proteins). Thus, the purpose of this solution is to prevent any human immunoglobulin in the sample from binding to unoccupied areas of the strip. Non-HIV derived immunoglobulin would produce a non-specific reaction when subsequent reagents were added.

Following this blocking step, the test sample is added and incubated for a specified time period. The plastic tray is placed on a rocker platform and gently moved during the incubation period. Incubation temperature varies with kit manufacturer as does incubation time. During this period, specific antibodies to the viral components will bind to their respective antigens. If the sample contains antibodies to p24 and gp41 for example, there will be binding only to those antigens. Subsequent washing will remove unbound serum components.

Conjugate addition follows and, again, incubation and washing. Most often, the conjugate is an anti-human immunoglobulin coupled to an enzyme (as in the ELISA screening tests). Other types of conjugates may consist of two separate components:

- an anti-human immunoglobulin coupled to a vitamin biotin (conjugate 1),

- followed by addition of a protein (derived from streptococcus) called avidin which is coupled to an enzyme (conjugate 2).

In this latter case, the biotinylated anti-immunoglobulin binds to the human antibody in the sample (if present) and the avidin-enzyme

reagent binds to this biotin. The advantage of this biotin-avidin system is an increase in sensitivity (detects smaller quantities of antibody) since many molecules of the second conjugate (enzyme reagent) can bind to the biotin - anti immunoglobulin reagent. This produces a greater visual signal that can be detected. An example of these WB conjugate reactions is depicted in Figure 14.

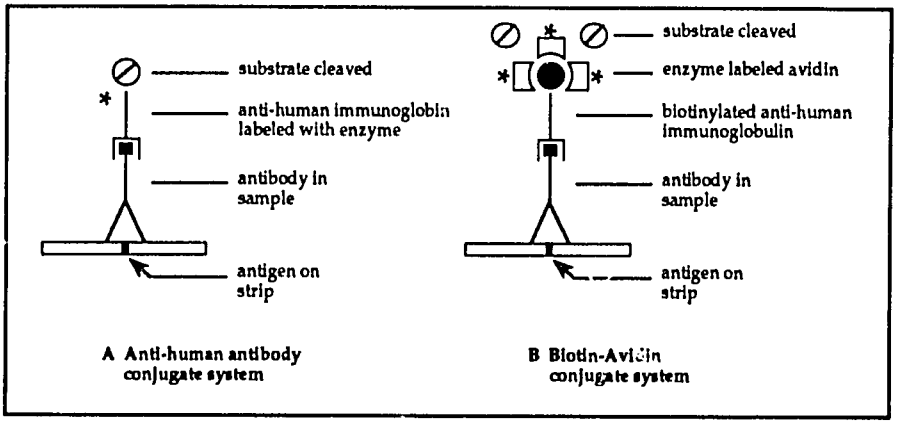


Figure 14: The Avidin - Biotin Conjugate System

Finally, a precipitable substrate is added to the system resulting in the formation of a colored end-product (a precipitate forms on the nitrocellulose strip) at the particular sites where specific antibodies have bound. Depending on the particular antibodies in the sample, band profiles will be produced. **The type of profile (the combination and intensity of bands that are present) will determine whether the individual is considered to be positive for antibodies to HIV** (see "interpretation of confirmatory test results".) Figure 15 is a schematic representation of positive and negative reactions by the WB.

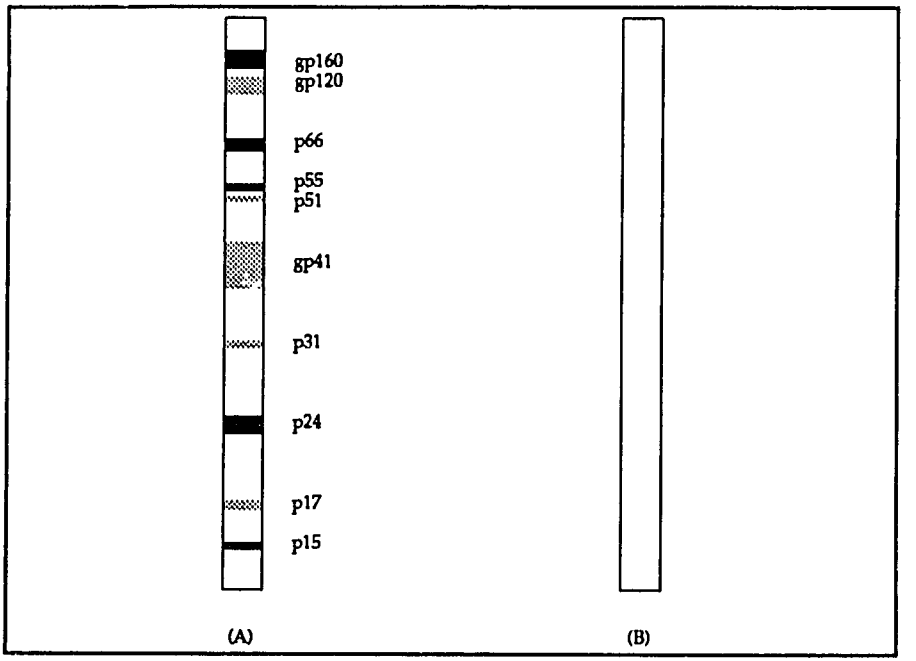


Figure 15: Schematic Representation of Western Blot Results
(A) Positive Result (Reactions to all Antigens)
(B) Negative Result

Care must be taken throughout the procedure to avoid cross contamination of test strips during pipetting and washing steps. As with other assays, clean plasticware is essential since chemical contamination can easily effect reactions.

As with all serological tests, the proper controls must be included with the WB assay. In particular, a weak positive control is used as a baseline for positivity (i.e. it produces the minimum intensity reaction required for a sample to be considered positive quantitatively). The controls must be run on the same lot of strips as the samples, since the relative position of antigens on strips may vary from lot to lot.

At the completion of each run, reactions should be read immediately, and then the strip can be stored after drying. If stored, strips should not be taped but enclosed in a plastic binder. Since the bands may fade with time, a written record of the reactive bands immediately after completion of the assay must also be included. The interpretation of WB results is discussed under "interpretation of confirmatory test results".

Line Immunoassays

The line immunosassays are second generation assays and have potential use as supplemental tests. Recombinant proteins and synthetic peptides are applied in band patterns on plastic support strips and tested in a manner similar to the immunoblot assay. These tests will be less expensive alternatives for validation and for differentiation of viral strains because protein bands representing more than one virus can be applied to the strip. This may be an important consideration where both HIV-1 and HIV-2 are present or suspected.

Indirect Immunofluorescence Assay (IFA)

The IFA test is used in many laboratories as the confirmatory test for HIV infection. However, it is also sometimes used as a screening test. This test is inexpensive, is simple to perform, and requires much less time to perform than many of the Western blot assays. The major disadvantages are the requirement for an expensive fluorescent microscope and the need for well-trained laboratory personnel to read and interpret the results. In principle, the IFA is similar to the ELISA and has the following characteristics:

- The solid support consists of a microscope slide containing cells (usually human T lymphocytes) that have been infected with HIV.
- As a control, cells are also included that have not been infected.
- The infected cells and control cells are fixed (attached) onto the slide in circumscribed areas referred to as "wells."
- Since reactions will take place on these fixed cells, the cells are referred to as substrates (not to be confused with enzyme substrates).
- The slides containing the substrate are usually packaged with four wells containing infected cells and four wells of uninfected control cells. Alternatively, a single well may contain both infected and non-infected cells. If all cells yield a positive reaction, this must be due to non-specific antibodies (see interpretation of results below.)

These slides should remain in their sealed packets and should be opened when at room temperature just prior to use; this will

prevent condensation from collecting on the cells, causing lysis of the cells. Also, care must be taken to prevent any contact with the surface of the wells, so that the cells are not disturbed. It is important to label each slide with a number or letter and to record on paper the location of each specimen on each slide (see Appendix). Diluted patient sera (usually 10-20μl of sample) are added to each well using a mechanical pipet.

- **An incubation** of usually 30 minutes at 37°C allows specific antibody (if present) to attach to the virus particles in the infected cells.

- **The slides are then washed** in a bath of phosphate buffered saline (PBS) for 15 minutes with one or two changes of PBS.

- **Following this, the slides must be dried** (by air or with a small fan).

- **Conjugate is then added** in the same manner as the serum.

The conjugate is an anti-human immunoglobulin labeled with a **fluorochrome** (fluorescein isothiocyanate, FITC). A fluorochrome is a substance that will fluoresce when exposed to UV (ultraviolet) light. This occurs when the molecules of the fluorochrome are excited to a higher energy level and emit light of a different wavelength as they return to the ground state. During the incubation with this conjugate (30 minutes, 37°C), the FITC-labeled conjugate will bind to the patient's antibody (if present in the sample). Another wash and drying step follows to remove any unbound conjugate. Buffered glycerol is added to each well (to decrease the refraction of light) and a coverslip is placed over the entire slide. The test should be read immediately but can be stored in the dark at 4°C overnight before reading. The slides are examined under UV light with the aid of a fluorescent microscope.

The viral particles are contained in the cytoplasm of the lymphocytes and therefore a positive (reactive) test will demonstrate cytoplasmic fluorescence. In contrast to the WB, the IFA will not identify antibodies to specific antigens; a positive reaction only indicates that antibodies to HIV are present. Since lymphocytes have large nuclei that occupy most of the cell, the fluorescence is generally seen as a small area in a wedge-shape. The intensity of fluorescence should be graded as 1+ (weak) to 4+ (strong). Appropriate serum controls will act as a guide. No fluoresence should be evident in non-infected control cells.

It is very important to recognize the staining pattern to identify possible fluorescence due to anti-nuclear, anti-mitochondrial, or other anti-cellular components. Several auto-immune diseases can result in the attachment of these antibodies, and will produce difficulty in reading, leading to the possibility of producing false-positive results (see interpretation of results below). A representation of the principle of the IFA method is depicted in Figure 16.

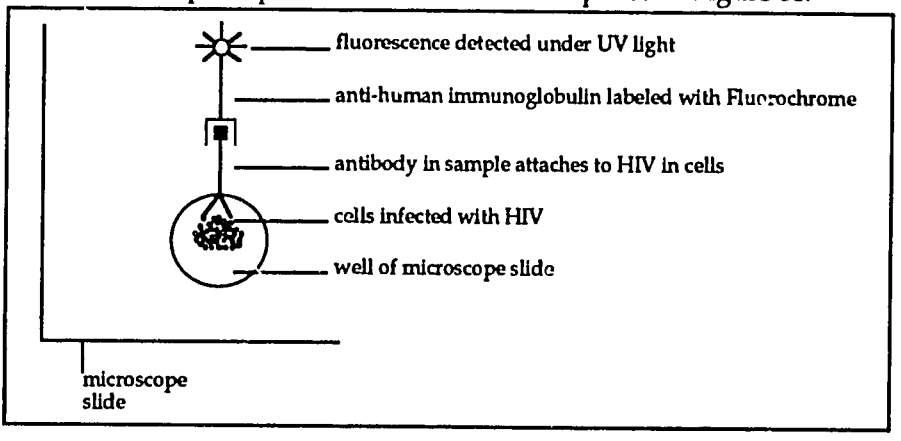


Figure 16: The Principle of the IFA Method

Radioimmunoprecipitation (RIPA)

The RIPA (or RIP) can be used in conjunction with, or as an alternative to the WB to confirm HIV infection. Since it is essentially a research technique and requires the use of radioactive substances, it is not used by many clinical laboratories. The RIPA is extremely sensitive. In addition, the antibody-antigen reaction occurs under non-reducing conditions, allowing the test to be very specific. In this technique, certain radio-labelled amino acids (e.g. cysteine) are added to cultures of HIV infected cells:

- As the virus replicates, it incorporates these labeled protein building blocks into its structural components.

- Subsequently, the viral particles that have now been labeled are collected, disrupted (lysed), and the proteins are solubilized.

- The viral lysate is added to the serum to be tested. The resulting immune-complexes are subsequently precipitated from solution by protein A (remember that protein A binds to human immunoglobulins).

- The labelled precipitate is then electrophoresed (SDS-PAGE) and

the separated labeled proteins are visualized by exposing them to a special film capable of detecting the label (a process called autoradiography).

Usually, the labeled antigens are the envelope glycoproteins gp160 and gp120. Both of these bands must be detected in order to ensure that the serum is positive. **The technique is sensitive for detecting antibodies to the envelope proteins, and therefore is very specific since antibodies to these components are present in virtually all HIV infected individuals after seroconversion.** Figure 17 indicates the RIPA principle.

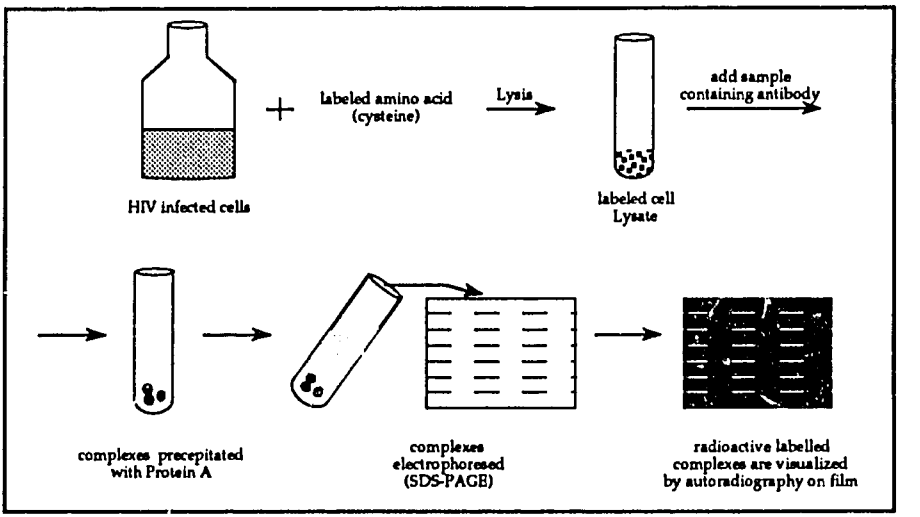


Figure 17: The RIPA Principle

Tests to Detect Circulating Antigen

The antigen test is most useful in detecting antigen in supernatants from cultures that have been inoculated with cells from a patient suspected of being infected (viral culture). In fact, the test was originally designed for this purpose. **The antigen test is the method of choice for detecting the presence of free antigen in culture, and it is two orders of magnitude more sensitive than the reverse transcriptase assay.**

The antigen test was not originally designed for the purpose of testing serum. Only about 50-60% of AIDS patients, 30-40% of ARC patients, and 10% of asymptomatic patients will be antigen positive. Nevertheless, this test is used occasionally for testing serum, and can be of value for the instances stated above.

As noted in Figure 5, HIV antigenemia does occur during certain times of infection. It is important to understand at what point during infection antigen can be detected, so that tests are not performed unnecessarily. A test for antigen may be useful in the following situations:

- early infection is suspected and the patient is seronegative;
- during late disease (when the patient is usually symptomatic);
- to monitor disease progression and/or therapy;
- to detect infection in a newborn where diagnosis is difficult due to the presence of maternal antibody (the detection of antigen in a newborn confirms infection and yields information of prognostic significance).

The most probable reason for the lack of sensitivity of this test in AIDS patients is that free antigen (p24) in serum may be complexed with p24 antibody. The test cannot detect complexed antigens. Currently, methods are being investigated that will break apart these complexes prior to antigen testing, in hopes of increasing the sensitivity of the assay. In addition, p24 antigen may not be readily detected by this test (particularly in asymptomatic infections) because the viral load (concentration of virus) may be low. If the virus is not present in high quantity or is not actively replicating, the amount of antigen in serum will be low.

The test is most useful for detecting viral antigen in culture fluids because there are large quantities of antigen in the fluid. The antigen test is relatively insensitive, being able to detect only between 50-100 pg/ml. This quantity of antigen is considered low, and these levels may not be present in the serum of infected individuals even when the virus is actively replicating.

The antigen test can be performed on fluids other than those of culture and serum. Evidence from the testing of cerebral spinal fluid (CSF) indicates that many patients with HIV dementia and encephalopathy have detectable antigen in CSF. This is most likely due to active replication of the virus in cerebral tissue.

It is important to realize that this test detects soluble p24 antigen, and does not specifically identify live virus. Just because a sample is negative for antigen does not indicate that the sample is non-infectious; similarly, the presence of antigen does not by itself

confirm that the sample is infectious. The only means available to demonstrate that a sample contains infectious virus is by isolating the virus using the culture method (see below).

The test to detect HIV antigen employs ELISA technology, but with the realization that antigen and not antibody is being assayed:

- A specific antibody (usually a monoclonal antibody to HIV p24) is attached to the solid phase.

- The serum is added, and if antigen is present in the serum, the antigen will attach to the monoclonal antibody.

- Following a wash step, conjugate (an antibody to p24 antigen coupled to an enzyme) is added and incubated.

A typical substrate reaction will subsequently detect the unknown antigen in the sample. Although all antigen tests utilize this principle, some require overnight incubations, while others are relatively short (total time of 3 hours). However, the tests are subject to false positive reactions, presumably due to interfering substances and other immune complexes.

The initial positive tests must be confirmed. This is accomplished using a specific antigen neutralization assay. This assay is relatively simple:

- The sample presumably containing the antigen is pre-incubated with a specific anti p24 antibody (neutralizing reagent).

- During this incubation, if antigen is truly present it will be complexed with the neutralizing antibody.

- Subsequently, the antigen assay is repeated (along with the same sample that has not been pre-incubated with antibody) and the O.D. readings are compared.

For the sample to be considered confirmed positive for antigen, the O.D. readings following the neutralization must be reduced by at least 50% (less than the O.D. readings of the non-neutralized aliquot). Since all of these assays are performed in duplicate or triplicate, nearly 1 ml of serum is required for a final result. The test is also relatively expensive. Figure 18 depicts the principle of the p24 antigen assay.

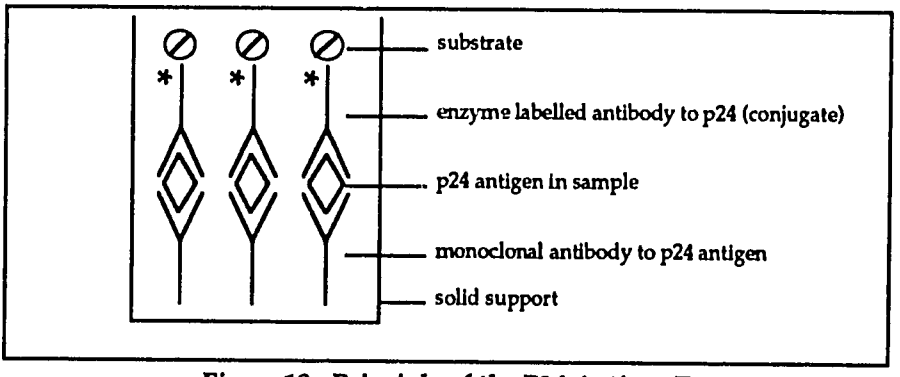


Figure 18: Principle of the P24 Antigen Test

In-Vitro Antibody Synthesis

Recently, success has been obtained by inducing HIV-infected cells in culture to produce antibody:

- Cells from the patient are separated, the resultant mononuclear cells are placed in cell culture and exposed to pokeweed mitogen (PWM).

- B cells can produce antibodies to antigens to which the cells have previously been exposed; therefore, if the patient has been infected with HIV, the antibody will be produced and exuded into the culture supernatant.

- Antibody can be detected after 4 days by routine antibody assays on the culture supernatants.

The technique may be valuable for detection of antibody during early infection before seroconversion. However, more evaluation is needed before this test is accepted as a standard procedure.

Viral Culture

Isolation of HIV from the blood is an unequivocal way of confirming infection. However, it requires time, is expensive, and is potentially dangerous. **The HIV isolation technique should only be performed by persons experienced in virology; it is <u>not</u> a standard diagnostic technique to be performed in routine laboratories.** Basically, isolation of HIV involves:

1. The co-cultivation of blood sample lymphocytes containing the virus with donor lymphocytes in the presence of mitogens and

interleukin 2, which stimulate and proliferate the CD4 cells.

2. Cell culture supernatants are then monitored for the presence of reverse transcriptase and/or HIV antigens beginning on day 7 (although peak virus expression occurs from 12-21 days post inoculation). The presence of RT or HIV antigens confirms the diagnosis; however; a negative result cannot rule out infection.

Even though viral isolation is ultimately confirmatory, the method's sensitivity is sub-optimal (i.e. some false negatives occur). Cultures must be held and monitored for about 4 weeks before a negative result can be reported.

Detection of Viral Nucleic Acids

Viral nucleic acids can be detected in HIV-infected lymphocytes by a technique known as **in situ hybridization.** This research technique takes advantage of the exquisite specificity between complementary strands of nucleic acid. Radiolabelled or enzyme labelled HIV RNA is usually used to probe mononuclear cells for the presence of HIV DNA. Due to the low number of proviral DNA sequences per cell, the technique is not very sensitive when testing peripheral mononuclear cells (i.e. infection may be present but the genomic copies are not detected since relatively few circulating cells are infected.) Therefore, **in situ hybridization is not sensitive enough to be used for routine diagnosis at this time.** It can be used effectively to detect HIV following the culture of infected cells in vitro; however, this does require several days.

Another technique can be incorporated to detect viral nucleic acid after amplification of the target RNA or DNA (viral), using a technique known as the **polymerase chain reaction (PCR).** This technique is extremely sensitive because a single copy of proviral DNA can be amplified and then be detected by the probe. The sensitivity of this method allows HIV infection to be detected prior to the detection by antibody assays. In fact, **PCR can detect infection before antigen tests and sometimes before viral culture. It is the most sensitive known method for identification of HIV infection.** PCR can be used to detect RNA or DNA, viral sequences in peripheral blood mononuclear cells, or in cells that have been cocultivated. In PCR, a pair of synthetic oligonucleotide primers (sequences of HIV nucleic acids) are used to detect a specific region and prime synthesis of DNA. The nucleic acids in the specimen are subjected to successive rounds of copying (perhaps 25-45 times) in order to produce multiple copies (millions) of the target DNA.

These multiple copies can then be detected using a probe, which is labelled with enzymes, radioisotopes or chemiluminescent signals.

The method can be simply explained in 4 steps:

1. denaturation of viral DNA

2. annealing of primers to DNA

3. primer extension

4. replication cycles

PCR is useful for:

- diagnosing early HIV infection,
- monitoring anti-viral therapy,
- diagnosing HIV infection in infants, and
- differentiating HIV-1 and HIV-2 infections.

In addition, quantitative PCR has the capability of determining viral load, which may give an indication of the prognosis.

The PCR technique is highly sensitive and specific, but is subject to technical false positives and false negatives if not performed with the utmost of care. It is not a simple procedure and requires careful and exact laboratory technique. The smallest amount of contamination can be amplified yielding false results. **At present, the PCR technique is not suitable for use in routine laboratories but it is an important research tool.**

Tests to Evaluate Host Lymphocytes

As described in Part 1, individuals with HIV infection and AIDS frequently demonstrate abnormalities in their immune system. This is not surprising since the virus infects cells and interferes with the normal interactions and regulation of immune cells. It is the T-4 (CD-4) lymphocyte that is most dramatically affected, and consequently brings about the severe immunodeficiency seen during the infection. **It is important to monitor infected individuals in order to determine their immune status and to gain some insight into**

the extent of involvement of the immune system. Often, the extent of involvement can be used as an indicator of disease progression. Severe changes can often be used to confirm HIV infection, if other reasons are ruled out.

There are several investigations that can indicate cellular immune status:

- Most often, these are cell quantitations for CD-4 cells, CD-8 cells, and sometimes CD-2 and CD-3 cells.

- In addition, the relative proportion of T-4 to T-8 cells (the T-4/T-8 ratio) can be a very valuable measurement.

In order to measure the quantities of these cells there must be a means to identify them. This is accomplished through the use of monoclonal antibodies. These monoclonals are specific antibodies with reactivities toward the various markers (T-cell antigens) on the surface of these cells:

- If a monoclonal against CD-4 is used, all T cells possessing this marker (i.e. T helper and T inducer cells) will be identified.

- Likewise, CD-8 monoclonals will identify T cytotoxic and T suppressor cells.

- Cells having the CD-3 marker are all T cells considered to be immunocompetent (functioning properly).

- CD-2 cells represent the total number of T cells, regardless of their capability to function.

By knowing the normal values for these subsets, it is easy to determine if the infection has compromised the patient's cells (and the immune system). The following list indicates the normal adult values (percents and absolute values) for each of the subsets, and their designations by different companies:

Cells	Marker	%	#
Total T cells	CD2, OKT-3	70-90	500-2200
Competent T	CD3	55-85	500-2500
T-helper/inducer	CD4, OKT-4	40-70	500-1600
T-suppressor/cyto	CD8, OKT-8	15-35	200-1030

Note that these numbers are only guidelines and that values may differ depending on the laboratory and the method used to quantitate. Normal values for these cells should be determined in each laboratory and in each population tested. Abnormal values in HIV infection and AIDS are noted in Part 1.

The methods used to quantitate these cells are generally referred to as **lymphocyte phenotyping** methods, with the phenotype or physical identification of the cells being determined. These methods are not easy to perform; they are also very expensive.

In general, specific monoclonal antibodies made against the specific antigen are labelled with a fluorochrome; these are incubated with an individual's mononuclear cells and the subsets are detected via a fluorescent instrument.

Some laboratories use a fluorescent microscope to count the numbers of each subset. In this case, one aliquot of cells labelled with one marker is counted first, followed by another aliquot using a different marker. This is a very time consuming and rigorous process.

Alternatively, a very sophisticated and expensive instrument called a flow cytometer can be used (flow cytometric analysis). This instrument is capable of automatically counting different subsets simultaneously as the cells flow through a detector. Usually, one cell type is labelled with one color fluorochrome, while another is labelled with a different color. The instrument can differentiate these fluorochromes and produce an accurate account of the various cell numbers. In principle, the flow cytometer uses a laser to identify cells as they pass through an orifice in a narrow stream of fluid. Cells can be identified not only by their markers but also by their size and granularity. Therefore, many different variables can be examined at the same time. The instrument is fully automated, but requires an operator thoroughly trained in its operation. The results obtained on the flow cytometer are much more accurate than the manual method because many more cells can be counted objectively, thus reducing error.

Lymphocyte phenotyping is a valuable investigation for individuals with HIV infection and AIDS. Unfortunately, the expense and the need for available experts to maintain and service a flow cytometer does not allow most laboratories to consider it for adoption.

INTERPRETATION OF CONFIRMATORY TEST RESULTS

Most diagnostic serologic assays produce results that can be characterized as positive, negative, or as yielding a titer. In some cases, a result may be close to the cutoff between positive and negative (**borderline reactor**). Even the determination of titers can yield a result that may be on the border between significant versus insignificant results; therefore, it is difficult to determine the true meaning of some serological results.

The serologic diagnosis of HIV infection is an example of a situation where the physician and the patient are expecting to obtain a conclusive result. Unfortunately, HIV results are not always either positive or negative. There are many times that a firm conclusion cannot be determined. In these instances, the results are labelled as inconclusive or **indeterminate.**

Since ELISA results are read spectrophotometrically, the results usually do not present a problem, unless the O.D. value is close to the cutoff (in which case additional testing is necessary). **Although there is not a consensus regarding the terminology for reporting of results, it is generally accepted that screening test results are reported as reactive or non-reactive, while confirmatory results are reported as positive or negative.** It is recommended that the terminology stated in the test kit package insert be followed.

Criteria for Positivity

Every serological test has guidelines or **criteria** to determine if the result fulfills the requirements to be labeled as positive, negative, or indeterminate. HIV testing has very stringent criteria, since results have such an important impact for the tested individual and society. These criteria are determined in two ways:

1. Manufacturers of test kits have predetermined the requirements for results based on studies of individuals who have been classified as positive or negative by other means (clinical status, PCR, etc.). They then adjust their controls so that the test will identify the maximum number of positives, while not producing many false positive results from individuals who are not infected. It is important that strict adherence to quality control measures be practiced to ensure optimal test efficiency.

2. Expert organizations exist that base criteria on large-scale evaluations. This is particularly true for the interpretation of WB

results, as is discussed below. Unfortunately, these organizations do not always concur on their recommendations.

Confirmatory assays are usually read subjectively by an individual. The individual must decide whether the result is positive, negative, or indeterminate. To complicate matters more, the WB assay incorporates separated antigens, so that there are many possibilities of reactions to identify and interpret. In addition, the interpretation of reaction intensities and overall patterns cannot be controlled objectively, and therefore variations in conditions and expertise among laboratories may cause further problems with results and interpretation.

The IFA test requires similar careful examination and interpretation to differentiate specific from non-specific reactions, and to detect very weak reactions.

1. Western Blot Criteria

The interpretation of Western blot results is usually definitive. It is now universally accepted that a negative result is the absence of all bands on the blot. Two organizations, including WHO, suggest that results can also be reported as negative if there is only a very weak p17 band. Unfortunately, sera from some noninfected individuals will show some reactivity to one or more antigens. This may occur in up to 15% of normal noninfected persons and many times occurs in persons who are negative by screening assays. Therefore, if ELISA negative sera are tested by Western blot, many will test as indeterminate. Most indeterminate results show only weak reactions to the gag proteins (mostly p17, p24 and/or p55); other patterns occur but are less frequent. Any Western blot reactivity that does not meet the requirements for being positive or negative (see below) must be considered as indeterminate. The significance of an indeterminate WB result is also discussed below (under inconclusive results).

Several organizations have formulated their own requirements for a Western blot result to be considered as positive. There are no universally accepted criteria. The non-consensus among these organizations is most probably due to uncertainty in the understanding of the antibody response to HIV. In some instances, there is inconsistency in the responses from some individuals. In addition, the incubation time may be long before

disease actually occurs; therefore, it is difficult to determine whether certain antibody profiles will eventually become indicative of true infection. For example:

- Some individuals who exhibit reactivity to p24 and p55 will later be shown to seroconvert, indicating that a p24 and p55 profile can be indicative of early infection.

- Conversely, there are other individuals who have the identical profile for long periods of time (years) and will never exhibit evidence of seroconversion or even seroprogression (ie. they are not infected).

Certain organizations such as the U.S. Food and Drug Administration (FDA) use criteria that maximize the specificity of the assay for persons in which there is little clinical or virologic information available. These criteria may not be ideal for other situations where patients have symptoms or are members of high risk populations. Inevitably, criteria that give the test a high specificity will sacrifice some sensitivity and vice versa.

In certain situations (e.g. institution of therapy for a patient), a high specificity is needed so that only HIV infected persons are treated. In other situations (e.g. detection of positive blood donors), a high sensitivity is mandatory so that no infected persons are incorrectly identified as negative.

Still other organizations, such as the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) and the Centers for Disease Control (CDC), choose criteria that yield the highest combined sensitivity and specificity (i.e. the highest correct percentage of positive results and the lowest number of indeterminate results). The FDA's criteria are the most specific, while those of ASTPHLD/CDC are the most widely used. **Occasionally, a specimen may be considered positive by one set of criteria, while being classified as indeterminate by another.** This should not be disturbing, since there are different reasons for adopting a particular criterion. Each laboratory must choose the criterion that best meets specified objectives for diagnosis.

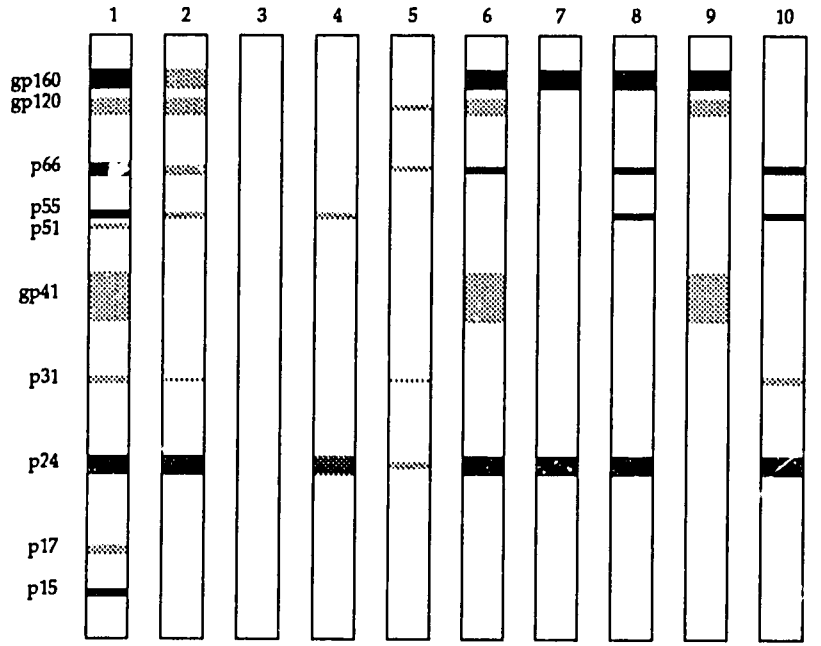
In the past, many different criteria existed for the interpretation of Western blot results. Table 3 provides a list of the different criteria currently in use and most widely adopted. Figure 19 shows a schematic representation of some WB results, and indicates the classification by the various organizations.

TABLE 3. Criteria for the interpretation of an HIV positive Western blot

Organization	Criteria
ASTPHLD CDC	Any two of: - p24 - gp41 - gp120/gp160
USFDA	p24 and p31 **and** gp41 or gp120/gp160
American Red Cross	At least 3 bands - 1 from each gene-product group: - GAG and - POL and - ENV
Consortium for Retrovirus Serology Standardization (CRSS)	At least 2 bands: - p24or p31, - **plus** gp41 **or** gp120/gp160
World Health Organization (WHO)	At least 2 ENV bands

The WHO Collaborating Group on HIV-2 has recommended a revision of previous WHO criteria for interpretation of HIV-2 Western blots. Their revised criteria for an HIV-2 positive Western blot are:

evidence of 2 or 3 ENV bands with or without Gag and/or POL bands



Key:

1. Strong positive control (positive by all criteria)
2. Weak positive control
3. Negative control
4. Indeterminate (p24 and p55)
5. Indeterminate (insufficient intensity of bands)
6. Positive by all criteria, except indeterminate by FDA
7. Positive by ASTPHLD and CRSS criteria, indeterminate by others
8. Positive by ASTPHLD and CRSS and Red Cross, indeterminate by FDA and W.H.O.
9. Positive by W.H.O. and ASTPHLD, indeterminate by others
10. Indeterminate by all criteria, suggestive of possible HIV-2 infection

Figure 19: Some HIV WB Profiles

2. Indirect Immunofluorescent Assay Criteria

The IFA test requires some expertise to read, but does not present the interpretative problems noted with the WB. Most laboratories follow the same requirements for positive, negative, and indeterminate results:

A negative result:

- A negative result can be reported if there is no cytoplasmic fluorescence at all in the infected or the non-infected control cells.

An indeterminate result:

- If the titer of reactivity is equal in the infected and the non-infected cells, or the fluorescence is in the nucleus, the result should be reported as indeterminate.

- Also, if the cytoplasmic fluorescence is only one two-fold dilution greater in the HIV infected well than in the control well, or equal to or at a higher titer in the control well than the infected well, the result is indeterminate. These latter possibilities would indicate non-specific reactivity.

A positive result:

- A positive result by IFA would be reported if:

- There is cytoplasmic fluorescence at the initial dilution in the infected well, but not in the control well.

- There is cytoplasmic fluorescence at two two-fold dilutions or greater in the infected well as compared to the control well.

Note: All reactive wells must exhibit at least a 1+ intensity reaction.

These criteria are the most widely adopted, but others may exist.

Inconclusive Results

As stated before, ELISA screening assays for HIV can almost always be reported as reactive or non-reactive since they are read objectively. Some screening tests such as the agglutination or dot-blot assays are read subjectively and may sometimes result in an indeterminable result. Confirmatory tests, however, can be positive, negative, or indeterminate. **An indeterminate result indicates that a specimen does not react in a manner that can be classified as positive or negative by that assay. It does not indicate that an individual is infected or not infected.** Therefore, it is important to realize that an individual with an indeterminate result should not be considered positive or negative until additional tests are performed, or until being retested and monitored at a later time.

Most authorities suggest that persons with indeterminate

results should be retested after several months, although a lesser period of time may yield a conclusive result.

The WHO recommends that persons be tested after 2 weeks if highly suggestive WB profiles exist.

If a person is retested over a period of 6 months and becomes negative or does not show an increase in reactivity, infection with HIV can be ruled out. For poorly understood reasons, many individuals continue to exhibit indeterminate results continuously, but are not infected.

If the individual does progress serologically (**sero-progression**) or converts to positive (sero-conversion), he/she was probably initially infected (early infection) and was most likely in the process of sero-conversion.

If a specimen exhibits sufficient reactions to antigens to be qualitatively positive, but the intensity of reactions is not strong enough to be considered positive, the person is probably in a state of sero-conversion and should be retested soon.

The significance of an indeterminate WB result varies depending on the risk factors and the clinical status of the patient, and the WB profile produced. For example, individuals with a history of high risk behavior are more likely to seroconvert later. In addition, some WB profiles are much more suggestive of early infection (eg. p24 and p55) than are others (e.g. p17 only). Many initially indeterminate results that subsequently become negative or remain indeterminate are probably a result of:

- Technical error (contamination or carry-over from pipetting or splashing);
- Non-specific reactions due to antibodies reacting to cell components of the infected cells (auto antibodies reacting to epitopes of human cells);
- Infection with an unknown related retrovirus.

The occurrence of non-viral specific bands presents difficulty in interpretation. It is recommended that these reactions be reported as inconclusive until follow-up testing is performed. Some organizations consider these reactions to be non-significant and report them as negative.

Also, it is known that some individuals with AIDS may lose reactivity to p24 later in disease, so that AIDS patients may have indeterminate WB results by some criteria. Ancillary tests may be helpful in resolving these indeterminate results. The laboratory should use all available tests to determine the true status of the individual. For example:

If the original result was produced by the WB, an IFA may subsequently be performed if available. Other tests may be helpful, including the newer synthetic peptide tests, antigen tests, and perhaps specific IgM assays. The latter may be positive if the patient is truly in early seroconversion (see Part 2). Additionally, tests such as the polymerase chain reaction (PCR), viral culture, and in-vitro antibody production assays can be performed (if available) to help elicit the patient's true status.

Some patients may be infected with HIV-2 and produce indeterminate results by HIV-1 assays; therefore, tests to detect HIV-2 infection should also be performed.

HIV-1 vs HIV-2

HIV-2 infections result in antibody responses similar to those of HIV-1 infections (i.e. responses to all major gene products). Although the actual molecular weights of the proteins and glycoproteins may differ slightly, both viruses have very similar structure and chemical composition. It is not unusual to expect cross reactions will occur. If an HIV-2 positive serum is tested on an HIV-1 assay, the results <u>may</u> be positive. In a number of HIV-2 positive sera, however, the results are negative or indeterminate by HIV-1 tests. By HIV-1 ELISAs, the O.D. readings of HIV-2 positive specimens may be high negative; by WB the results may be indeterminate. Therefore, it is important to recognize slightly high negative readings and suggestive indeterminate results, and consider testing the patient for HIV-2. When using HIV-2 tests, similar cross reactions may be observed when testing HIV-1 sera.

The most common antibodies that cross react between these two viruses are those directed toward the core proteins (eg. p24 and p55). Similarly, antibodies to the pol antigens may also produce cross reactions. The env antigens are more unrelated and therefore generally will not produce cross reacting antibodies.

It is estimated that 30 to 50% of patients infected by HIV-2 will have sera that cross react with HIV-1 antigens, depending on the assay used.

It is the policy in the laboratory of the authors to test all indeterminate sera by assays for HIV-2, unless there are only minor reactions to a few antigens (eg. p17, or p55). **Any indeterminate result with a reaction to polymerase antigens or envelope antigens constitutes a reason to test for HIV-2 infection** (see testing algorithms below). Figure 20 shows a representation of an HIV-2 positive serum tested on both HIV-1 and HIV-2 WBs and an example of OD readings by ELISAs.

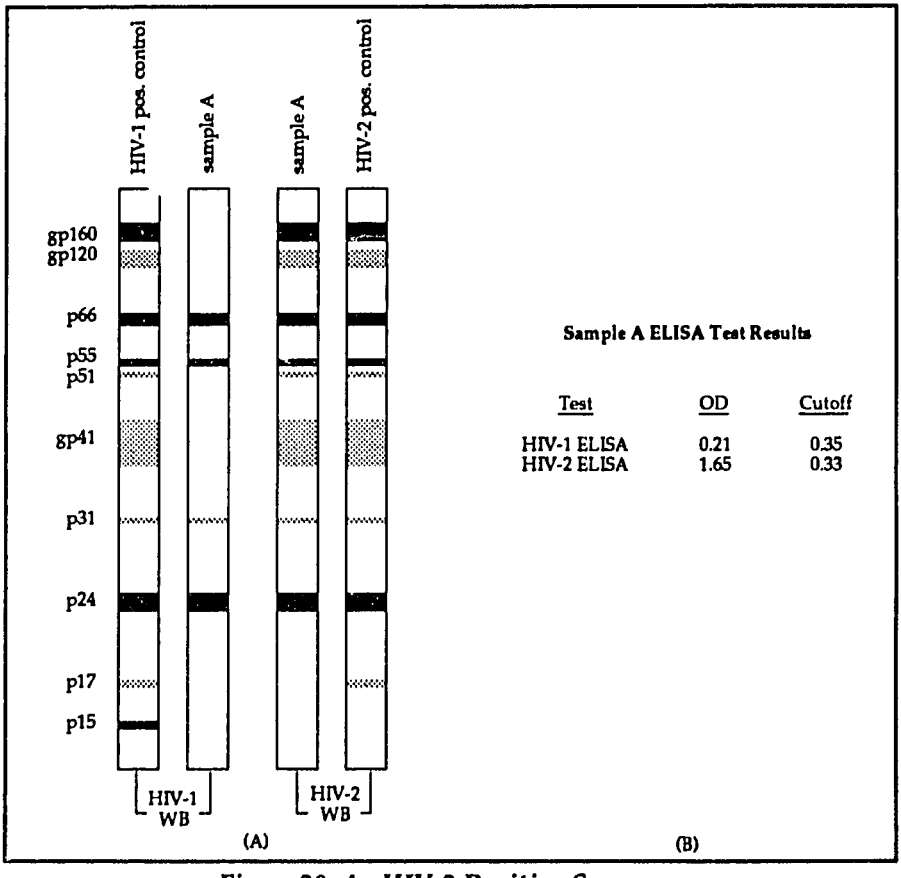


Figure 20: An HIV-2 Positive Serum:
(A) on HIV-1 and HIV-2 WBs,
(B) and by HIV-1 and HIV-2 ELISAS

ADVANTAGES AND DISADVANTAGES OF CONFIRMATORY TESTS

Confirmatory tests to detect antibodies to HIV are, in general, more expensive and more time consuming to perform than screening tests. Also, most tests accepted as effective confirmatory assays are read subjectively, and therefore require a certain level of technical expertise (experience and knowledge). The major advantage of confirmatory assays is their excellent specificity. If the tests are performed correctly, and with no technical errors, there should be few false positives. This specificity of the confirmatory tests is an absolute necessity, since the result of these tests will ultimately determine the fate of the individual. In addition, some confirmatory tests (e.g. the WB) can be used as a prognostic indicator. For example, profiles that show a decrease in anti-p24 over time may indicate disease progression.

The positive predictive value (see part 5) of a confirmatory or supplemental test is greatly increased because the prevalence rate of the sera being tested has increased, as only those sera reactive during screening are submitted for confirmatory testing.

Although the Western blot assay has the advantage of being able to indicate the actual antibodies that react with the specific HIV antigens, it is also the most expensive, the most time consuming, and the most difficult serologic test to interpret. In addition, it is an assay that is very technique dependent, since cross contamination can easily occur. **Although there are several disadvantages of the WB, it does yield the most information and is considered by many as the best and most reliable confirmatory test to detect HIV infection.**

The IFA test is also widely used by many testing centers and offers several advantages over the WB.

- It is easier to perform, is less expensive, and can be completed in a relatively short time period.

- Unless autoantibodies are present in the sample, the test is easy to read.

The IFA does require an expensive fluorescent microscope, some expertise to interpret the results, and more time to read the results (if large numbers of specimens are tested at one time). The sensitivity and specificity of the IFA are equivalent to the Western blot. As with the WB, standardized kits are available from commercial

manufacturers. When tests are produced "in house," the consistency of the reagents cannot be ensured, and results may differ between laboratories.

Certain screening tests exhibit a high degree of specificty and may be considered for use as confirmatory or supplemental tests. These tests are less expensive and often can be performed in peripheral laboratories, thus reducing the time waiting for confirmatory results. These tests do not require additional equipment or expertise. A test principle different than the screening test should be used for confirmation. For example, if an ELISA using a viral lysate is used for the screening test, a test incorporating a recombinant antigen should be considered for supplemental testing. Likewise a combination of indirect and competitive ELISA's may be considered. Usually, the non-competitive (indirect ELISA) is used as the screening assay followed by a competitive assay that is considered to be more specific. If the first test is positive while the second is negative, additional confirmatory test must be performed.

TESTING ALGORITHMS

Algorithms refer to the sequence in which tests should be performed. Although each laboratory must determine its own algorithms based on the available tests, there are some general guidelines for HIV testing. The two important points are:

Screening tests must be repeated in duplicate on all initially reactive samples. This is a measure to ensure that technical error did not occur (e.g. handling errors, or the testing of the wrong sample). The optimal situation would be to have the repeat tests performed on serum from the original specimen tube. For the sample to be considered as truly reactive, it must be reactive on at least two of the three tests.

All results that are repeatedly reactive on screening tests must be confirmed by a recognized confirmatory test. This is to ensure that the result was not due to a biologic false positive (e.g. due to non-specific antibodies such as those that may occur in some autoimmune diseases).

The most commonly used testing algorithm is to screen using an ELISA and to confirm repeatedly reactive specimens using the WB. This conventional strategy does have some technological, practical, and cost disadvantages:

- ELISA is not efficient for screening single specimens
- WB is expensive.
- ELISA is not appropriate for many small, poorly equipped labs.
- Western blot often results in indeterminate results.
- Both ELISA and WB often result in long delays for test reports.

Alternative algorithms to the conventional ELISA - Western blot have been evaluated by several laboratories and are currently being evaluated by WHO. Many labs have adopted these alternatives after careful evaluation. These alternatives include:

1. Use of ELISA and IFA;
2. Use of two ELISA tests (i.e. competitive and non-competitive, or a test using a viral lysate antigen combined with one using a recombinant antigen);
3. Combination of a dot blot or agglutination test with an ELISA;
4. Any of the screening tests supplemented with a line immunoassay;
5. Combination of two simple, rapid assays.

In order to establish an algorithm with two different ELISA assays, the screening ELISA (the first assay) should be the ELISA with the highest positive delta value. This delta value helps in selecting among ELISA's with equal sensitivity. The higher the positive (delta+) values, the higher the probability that this test will correctly identify antibody positive sera. A positive delta value (delta+) is a quantitative measure to express sensitivity. It helps in defining which serum is positive and how strongly positive it is, because it takes the optical density/cutoff (OD/CO) ratio into consideration.

The second ELISA used in this algorithm should be that ELISA which has the greatest negative delta value. The greater the negative delta value (delta-), the higher the possibility that this assay will correctly identify the true negative sera. For example: a negative delta value of -4 would have a higher possiblity of correctly identi-

fying the true negative sera than a negative delta value of -1.

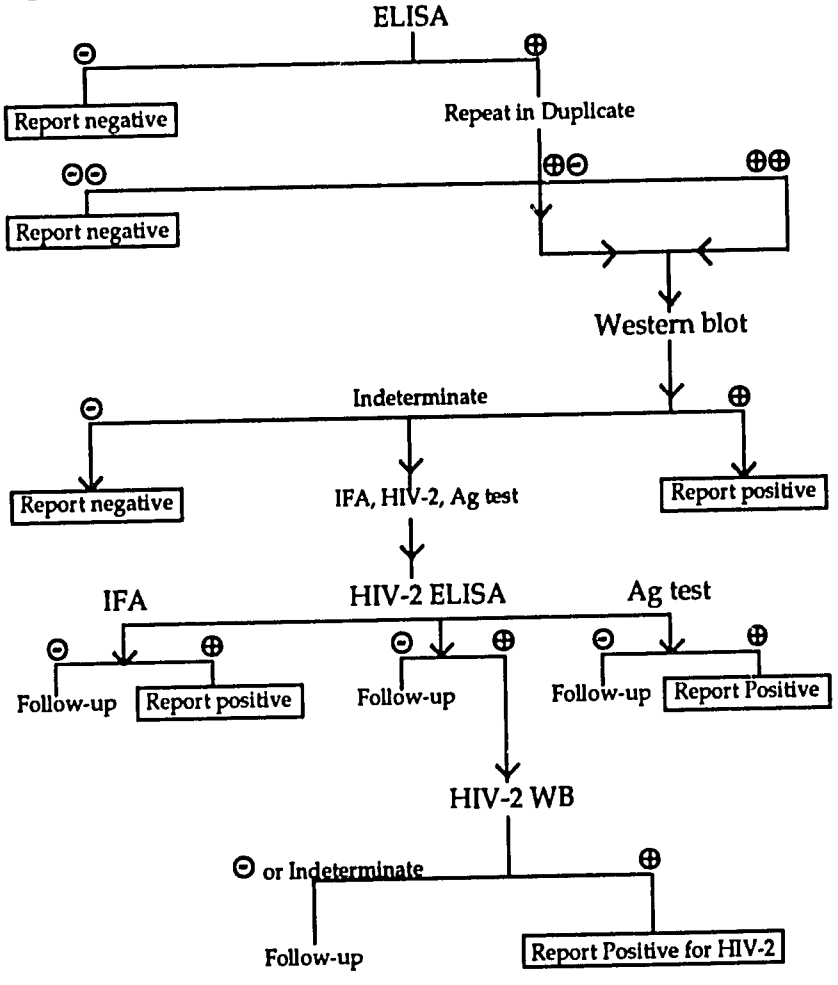
To calculate delta values, test sera on two different ELISAs until 50 sera have been found to be confirmed positive. The positive delta value for positive samples is calculated by dividing the mean OD ratio (log 10) of the positive samples by the standard deviation of the positive samples:

$$\text{OD ratio} = \frac{\text{OD sample}}{\text{OD cutoff.}}$$

The negative delta value for negative samples is calculated by dividing the mean OD ratio (log 10) of the negative samples by the standard deviation of the negative samples.

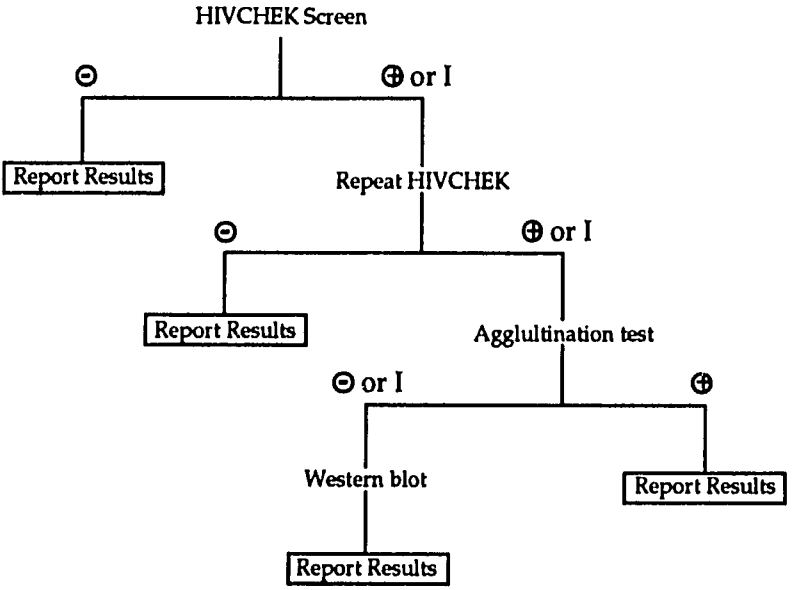
When establishing an algorithm, the screening test should be the most sensitive. The confirmatory test should be sensitive and extremely specific with a high positive predictive value. It is best to choose tests that are based on different principles or antigens. The presence of HIV-2 must be considered when adopting a testing algorithm.

Two examples of the many options for HIV testing strategies are presented below. The first is a guideline depicting th testing algorithm that is used by our laboratory:



NOTE: SPECIMENS FOR FOLLOW-UP SHOULD PROVISIONALLY BE REPORTED AS INDETERMINATE.

The second testing algorithm shown here is based on the need, in some situations, for a less expensive testing strategy. This alternative strategy, illustrated below, was evaluated by AIDSTECH/FHI and collaborators in Kenya, Ghana and Senegal, and Zaire.



It has been shown that up to 80% cost savings can be gained by using two rapid, simple tests in combination (as opposed to the conventional ELISA - WB) with minimum loss of sensitivity and specificity. However, it is critical that each laboratory or national program carefully evaluate a testing algorithm in their own setting before adopting it for routine use. Careful monitoring through quality control and proficiency testing is essential.

Table 4 indicates a partial list of conventional confirmatory tests.

Table 4
Conventional Confirmatory Assays for HIV

Test Manufa .urer	Envacor HIV-1 EIA Abbott	Novopath Immunoblot Bio Rad	Retro-tek IFA/HIV Cellular Products	Page-blot HIV-1 Genetic Systems	InnoLia Innogenetics	HIV Western blot Ortho Diagnostics
Principle	Competitive EIA	Western blot	Indirect immuno fluorescense	Western blot	Immuno-binding	Western blot
Format	beads	nitrocellulose test strips	fixed cells on slides	nitrocellulose sheet	line immunoassay	nitrocellulose test strips
Antigen	recombinant ENV and core antigens	purified separated HIV proteins	HIV-infected cells	purified separated HIV proteins	recombinant proteins, synthetic peptides HIV-1/HIV-2	purified separated HIV proteins
Additional supplies or equipment required	Abbott reader, washer micropipettor	rocker platform, micropipettors vacuum pump	incubator, micropipettor, fluorescent microscope	G.S. Integra micropipettors water bath	agitator aspirator micropipettor	rotary shaker, aspirator, micropipettor
Time required	21 hours	2.75 hour	2 hours	2 hours	4 hours	24 hours
Relative cost US$	8.0	20.0	8.0	18.0	20.0	40.0

This table is reflective of different types of technology available and is not meant to be all inclusive nor endorse these specific tests

The operational characteristics of over 35 HIV assays have been evaluated and the results are contained in a series of WHO reports. GPA/BMR/89.4 entitled "Operational Characteristics of Commercially Available assays to Determine Antibodies to HIV-1, Geneva, March 89 and GPA/BMR/90.1 "Operational Characteristics of Commercially Available Assays to Determine Antibodies to HIV-1 and/or HIV-2 Report 2", Geneva, April 90. These reports are available from WHO, Global Programme on AIDS

PART 5
INDICATORS OF THE VALUE OF DIAGNOSTIC TESTS

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INDICATORS OF THE VALUE OF DIAGNOSTIC TESTS

It is appropriate to once again question "Which test is the best?" (see part 3, selection of a screening test). The answer is: "It depends on the specific testing situation." Obviously, **there is no best test, otherwise, everyone would use that test exclusively.** Certain tests perform better in some geographic locations. For example, certain sera may contain antibodies to a variety of parasitic organisms and may produce a significant number of false positive results. Our laboratory has noted differences in test performance depending on the origin of the sera. The value of other tests may depend on the expertise of the laboratory workers. If a laboratory worker has not had the proper training for conducting the IFA for example, the results may not coincide with other tests and therefore the IFA will be considered as an inferior test. Other tests may not perform optimally if the conditions in the laboratory are not optimal (e.g. ambient temperature varies from the requirements.)

There are general guidelines for conducting evaluation studies and controlling for these differences (see Part 8). There are parameters that can be used to determine the relative usefulness or efficiency of a particular test, assuming that each test is:

1. evaluated by properly trained individuals;

2. performed exactly as required by the manufacturer;

3. evaluated on the same population of sera; and

4. performed under the same testing conditions.

In HIV testing, the presence of infection is not obvious; therefore, it is important to realize in determining the value of a test, it must be compared to a **reference test** or "gold standard." It may be that the new test is actually better than the reference test, and the seemingly false positives and false negatives may actually be true positives and true negatives. Therefore, additional testing may be necessary to resolve discrepant results. **The test parameters to describe the usefulness of a test are sensitivity, specificity, efficiency, and predictive values.**

SENSITIVITY

The sensitivity of a diagnostic assay can have two different meanings:

It can explain the ability of a test to detect very small amounts of the analyte (e.g. antibody). For example, if one test can detect antibody while another cannot, the former is more sensitive. Similarly, if one test produces a result of a titer of 256 while a second test only detects a titer of 64, the first test is detecting more antibody and is more sensitive. Many laboratories will dilute positive sera and try to determine which test is more sensitive (able to detect smaller quantities of antibody which might therefore detect seroconversion at an earlier time).

The second meaning of sensitivity is the one that we will be interested in for our discussion; that is: the ability of a test to detect positive cases (the absence of false negatives). This differs from the first definition in that a test may be able to detect very small quantities of certain antibodies, but may still miss some positive cases due to the absence of the proper antigens in the test.

The sensitivity of an assay can be calculated relatively easily using the following formula:

$$\text{SENSITIVITY} = \frac{\text{TRUE POSITIVES}}{\text{TRUE POSITIVES} + \text{FALSE NEGATIVES}} \times 100$$

For example:

100 sera are tested
5 sera are true positives (infected)
95 sera are true negatives (non-infected)

If the test reveals only 4 positives and one false negative as compared to the reference test:

$$\text{Sensitivity} = \frac{4}{4+1} = .80 \times 100 = 80\%$$

Note: true positives in the formula indicate the number of true positives detected by the test.

SPECIFICITY

The specificity of an assay is the ability of the test to identify all negatives correctly (i.e. produces no false positives). Most screening assays for HIV are not 100% specific. The specificity of an assay can be calculated from the following formula:

$$\text{SPECIFICITY} = \frac{\text{TRUE NEGATIVES}}{\text{TRUE NEGATIVES} + \text{FALSE POSITIVES}} \times 100$$

For example:

100 sera are tested
5 sera are true positives (infected)
95 sera are true negatives (non-infected)

If the test reveals 6 positives (one false positive) as compared to the reference test, the specificity is calculated as follows:

$$\text{Specificity} = \frac{94}{94+1} = .989 \times 100 = 98.9\%$$

Note: true negatives in the formula indicate the number of true negatives detected by the test.

TEST EFFICIENCY

Test efficiency refers to the overall ability of a test to correctly identify all positives and negatives (the absence of false positives and false negatives). It is a combination of the sensitivity and the specificity of an assay and gives an idea of the total effectiveness of the assay. It is determined as follows:

$$\text{TEST EFFICIENCY} = \frac{\text{TRUE POS.} + \text{TRUE NEG.}}{\text{TRUE POS.} + \text{FALSE POS.} + \text{TRUE NEG.} + \text{FALSE NEG.}} \times 100$$

In our example above:

100 sera tested
5 true positives (infected)
95 true negatives (non-infected)
1 false positive (test results)
1 false negative (test results)

Therefore:

$$\text{Test efficiency} = \frac{4+94}{4+1+94+1} = .98 \times 100 = 98\%$$

$$NPV = \frac{945}{945 + 5} = 99.5\%$$

Note: True positives and true negatives in the formula indicate the number detected by the test.

With the same test results (same number of false positives and false negatives) in a different population, the following is noted:

Population #2: where the prevalence of infection is low (0.7%)

1000 sera tested
7 True positives (infected)
993 True negatives (non-infected)
5 False positives (test results)
5 False negatives (test results)

Therefore:

$$PPV = \frac{2}{2 + 5} = 28.6\%$$

$$NPV = \frac{988}{988 + 5} = 99.5\%$$

Note: True positives and true negatives in the formula indicate the number detected by the test.

As is dramatically shown, using the same test that yields the same number of false positives and false negatives produces a different positive predictive value when testing two different populations.

The specificity of the test in population #2 is an excellent 99.5% (988 / 988+5).

However, the chance of a positive result being a true positive is only 28.6% (2 true positives detected by the test and 5 false positives).

This means that if a patient has a positive result, only one chance out of four will a result indicate a truly infected individual (a guess would yield a better chance!). Therefore, the specificity of the test does not indicate the usefulness of the test in this low prevalence population; the predictive value must be determined.

PART 6
QUALITY CONTROL AND QUALITY ASSURANCE

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NECESSITY AND IMPORTANCE

The validity of diagnostic test results produced in each laboratory is entirely dependent on the quality of the measures employed before, during, and after each assay. **Consistency in the production of good results requires an overall program that includes quality assurance, quality control, and quality assessment.**

- Measures to control quality in an HIV diagnostic laboratory are extremely important, because the consequences of either a false positive or a false negative result can be extremely destructive:

- a person falsely labeled as negative for antibodies to HIV may unknowingly continue to infect other individuals;

- a person who is falsely labeled as positive may be subjected to discrimination, may lose employment, may be outcast by family, and in some cases may even commit suicide.

The entire effectiveness and reputation of the laboratory are dependent upon the quality of work generated. There are many variables that can (and will) effect quality. Examples include:

- the competence of the personnel;

- the quality of the kits and equipment;

- the conditions of the specimens;

- the controls used with the runs;

- the interpretation of the data;

- the transcription of results; and

- the reporting of results.

QUALITY ASSURANCE

Quality Assurance (QA) is defined as the overall program that ensures that the final results reported by the laboratory are correct (as accurate as possible). QA includes every parameter affecting test results and the final reports. Quality assurance is dependent on a good quality control program and is verified by a good quality assessment program. QA also includes such factors as:

- reporting results in a timely manner;
- being sure that the results are reported to the appropriate individual;
- making sure the laboratory is functioning in the most efficient way;
- using the most reliable tests;
- reviewing transcriptional measures;
- inspecting specimens; and
- verifying final reports.

QUALITY CONTROL

Quality Control (QC) refers to those measures that must be included during each assay to verify that the test is working properly. Thus, QC indicates that each run produces acceptable results. It includes those measures indicating that all assay conditions are met satisfactorily.

All controls must be treated in exactly the same manner as unknown samples to validate assay performance. The controls are run simultaneously, and under the same conditions as the unknown samples. Upon completion of the assay procedure, the controls and samples are examined using the same criteria for interpretation. When the known controls produce acceptable results the QC indicates that:

- the assay is valid;
- all test conditions have been met;
- all test results are reliable.

QC does not, however, indicate that your results are accurate. This depends on the characteristics of the tests themselves. QC does not indicate that the results have been reported properly, or reported on the correct patient.

QUALITY ASSESSMENT

Quality assessment is a means to determine the quality of results. It is usually an external evaluation of a laboratory's performance by incorporating proficiency panels as the means of evaluation. Quality assessment is a challenge to the effectiveness of the quality assurance program. A good QC/QA program may be a substitute for quality assessment in some situations; however, quality assessment is never a substitute for good QC/QA.

For a laboratory to be considered a respected testing facility, it must be a laboratory that can always produce excellent results, without error. If good quality control and quality assurance are diligently practiced, the laboratory will be able to meet the needs and expectations of the medical community by producing highly reliable results. A discussion of methods for quality assessment is presented later.

GUIDELINES FOR QUALITY TEST RESULTS

1. Each test run must include QC.

2. Each test run requires a set of controls to validate the results; these controls must perform within the limits of the manufacturer's criteria for acceptability.

3. Any run not having at least the minimum number of controls falling within the acceptable range is invalid and MUST be repeated (see kit insert).

4. All test kits MUST be used within the expiration date stated, to ensure valid results.*.

5. Physical parameters of the test such as incubation time and temperature must be followed to insure proper performance.

6. All specimens to be tested must be inspected upon receipt, and before testing, to ensure that they are suitable. Lipemic, hemolyzed, or contaminated samples should not be used. If they are used, a note must be included on the reporting form and should indicate that the results may not be valid. A new specimen

should be requested for repeat testing.

7. Serum or plasma are the specimens of choice. Some laboratories save the original tube (with the original label) for retesting in the case of a positive HIV result.

8. Insure that all tubes of blood received in the laboratory are properly labeled before acceptance. Information on the specimen label should include the full patient name at the minimum, but preferably also the collection date, submitting physician, and patient identification number if applicable.

9. If a serum sample is frozen before testing, it is essential that after thawing, the sample be well-mixed before testing.

10. All test results, controls, and records must be checked and re-checked before reporting results.

*NOTE: In some testing situations, expired kits may be the only resources available, especially in more remote testing areas. In these situations expired kits may be used as **a last resort as long as strict QC is practiced.** If all test parameters and controls still fall within expected limits, the results can be assumed as valid. It should be understood that this practice is not acceptable under ordinary circumstances , and indeed, may not be permitted by the regulatory agencies of some countries. Therefore, this practice is only considered as a last resort when no other supplies are available or expected in the near future. If expired kits are used and the QC is adequate, positive individuals should be confirmed as is customary. All test results using expired tests should be carefully noted in records and reports, and retested if possible. Even with adequate QC, results may not be valid.

MONITORING QUALITY

Internal Controls and External Controls

Ordinarily, each HIV test kit has a set of positive and negative controls that are to be included in each test run. These controls included with the test kit are considered **internal controls,** while any

other controls included in the run are referred to as **external controls:**

- Internal controls are essential for quality control measures for each run and are intended for use only with the lot number of the kit from which they originated.

- External controls should be included on each HIV run to monitor consistent performance and lot to lot variation between kits. These controls may be purchased commercially, made from pooled test kit controls in the laboratory, or made from pooled sera. In some testing situations, pooled test kit controls or pooled sera (in-house controls) may be the best choice for economic considerations. If pooled human sera are used for HIV positive or negative controls, the sera should be filtered using a 0.2 um. biological filter to remove contaminating bacteria. Heat inactivation at 56°C for 30 minutes reduces residual HIV infectivity to below detectable limits (although it is uncertain if all virus is destroyed).

Since the test controls are included with all runs, the mean optical density (O.D.) readings of internal controls, external controls, and cutoff values during each run allow for monitoring of lot to lot performance (see HIV quality control chart). The most important external control to include is a borderline reactor. This calls attention to any minor changes, especially around the cutoff. **Any change in the status of a borderline control will indicate the potential for unknown samples with O.D. values near the cutoff to be falsely labeled as positive or negative.** Ideally, strong positive and negative external controls should also be included.

The borderline or low positive reactor can easily be produced by serially diluting a known positive serum and assaying the dilutions (see dilutions in Part 7). Use normal serum (HIV negative) as the diluent rather than normal saline (or other serum diluents) to keep the proteins in a natural environment. Once the titer is determined, the dilution is selected that produces a value just slightly above the cutoff for indirect ELISA:

Do not select a sample with a value so low that the O.D. fluctuates above and below the cutoff due to normal variations.

Do not select a sample that is too strong, since a control with a strong O.D. (well above the cutoff) would be of limited use for borderline monitoring.

Once the selection is made, aliquots are prepared and stored as indicated below:

1. Serum or plasma to be used for external controls should be in sufficient quantities to last for 6-12 months.

2. Sera should be homogeneous, sterile, and contain no preservatives.

3. These controls should be aliquoted and stored at -20°C in a non-self defrosting freezer. The aliquots should contain sufficient volume for one week's testing.

4. Once thawed, the aliquot should be stored at 2° - 8°C and discarded at the end of the week, if not completely used.

Another method to monitor quality using internal and external controls is to repeat one control (either internal or external) in triplicate on the same run and compare the results; this will show **"intra-run reproducibility"**. Differences usually indicate pipetting errors, but may indicate equipment failure. Also, a few controls could be run on three consecutive days to evaluate **"inter-run reproducibility"**. By either of these methods variations should not exceed 10%.

How to Determine Acceptance of Control Values Using Statistical Methods

Values for the internal (test kit) controls, the external control, and the cutoff should be monitored by quality control charts. **Subtle changes in control relationships are more easily observed with a visual presentation of control values over time rather than data from a single day's run.** The discussion below will describe the method and necessity for monitoring runs by using external controls.

It is beyond the scope of this chapter to present a comprehensive review of statistics, but several basic statistical tools that may be used to determine if external control O.D. values are acceptable are presented here. Acceptable OD values can then be used to monitor laboratory performance.

Linear graph paper is used to plot quality control values over time:

The y-axis represents optical density (O.D.) units or O.D./cutoff ratios.

The x-axis represents either the run date or run number.

Many test kit inserts establish the range of internal controls and define how to handle out-of-range values. However, several statistical values need to be established for external controls. The objective is to monitor the external control during each run to determine if results are as expected and are acceptable. To accomplish this, limits of acceptability must be determined. These limits are determined by using several statistical tools:

the mean;

the standard deviation;

the coefficient of variation.

Mean

The arithmetic mean ($\overline{X}$) represents the average value of a set of O.D. values and is easily calculated. Let the symbol "X" represent O.D. values, and the subscript number represent each repeat of the control. Therefore, the sum of the O.D. values may be represented as $X_1, X_2, X_3 \ldots, X_n$, and the symbol (N) will represent the total number of O.D. values. The sum of all X (or ΣX) values divided by the total number of values (N) equals the mean, and is represented by the formula:

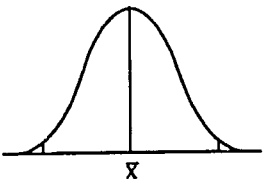
where the mean $\overline{X} = \dfrac{X_1 + X_2 + X_3 \ldots, X_n}{N} = \dfrac{\Sigma X}{N}$

example: if ($X_1 = 0.675, X_2 = 0.598, X_3 = 0.702$)

$$\frac{0.675 + 0.598 + 0.702}{3} = 0.658$$

To be statistically significant, the mean value of an external control should be obtained after testing the control on at least 15 to 30 runs. However, positive controls can be extremely variable and may require 100 or more values to establish a reliable mean. In general, the greater the differences between particular O.D. values and the mean (referred to as deviations from the mean), the greater are the number of values required to get a valid control mean.

The degree to which the numerical data tend to disperse about the mean can be represented as a bell shaped curve called a gaussian (or normal) curve. The curve gets its bell shape from the fact that more values are found near the mean and increasingly fewer values are found away from the mean.

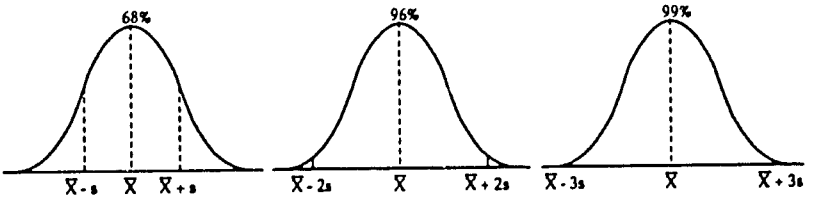


Standard Deviation

The standard deviation (s) is a measure of this variation or dispersion and defines the expected range of a control in relation to the mean value. The standard deviation of a set of O.D. values can be represented as "s" and is defined by the formula;

$$s = \sqrt{\frac{\Sigma(Xn - \overline{X})^2}{N}}$$

To establish an acceptable range for external controls, first determine the mean value of the control and then calculate the standard deviation. The numeric value obtained by this formula represents one standard deviation. The mean value plus or minus one standard deviation establishes a range around the mean where approximately 68% of all values would be expected to fall. Similarly, the mean plus or minus two standard deviations and three standard deviations represents the range where 95%, and 99% of all values would be found respectively. The figure below represents the approximate normal distribution of an external control value that can be expected at different standard deviations.



Specifying an interval within which it may be asserted with some known degree of confidence that the population mean lies is what is commonly referred to as a **confidence interval**. The confidence interval of one standard deviation is approximately 68%. This means that on any given run an external control would be expected to fall in this range 68% of the time, while 32% of the time it would fall outside of this range due to **random error** (or chance), even if

the run is performed perfectly. Any control outside one standard deviation may or may not represent a significant QC problem, since 32% of the time it is random error while 68% of the time it is significant error.

However, if the external control range is based on one standard deviation, an external control falling outside of the range of one standard deviation would have to be rejected, as it is more likely that the aberrant control value was due to an actual or non-random error (occurring due to a problem with the assay).

We recommend a range of two standard deviations above and below the mean for evaluation of external controls. Thus, any control that falls within this range will have the level of confidence of at least 95%; only 5% of any set of control values will fall outside of this range due to chance (random error). This means that when compared to a control outside one standard deviation, such a value outside two standard deviations has a greater chance of representing a significant quality control problem rather than random error alone (95% vs. 68%). It is permissible to use three standard deviations as well; however, any run with an HIV external control value that falls between two and three standard deviations should be carefully scrutinized.

Coefficient of Variation

The Coefficient of Variation (c.v.) is determined by dividing the standard deviation by the mean, and then multiplying by 100. The value obtained is a reflection of the relative dispersion of O.D. values around the mean, and is useful as an indicator of reproducibility of control values.

$$\text{c.v.} = \frac{s}{\bar{X}} \times 100 = \frac{0.136}{0.699} \times 100 = 19.5\%$$

The standard deviation, and thus the coefficient of variation are affected by aberrant O.D. values. When determining a range for a given control, a minimum of 15 to 30 O.D. values are necessary to establish a control range. Of course, the reliability of the range increases as the number of determinates increases. An outlying value would have a greater effect on a set of 15 values than on 35 values.

Calculate the c.v. after the first 15 determinates, and if the value is approximately 15% or less, then this is a sufficient number to

establish a range for the control. If the c.v. is approximately 20% or greater, then additional values should be collected to establish a more accurate range. A range is selected based on the number of standard deviations around the mean; this determination is a decision made by each individual laboratory, depending on the confidence interval desired. **Once a range is determined, any run with a control that falls outside of this range must be rejected and the run repeated.**

To illustrate the use of these three formulae, the mean ($\overline{X}$), the standard deviation (s), and coefficient of variation (c.v.) given the following hypothetical low positive external control O.D. values, performed on 16 different occasions, we will calculate as follows:

NOTE: We will refer to this set of data again to illustrate several graphing alternatives.

Low positive external control O.D. value
$X_1 = 0.698$
$X_2 = 0.754$
$X_3 = 0.656$
$X_4 = 0.715$
$X_5 = 0.732$
$X_6 = 0.707$
$X_7 = 0.757$
$X_8 = 0.686$
$X_9 = 0.712$
$X_{10} = 0.635$
$X_{11} = 0.656$
$X_{12} = 0.734$
$X_{13} = 0.753$
$X_{14} = 0.654$
$X_{15} = 0.640$
$X_{16} = 0.695$

$$\text{Low positive control Mean} = \frac{\Sigma X}{N} = \frac{11.184}{16} = 0.699$$

To calculate the **standard deviation** of the low positive control mean:

First find the absolute value of each given O.D. value (X_n) minus the mean ($\overline{X}$) of all values, set up a table as follows:

X_n		$\lvert X_n - \overline{X} \rvert =$	absolute value
X_1	= 0.698	0.698 - 0.699	= 0.001
X_2	= 0.754	0.754 - 0.699	= 0.055
X_3	= 0.656	0.656 - 0.699	= 0.043
X_4	= 0.715	0.715 - 0.699	= 0.016
X_5	= 0.732	0.732 - 0.699	= 0.033
X_6	= 0.707	0.707 - 0.699	= 0.008
X_7	= 0.757	0.757 - 0.699	= 0.058
X_8	= 0.686	0.686 - 0.699	= 0.013
X_9	= 0.712	0.712 - 0.699	= 0.013
X_{10}	= 0.635	0.635 - 0.699	= 0.064
X_{11}	= 0.656	0.656 - 0.699	= 0.043
X_{12}	= 0.734	0.734 - 0.699	= 0.035
X_{13}	= 0.753	0.753 - 0.699	= 0.054
X_{14}	= 0.654	0.654 - 0.699	= 0.045
X_{15}	= 0.640	0.640 - 0.699	= 0.059
X_{16}	= 0.695	0.695 - 0.699	= 0.004

Next find the sum of the absolute values:

$$\Sigma(X_n - \overline{X}) = 0.544$$

N = total number of values (there are 16), thus the standard deviation of these control values is calculated as follows:

$$s = \sqrt{\frac{\Sigma(Xn - \overline{X})^2}{N}} = \sqrt{\frac{(0.544)^2}{16}} = 0.136$$

Calculate the range based on the standard deviation:

one standard deviation:

upper limit = (0.699 + 0.136) = 0.835
lower limit = (0.699 - 0.136) = 0.563

* the range of (0.563 - 0.835) represents one standard deviation.

two standard deviations:

upper limit = [0.699 + (2 x 0.136)] = 0.971
lower limit = [0.699 - (2 x 0.136)] = 0.427

* the range of (0.427 - 0.971) represents two standard deviations.

three standard deviations:

upper limit = [0.699 + (3 x 0.136)] = 1.107
lower limit = [0.699 - (3 x 0.136)] = 0.291

* the range of (0.291 - 1.107) represents three standard deviations.

Please Note: Two methods of calculating control ranges (O.D. and O.D. / Cutoff Ratio) are described in the next section.

How to Plot QC Graphs

A QC graph is relatively simple to formulate and is easy to maintain. There are two methods that may be used. The first described below is acceptable, but not preferred. It is represented here for the purpose of illustration and comparison.

The absorbance or Optical Density (O.D.) value obtained from each external control is plotted on the Y or vertical axis.

The X axis is used to indicate the date of the run, or if your laboratory does more than one run per day, the X-axis can be designated as the run number.

If values are plotted as run number, this number must be indicated on your worksheet. All graphs shown here are adaptations of what is known as a Levey-Jennings chart. Figure 21 shows a simple QC graph indicating the low positive control and cutoff values given previously, plotted with each run. A similar chart can be adapted to show all internal and external controls, and the cutoff, allowing for a visualization of all test controls simultaneously.

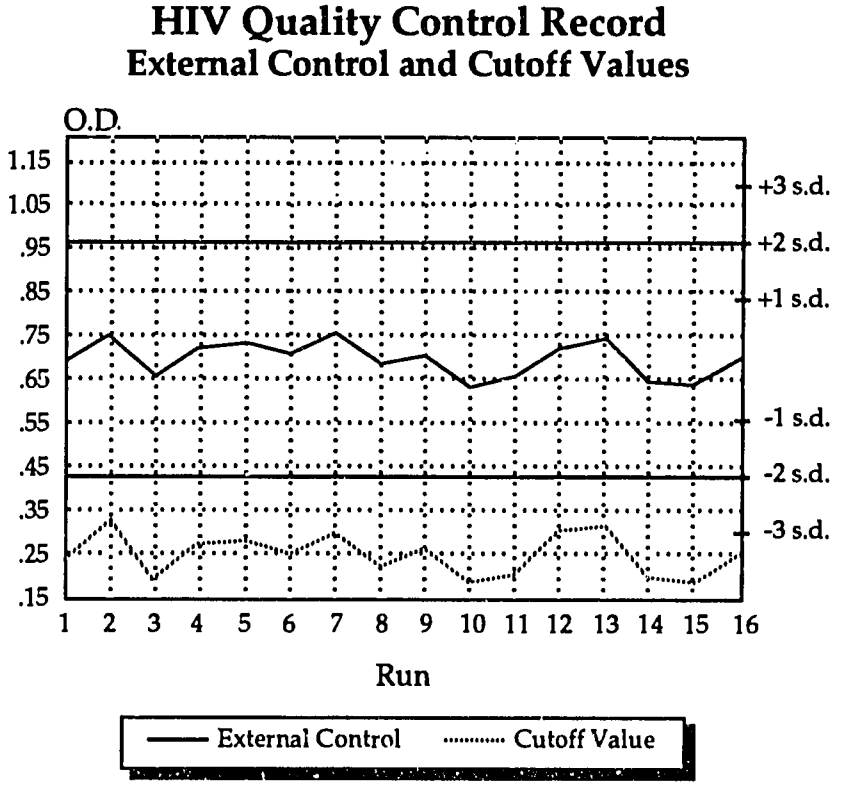


Figure 21. A Simple Quality Control Chart

A second, and more accurate alternative is to plot the O.D./cutoff ratio instead of the raw O.D. values on the Y axis. This is the preferred method since the control values are expressed relative to the cutoff value (i.e. cutoff values will change slightly between runs therefore controls should be compared to each calculated cutoff.) Using the 16 runs from the previous examples including their corresponding cutoff values, we will set up a similar table as before using ratios instead of raw O.D. values. A mean and standard deviation are calculated from the O.D./cutoff ratio (see below). The O.D. to cutoff ratio can be graphed as noted in Figure 22. Notice the change in values representing the y-axis; negative numbers are now used since a control O.D. value below the cutoff may be reflected as a negative number.

The calculation for the (1) mean and (2) standard deviation and control ranges are basically as before with the exception of the deviation is being based on the O.D./cutoff ratio instead of the raw O.D. values. Set up a table as follows:

Low pos. external control O.D. value	Cutoff O.D. value	O.D./cutoff ratio
$X_1 = 0.698$	0.248	2.814
$X_2 = 0.754$	0.318	2.371
$X_3 = 0.656$	0.194	3.381
$X_4 = 0.715$	0.267	2.678
$X_5 = 0.732$	0.280	2.614
$X_6 = 0.707$	0.251	2.817
$X_7 = 0.757$	0.285	2.656
$X_8 = 0.686$	0.218	3.147
$X_9 = 0.712$	0.266	2.677
$X_{10} = 0.635$	0.179	3.547
$X_{11} = 0.656$	0.192	3.417
$X_{12} = 0.734$	0.296	2.480
$X_{13} = 0.753$	0.313	2.406
$X_{14} = 0.654$	0.204	3.206
$X_{15} = 0.640$	0.181	3.536
$X_{16} = 0.695$	0.254	2.736
		sum = 46.483

(1). Calculation of the mean of the O.D./ cutoff ratio.

$$\overline{X} = \frac{46.483}{16} = 2.905$$

(2). To calculate the standard deviation, first calculate the absolute value derived from the difference of each ratio (third column below).

Xn (OD./cutoff)	$(X_n - \overline{X})$ (OD./cutoff-mean)		Absolute value
X_1 = 2.814	2.814 - 2.905	=	0.091
X_2 = 2.371	2.371 - 2.905	=	0.534
X_3 = 3.381	3.381 - 2.905	=	0.476
X_4 = 2.678	2.678 - 2.905	=	0.227
X_5 = 2.614	2.614 - 2.905	=	0.291
X_6 = 2.817	2.817 - 2.905	=	0.088
X_7 = 2.656	2.656 - 2.905	=	0.249
X_8 = 3.147	3.147 - 2.905	=	0.242
X_9 = 2.677	2.677 - 2.905	=	0.228
X_{10} = 3.547	3.547 - 2.905	=	0.642
X_{11} = 3.417	3.417 - 2.905	=	0.512
X_{12} = 2.480	2.480 - 2.905	=	0.425
X_{13} = 2.406	2.406 - 2.905	=	0.499
X_{14} = 3.206	3.206 - 2.905	=	0.301
X_{15} = 3.536	3.536 - 2.905	=	0.631
X_{16} = 2.736	2.736 - 2.905	=	0.169
	sum	=	5.605

Second, calculate the sum of the absolute values.

$$\Sigma\ (Xn - \overline{X}) = 5.605$$

Third, calculate the standard deviation.

$$s = \sqrt{\frac{(5.605)^2}{16}} = 1.401$$

The range based on the standard deviation (s) becomes:

1s = 2.905 +/- (1 x 1.401) = (1.504 to 4.306)
2s = 2.905 +/- (2 x 1.401) = (0.103 to 5.707)
3s = 2.905 +/- (3 X 1.401) = (-1.298 to 7.108)

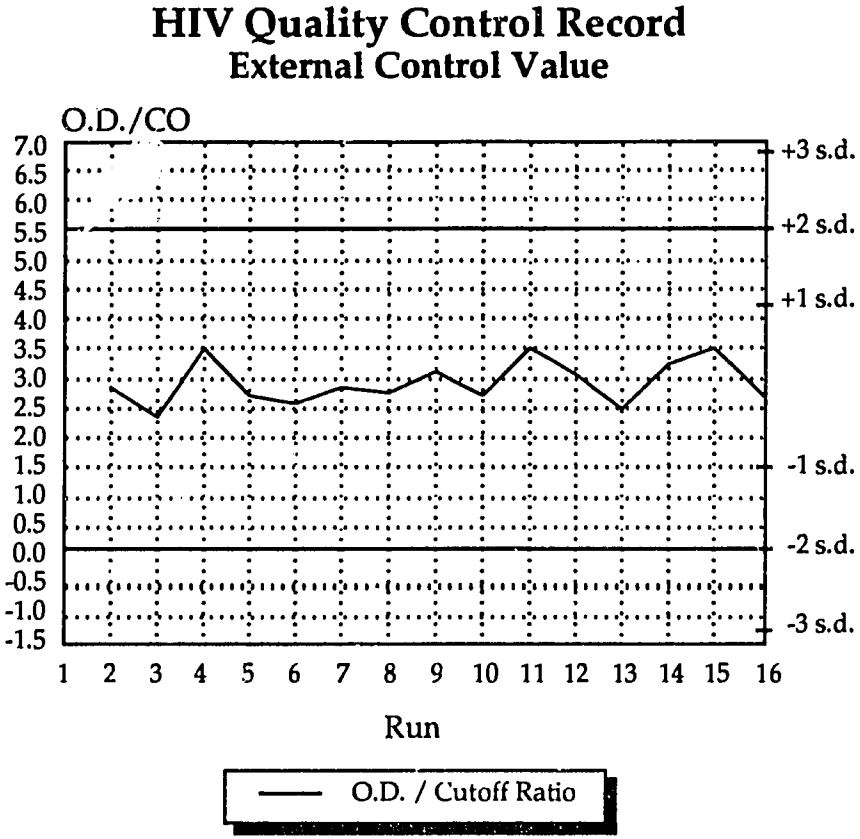


Figure 22. Illustration of O.D./Cutoff vs. Run

Notice that the O.D. values shown as two lines on the Figure 21 are now combined and shown as one line on Figure 22. This same technique can be used to graph the high positive and negative values as well.

Exercise 1: Given the statistical values calculated in the previous examples, plot the following external low positive control O.D. values on a QC graph. Then determine which of the six values you would accept.

Run #	External cont. O.D. Value	Cutoff value
1.	0.426	0.202
2.	0.869	0.268
3.	1.108	0.457
4.	0.638	0.317
5.	0.970	0.140
6.	0.563	0.201

Solution: To plot a simple graph using these raw values, calculate as before:

The range of the raw O.D. values for the low positive based on 1, 2, and 3 standard deviations (s) should equal:

1s- (0.563 - 0.835)
2s- (0.427 - 0.971)
3s- (0.291 - 1.107)

Therefore, the simple plot would appear similar to Figure 23:

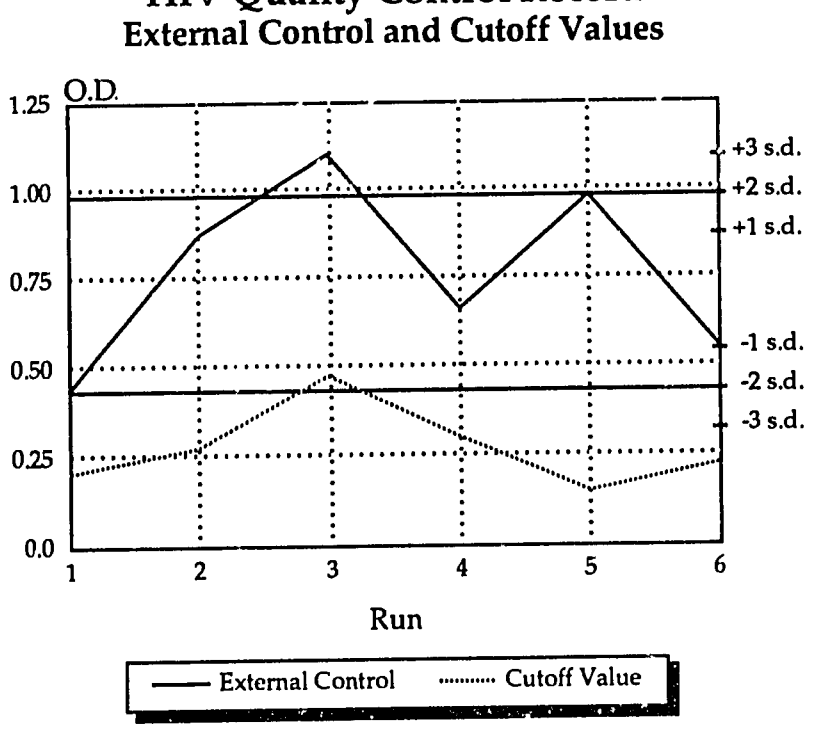


Figure 23 - Simple Graph Using values from Exercise #1 (see text)

As noted in Figure 23, it is readily apparent that the range selected affects whether the external control value is acceptable or not.

By choosing a one standard deviation range, runs 4 and 6 show the only acceptable control values.

Based on two standard deviations, runs 2, 4, 5, and 6 are acceptable.

By increasing the limit to three standard deviations, all values are in range except #3.

Therefore, if two standard deviations are chosen, runs 1 and 3 would be rejected since the external controls did not fall within acceptable limits. As more standard deviations are selected, more values will be considered as acceptable; however, there is a greater chance that these values were due to significant errors.

Exercise 2: With the six run values from above, set up a graph of O.D/cutoff ratios using these O.D./cutoff ranges:

1s = (1.571 - 4.205)
2s = (0.254 - 5.522)
3s = (-1.063 - 6.837)

Set up a table as before:

Run #	O.D. Value	Cutoff	O.D/Cutoff
1.	0.426	0.202	2.109
2.	0.869	0.268	3.243
3.	1.108	0.457	2.424
4.	0.638	0.317	2.013
5.	0.970	0.140	6.922
6.	0.563	0.201	2.800

Now that you have set up the graph with ranges, chart the six values and observe which of the O.D./cutoff values are in range.

Your QC graph should be similar to the one depicted in Figure 24:

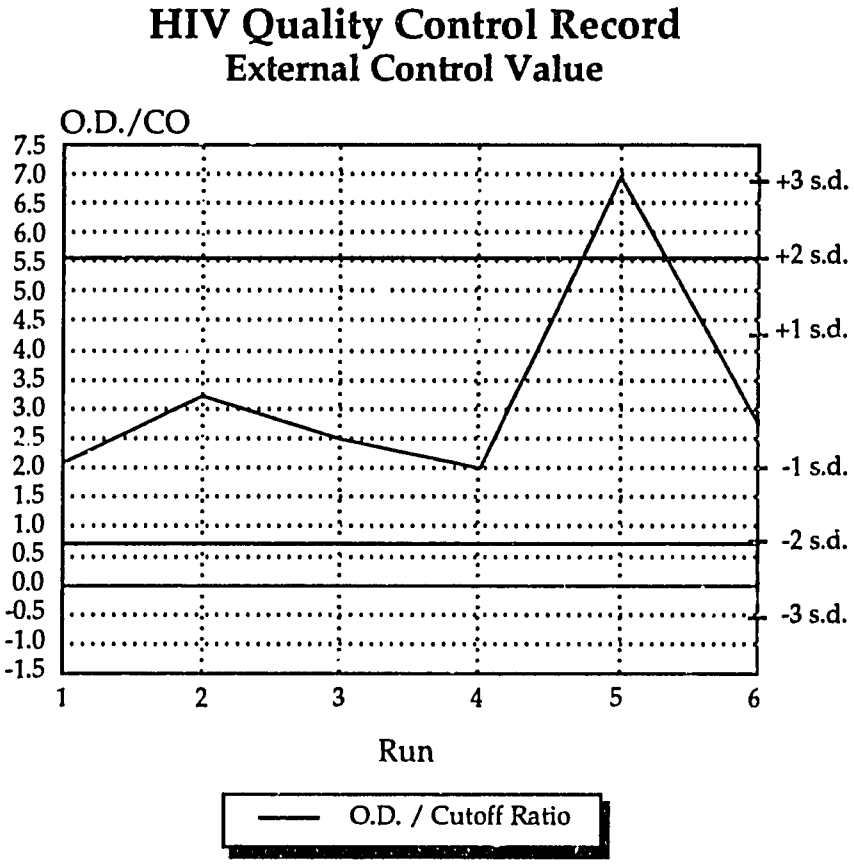
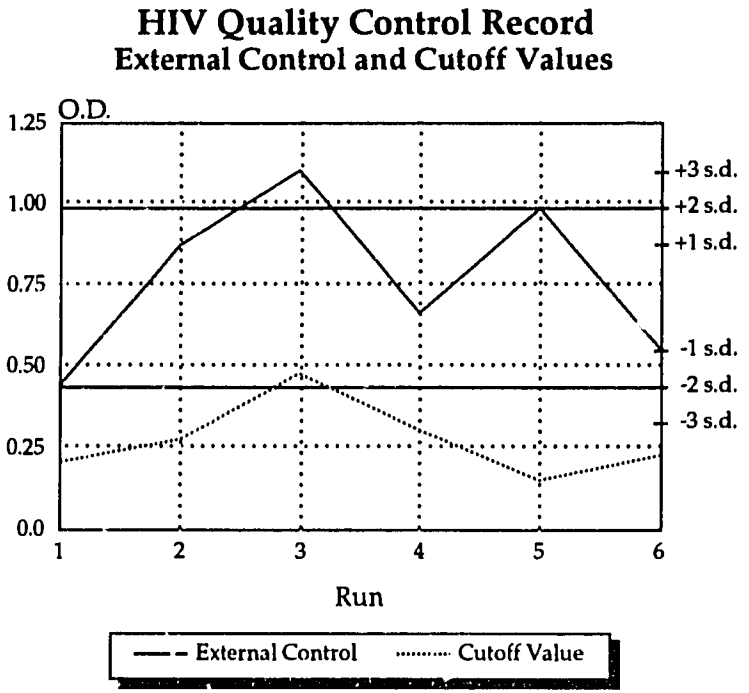


Figure 24: QC Graph



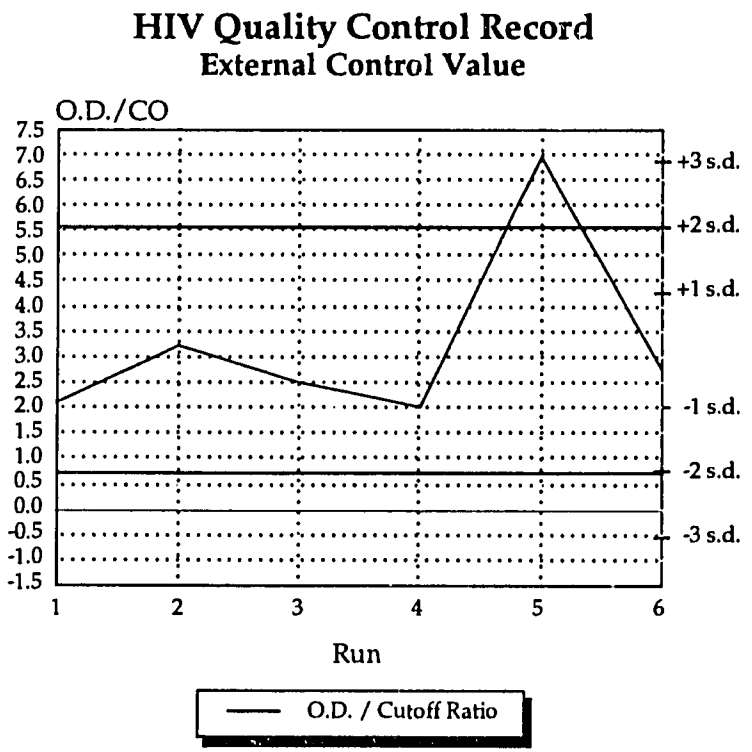


Figure 25: Comparison of figures 23 and 24

By comparing the six run results by two different graphing methods (Figure 25), it is evident that the same external control may be accepted using one method, but not accepted using the other:

By plotting raw O.D. values alone, the control in run #1 and #3 is rejected while the control in run #5 is accepted (using either two or three standard deviations as limits).

In contrast, when plotting O.D./cutoff values, the control in run #1 and #3 now become acceptable, while the control in run #5 is now unacceptable.

As noted on run #3, the control value was outside of three standard deviations when plotting simple O.D. values, and therefore the run should have been rejected. But when this same value is compared in relation to the cutoff (O.D/cutoff), its net value then becomes acceptable within two standard deviations (because the cutoff on that run was very high). Likewise, on run #5 the control fell inside of two standard deviations by the simple graph, yet was rejected when plotting O.D./cutoff (because the cutoff value was very low on this run).

This hypothetical set of values illustrates two QC graphing alternatives. Both methods can be effective quality control tools, although the second method can give a more accurate assessment by taking into consideration daily fluctuations in O.D. values. It may take several months to establish ranges for external controls, and it does take some time to initiate the use of these control systems. However, this type of control system can be very effective in helping to identify potential problems and inaccuracies in the laboratory setting.

Shifts occur when control values of six consecutive runs fall on one side of the mean line. This usually indicates that a major change has occurred. Recall that a normal distribution should fall randomly above and below the mean. Examples of situations that can cause shifts include:

- switching to a new lot of kits;
- new reagents;
- changes in incubation temperatures (perhaps the water bath was not turned on, or the temperature was changed);

- a new technician (who may have different pipetting techniques);

- changes of equipment (pipets, etc.).

Trends occur when six successive points become distributed in one general direction. This is usually the result of slowly changing parameters such as:

- deterioration of reagents;

- faltering equipment (a routinely used pipete slowly losing its calibration for example).

The most common reasons for shifts and trends are deteriorating reagents or controls. Conjugates are generally the first reagents to deteriorate as a kit ages. Figure 26 (next page) illustrates the concepts of shifts and trends.

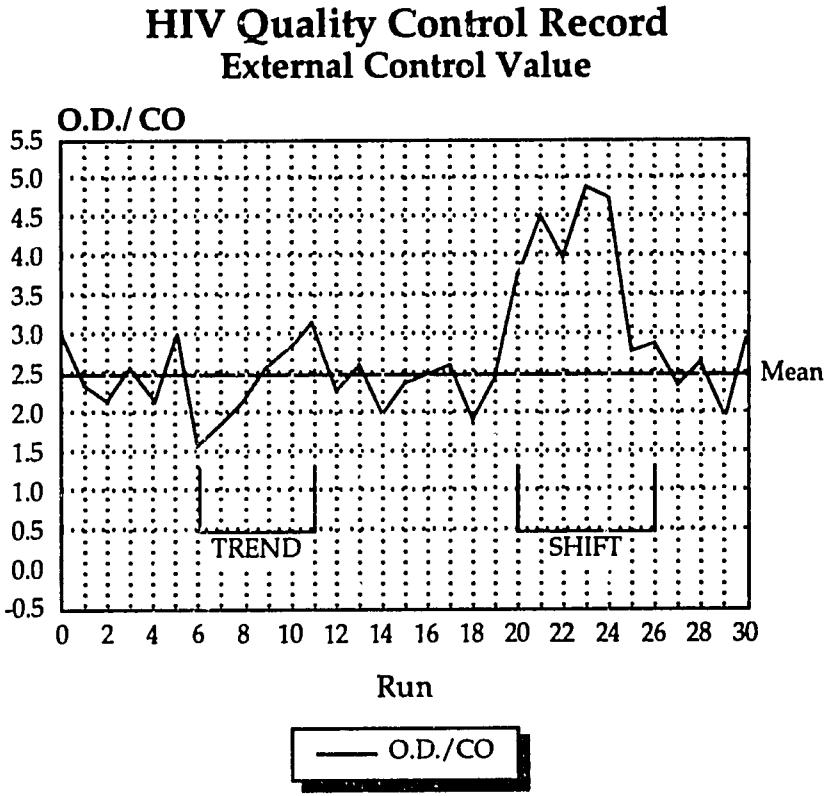


Figure 26. (a) Shifts and (b)Trends

Sometimes a manufacturer produces reagent lots that pass their quality control requirements but fail to perform properly in the field. A contributing factor may be shipping or storage conditions. These reagents must be identified as quickly as possible. A comparison of performance of new lots of kits with the previous lots via a common control material (external controls) is essential for this purpose. This comparison is called "parallel testing" and involves including external control, and the controls from the previous lot on the first run of the new lot. If all controls produce the expected results, then the new lot has passed the parallel test, and the new lot of kits is ready for routine use.

1. **When a new lot of test kits is introduced into the testing process the date should be noted on the control chart.** Be especially vigilant for sudden shifts in all control values. Some variation, either up or down, is expected. Any misclassification of controls or extreme shifts in values should be recorded and the manufacturer notified.

2. **Monitor the performance of the external positive control versus the kit positive control.** Internal kit controls are adjusted by the manufacturer so that an expected range of values are obtained with each lot of kits. We have seen kits where the internal kit controls give the same value as the previous lot, but the external controls show a significant shift in values. This is most likely due to the artificially stabilized kit controls. Differences, however, should be minimal.

3. **Monitor the external and internal negative controls for trends toward higher values.** Such an increase may suggest reagent problems but may also indicate technique or equipment problems (e.g., washer out of alignment or needs cleaning).

4. **Review the average absorbance value for the specimens yielding negative results.** An increase in this value may indicate problems with technique, equipment, or reagents. Similarly, increases in the number of initially reactive specimens that do not repeat or that do not confirm by Western blot are signals of the same type of problem.

CALULATION OF "GRAY ZONE" REACTORS

The ideal HIV test should have only two possible results, either positive or negative. Unfortunately, problematic samples invariably occur during routine testing. For example, some samples technically classified as negative may have an elevated O.D. reading suggestive of some antibody activity. During early seroconversion only slightly elevated O.D. readings may be found.

It is the policy in most laboratories to repeat any HIV reactive result having O.D. values greater than or equal to the cutoff value. Any repeatedly reactive result is then verified using a more specific confirmatory assay. Some assay protocols require that samples reacting relatively close to the cutoff value be repeated. A specimen producing an O.D. value close to the cutoff, yet on the negative side, is often referred to as a "gray-zone reactor."

A value of 10% is most often calculated below the cutoff, and all samples having O.D. values falling within the range are repeated or further tested using other screening tests or confirmatory assays. The calculation of 10% for the gray zone may be somewhat arbi-

trary, but there is a reasonable chance of finding some early HIV infections (seroconversion) by using this approach. By adopting this policy, more individuals may be revealed producing indeterminate HIV results by confirmatory assays. However, these persons should be identified so that they can be informed and monitored for seroprogression. In the case of blood banking, they are prevented from donating blood. One possible disadvantage is that many individuals (perhaps 20%), with completely negative screening test results will produce some bands by Western blot.

PROFICIENCY PANELS AND BLIND TESTING

Several agencies have proficiency testing programs and services for laboratories interested in quality assessment. These include the World Health Organization (WHO), the College of American Pathologists (CAP), the Centers for Disease Control (CDC) and the American Association of Blood Banks (AABB). These and other agencies provide panels of known HIV positive and negative sera to participating laboratories for HIV proficiency testing.

Participating laboratories receive the panels on a regular basis (approximately one per quarter year). The panels consist of a set of sera, a report form, and a return envelope. It is important that these panels be processed and tested in a timely manner so that results can be analyzed along with panels from other participating facilities. It should be stressed that these proficiency panels are to be handled routinely and objectively, and in exactly the same manner as other routine samples for HIV testing, as the purpose of these panels is to identify problems within a quality control or quality assurance system.

The results of each proficiency panel are analyzed along with the results from other participating laboratories. A summary analysis is prepared, and a confidential report as well as the comprehensive report are distributed to all participants. The comprehensive report will not contain references to any specific laboratory by name or location; however, it does include the general type of laboratory (i.e., hospital, private physician, or research lab, etc.) and the results obtained. With a copy of these reports, the laboratory manager can then assess the adequacy of the quality control/quality assurance in their own laboratory, as well as examine a comparison of the results obtained by other similar laboratories. If a problem is identified, investigation and appropriate action can be taken.

Participation in proficiency testing requires relatively little time and few resources from the laboratory; the burden of analysis is born by the agency providing the panel. The increase in the laboratory work load is insignificant compared to the advantage of increased laboratory performance. The organizations listed below can provide information regarding proficiency programs:

WHO Anti HIV Quality Assessment Scheme
Global Program on AIDS
World Health Organization
20, Avenue Appia
CH-1211 Geneva 27, Switzerland

College of American Pathologists (CAP)
325 Waukengan Road
Northfield IL 60093-0800, USA

American Association of Blood Banks (AABB)
1117 N. 19th Street, Suite 600
Arlington, VA 22209, USA

CDC Model Performance Evaluation Program
MPEP Survey Coordinator
Program Resources, Inc.
Science & Technology Center
P.O. Box 12794
Research Triange Park NC 27709, USA

A second alternative for self evaluation is to create an unknown panel of sera for "blind testing". This panel should include several low positive or borderline samples, and should be submitted along with routine test samples. Certainly the clear positive and negative samples should pose no diagnostic difficulty for the laboratory; however, by including borderline samples in a panel, a more effective challenge to quality control is assured by verifying consistency of assay performance.

The purpose of blind testing and proficiency testing is to evaluate the effectiveness of a QC/QA program. **Participation in external proficiency programs, and comprehensive quality control measures in the laboratory are elements of a good overall quality assurance program.**

MONITORING LABORATORY STAFF

Laboratory managers may wish to periodically monitor the performance of their laboratory workers. A policy may be created where samples with known results are occasionally resubmitted discreetly along with the routine workload, to monitor performance. To be effective, the laboratory workers should not realize when these samples are submitted and the testing becomes part of the routine run. This is done so that the laboratory director, who is ultimately responsible for all results leaving the laboratory, can monitor the accuracy and reproducibility of laboratory results. This monitoring should not be done to intimidate or trick the workers, but rather to help them feel confident about their results. They should be told that these blind panels will be periodically included in their work load.

VIGILANCE

Vigilance refers to watchfulness. **Scrutiny of every aspect of the laboratory and every step during the testing of specimens will help to assure that the final results will be accurate.** This begins when the specimens and requisition slips are received in the laboratory, and ends when the final report is submitted. Particularly important is the ability to recognize a mistake or problem and report it to the other workers or supervisor. Of course, corrective action must be taken.

The testing and reporting of accurate results is of primary concern when testing for HIV. To fulfill this obligation, a quality assurance program is of utmost importance. QA measures will help ensure a consistent accuracy of results; however, accuracy requires an extra measure of vigilance on the part of every laboratory worker in order to maintain high standards. The laboratory must function as a team with each worker taking the responsibility that ensures that all results are reported correctly. QC discrepancies must be brought to the attention of the supervisor and any resulting corrective action documented for further reference.

Specifically, vigilance must be practiced by:

1. Always watching to see that the identification on the specimen matches that of the requisition slip.

2. Noting conditions of specimens as they are received (i.e. are they adequate for testing, are they blood, urine, or CSF).

3. Reviewing QC charts each day, noting small changes that may indicate a problem is developing.

4. Observing co-workers, and identify potential problems or inaccuracies. Discuss these matters in a diplomatic manner.

5. Always looking to see if the laboratory is remaining a safe place to work.

6. Always re-checking paperwork and worksheets before reporting a positive result. False positive and false negative results can have a disasterous impact.

VERIFICATION OF TRUE POSITIVITY AND TRUE NEGATIVITY

A positive HIV result is a serious concern. Each laboratory must be absolutely certain that each positive result is correct. Once a sample is found to be "reactive" by a screening test, an aliquot from the initial "specimen" tube should be retested. To be certain that the sample is truly reactive, the specimen can be re-tested using an assay based on an alternative principle.

A current trend in HIV testing is to classify a sample as "reactive" by a screening test and then "positive" only after testing with a confirmatory assay. In most cases, a repeatedly reactive result by a screening assay and a positive result by a confirmatory assay are sufficient to verify positivity; however, the sample may be tested using a second confirmatory assay if desired. For example, if Western blot is used to initially confirm, an IFA can subsequently be used to verify positivity. Whenever possible, a second sample should be collected and retested to eliminate any possible handling or transcriptional errors.

Verification of negative results is also improtant, especially in screening blood for transfusion. A representative sample (10%) of initially non-reactive specimens should be retested to verify the results.

RECORD KEEPING

An efficient laboratory will be able to monitor the records of specimens from the time the samples arrive until the time that results are released. Log books are an essential step in the recording of laboratory specimens. **It is imperative that log books containing**

names be kept confidential. The log book should include:

1. the name and/or specimen ID;

2. the date that the sample was collected;

3. the date received in the laboratory;

4. the name of the requesting physician;

5. the specific tests requested.

Any specimen inadequate for testing or that does not contain the essential information (including labeling), should not be tested and a note should be entered in the log book.

A worksheet must accompany each test run performed and should be completed before the first sample is added. The worksheet may then serve as a guide when adding samples to the run, thereby avoiding errors during addition. Worksheets should contain the kit lot number, expiration date, date performed, and the technician's initials. Completed worksheets with results can be filed for a permanent record. If available, records can be stored in a laboratory computer system (always keep a backup disk or hard copy of results). Examples of worksheets are included in the appendix of this guide.

Quality control records are also important in validating laboratory results. Daily log sheets for temperatures of waterbaths, incubators, refrigerators, and freezers document that kits and samples were stored under proper conditions and that all tests were performed as recommended.

A **standard operating procedure (SOP)** book should be kept in the laboratory at all times. The SOP contains all procedures (package inserts and/or detailed explanations of procedures) performed in the laboratory. This can act as a quick reference for any procedure, and as a teaching source for new employees and students. The SOP book should be reviewed and updated frequently. Periodically, the lab manager should monitor technicians to assure that they are following SOP.

EQUIPMENT MANTENANCE AND CALIBRATION

Preventive maintenance schedules should be included in the

operating instructions of laboratory instruments such as ELISA washers and readers. It is recommended that these maintenance and calibration procedures be followed regularly for optimal performance of equipment. Preventive maintenance is the best measure to ensure that the equipment will function properly. Results of scheduled maintenance activities should be filed for future reference.

Ordinarily, maintenance schedules are set up as tasks to perform daily, weekly, monthly, every six months, and yearly. Examples of maintenance and calibration schedules with activities that can be performed are listed below.

A record or check-sheet of such activities can be adopted for any given laboratory to help the laboratory manager keep track of necessary tasks. Of course these activities can vary greatly depending on such factors as work load, types of instrumentation, number of employees, and environmental conditions such as humidity or dust.

Daily: Controls or calibrators are added to each run, recalibrate instruments if necessary; for ELISA readers, check that the proper filter is in place, empty waste containers, rinse sample ports, etc. with distilled H_2O, or acceptable cleaners (see operators' manual). Clean up all spills, check reagent levels; flush ELISA washers; review safety measures, dispose of biohazardous waste.

Weekly: Keep optical components, etc., free from dust, clean surfaces of instruments, prepare fresh batches of reagents as needed, recalibrate instruments if necessary.

Monthly: Perform electronic or optical checks on all components. Many automated and semi-automated instruments have built-in programs for calibration (see operator's manual).

Every 6 months: Clean or change filters, check all fluid lines and tubing for signs of deterioration and replace as needed; regrease all pipets as needed (grease is usually provided with the pipet. See maintenance instructions).

Yearly: Change all fluid lines and tubing for major instruments; arrange for a service call by a factory representative if possible; recalibrate pipets.

Pipets are precise and important basic instruments of the laboratory, and as such, need to be calibrated at least annually. Several methods of pipet calibration are available, including the pipetting of colored solutions followed by spectrophotometric analysis, the weighing of volumes of distilled water, or pipetting volumes of radioisotopes. One commercial company (MLA, Pleasantville, NY) markets a calibration kit for mechanical pipets.

When selecting a pipet for a routine procedure, choose the pipet in the most appropriate range for the volume needed. Never exceed the range of the pipet as damage may result and precision may vary. Technicians should watch carefully while pipetting to assure that accurate amounts are delivered. Multi-channel pipettors warrent careful attention to assure that each tip is delivering consistently and accurately.

For convenience, many laboratories make batches of wash buffers or stop solutions and store them in containers that dispense fixed volumes. When changing these solutions it is easy to accidentally disturb the volume setting, thus altering the delivery volume. To ensure accurate reagent delivery, recheck the volume dispensed after any disturbance to assure consistent performance. Also clean these containers periodically before adding new reagent.

Most photometric instruments require calibration to ensure accuracy and linearity of their readings. This is usually accomplished using special calibration plates, available from the manufacturer. The plates consist of different wells, each capable of producing a different O.D. reading. Observed readings are compared to theoretical values and evaluated using confidence limits. Likewise, automated diluters must be calibrated. This is usually accomplished by diluting a standard color solution and reading O.D. spectrophotometrically. Results must be within 10% of the expected. The manufacturer of any automated instrument can be contacted for details of these procedures.

COMMON ERRORS IN HIV TESTING

Here are a few of the more common errors that occur during HIV testing:

1. **Dilution errors.** Carefully calculate and perform dilutions of all reconstituted components. Most ELISA kits contain instructions for dilutions, but many are based on volumes required per plate. When working with smaller numbers of strips or wells, or two or more plates, the required volumes are different, but the proportions remain the same. (See part 7 for a discussion of dilutions.)

2. **Scratching the coated antigen during sample addition.** This is a common problem in both microtiter plate ELISA and Indirect Fluorescent Antibody techniques. Do not touch the plate or slide well while adding samples.

3. **Improper dropper use.** In some agglutination assays, as well as some of the dot blot tests, a dropper is supplied for adding reagents. There are two important considerations to remember while using these droppers: First, these droppers are to be used in a vertical position while adding reagents and secondly, free falling drops are essential. If either of these practices are not employed, the volumes dispensed could be inaccurate.

4. **Using improper pipet tips.** Be sure to use the proper pipet tips for the pipets, otherwise inaccurate volumes may result.

5. **Inconsistent technique.** Each specimen and control must be treated exactly the same. Do not treat controls more carefully than unknowns. Also, be sure that the same technician adds all controls and samples.

6. **Use of improper equipment.** The right equipment must always be used (eg. high intensity lamps for agglutination tests). Also, be sure that all rotators are calibrated to the proper speeds.

7. **Improper temperature.** Allow kits and specimens to reach room temperature before use; adding a cold conjugate to a competitive binding assay would severely alter reaction kinetics, possibly producing false positive results.

8. **Mixing of components from different lots of kits.** Reagents of each lot are titrated to work optimally as a kit. Many WB kits have conjugate dilutions that fluctuate from 1:500, 1:1000, 1:1500, etc., depending on the given lot. In addition, the avidin-biotin conjugate systems may require different dilutions for each conjugate.

TROUBLESHOOTING

When the failure of a test run occurs, the reason may be readily apparent in some cases, but not so obvious in other instances. "Troubleshooting" refers to the measures used to determine why a run has failed.

The most effective way to prevent the failure of a run is to review the procedure carefully before beginning. Anticipate procedural steps ahead of time. For example, many conjugates require reconstitution and time to equilibrate before use. Insure proper incubation temperatures and times. And most importantly follow the manufacturers' protocol precisely.

If an HIV test run fails after these precautions, an investigation must be conducted to determine the most probable cause. The following are suggestions that could help discover the problem:

1. Review instructions with a technician, carefully checking for procedural omissions or changes.

2. Double check component expiration dates and the master lot expiration date.

3. Were all physical parameters of the assay followed (time and temperatures)?

4. Is the support equipment such as pipets, plate washing and reading systems, etc., working properly? Has the preventive maintenance and servicing schedule been performed?

5. Is the plate reader on the proper wavelength?

6. Are the pipets calibrated? If the assay calls for 10ul of specimen, there is very little room for error in the volume amount for the assay. If a pipet only delivers

9 ul rather than 10, this constitutes a 10% error which can be sufficient to cause the controls to fall outside of acceptable limits. Proper calibration of pipets is essential for accurate results.

7. Check the quality of the distilled water.

8. Check that regents are not contaiminated and were prepared and stored properly.

There are several situations where characteristic appearances in your test system may indicate typical problem situations. Examples include:

Problem #1. All wells in an ELISA are colorless upon completion of the procedure.

Possibilities: The conjugate or substrate may have deteriorated or may not have been added. Note that the conjugate usually will be the first reagent to deteriorate in an ELISA assay as you approach the expiration date. Conjugates can also deteriorate if your test kits become "heat spiked" during delivery, or otherwise compromised due to improper storage conditions, etc. Also reconsider the conjugate dilution, a critical factor in assay performance. Substrates are usually stable if stored properly; however, if the reconstitution is done improperly or the dilution is incorrect, color may not develop in the assay. Alternatively, all samples may be negative, with the positive controls not added. This possiblity represents another value of incorporating an external control.

Solution: Review procedure to assure all steps were followed. Try another kit from the same lot number; if this also fails, contact the manufacturer for a possible replacement kit.

Problem #2. All wells in an ELISA are the same color evenly in each row.

Possibilities: It is important to inspect the color of your substrate before addition to the wells. If it is not colorless or slightly yellow, or if it is otherwise not the color stated in the package insert, your entire run may be compromised, resulting in a uniform color production.

Solution: Scrupulously clean glassware is highly important as residual material remaining in previously used glassware can react

with the substrate. Even small volumes of remaining conjugates can react with the substrate before it is added to the ELISA wells. This is why it is important to insure a good wash of the ELISA plate or wells following the manufacturer's protocol, and ensuring that no residual material remains to react.

Problem #3. Upon visual inspection of your assay, there is a normal appearance in the reaction wells (i.e. the blank well is clear, positive and negative control wells are darker and lighter respectively) yet when read on a spectrophotometer, all of the absorbances are nearly identical.

Possibilities: Either the detection or reference wavelength (or both) of the spectrophotometer are incorrect.

Solution: Correct the wavelength and then re-read.

Western blot assays are also susceptible to technical problems (see WB troubleshooting guide in the appendix). Here are a few of the most common problems in performing WBs:

Problem #4. WB strips fail to develop.

Possibilities: Conjugates added in the wrong order, or the dilution was not correct. Substrate may have become non-functional. Positive controls may have been omitted.

Solution: If the substrate was suspected, re-wash the strips and add fresh substrate. Otherwise, repeat the entire test.

Problem #5. Negative control strip has an unexpected band.

Possibilities: Contamination between wells or the reaction was allowed to develop too long.

Solution: Repeat the procedure with the same controls plus the negative control from another lot number. Relocate the controls on the tray. If both negative controls have the same band, the problem is most likely contamination; if the negative control from the previous lot is still working properly the new negative control should be discarded. If a contamination problem occurs in a Western blot system, and it can not be attributed to carry-over, all support equipment, including vacuum line hoses, automatic dispensing equipment and any other item that comes in direct contact with the blotting system will either have to be thoroughly

washed or replaced to eliminate any possible source of contamination.

REPORTING OF RESULTS

The nature of HIV infection and the impact on the individual and society are emotional issues, so the handling of results must be controlled. Ideally, HIV testing results are reported to the submitting physician who in turn can appropriately inform and counsel the tested individual. Every effort should be made to keep results confidential.

At this point, country policy determines how HIV testing results are reported by the physician to other public health authorities. A policy decision on the handling of HIV testing results must be established and uniformly enforced in any laboratory where HIV testing is performed.

CONFIDENTIALITY

Specific HIV laboratory results should never be a topic of loose discussion. The privacy and rights of an individual can be severely compromised by misinformation from overheard conversation. HIV testing results should not be available for general viewing, and must be kept in a locked file cabinet to prevent access by unauthorized individuals. Lab reports should be submitted to physicians in a sealed envelope to preserve confidentiality.

INTERACTION WITH PHYSICIANS

The relationship between laboratory personnel and submitting physicians should be one of mutual trust and respect. Communication is important in establishing rapport, since many times there will be questions about interpretation of laboratory results. The laboratory must earn trust and respect by being understanding and courteous while explaining the assay principles and the significance of the interpretation of the results. There will be instances where the laboratory worker will know more about the significance of the laboratory findings than the physician. The relationship can also be one of mutual education, as diagnosis should be made from a combination of clinical and laboratory findings.

STORAGE OF SPECIMENS FOR FOLLOW-UP

Organization in the laboratory is essential. When a specimen is

submitted for HIV testing the sample is accessioned, all information is entered into the laboratory log book, and the sample is centrifuged and separated into a vial suitable for testing and storage. This vial should ideally be able to hold at least 1 ml. of serum for screening and confirmation. Prior to testing, samples may be stored up to one week at 4°C. Once the sample has been tested, it may be stored at -20°C for years. A serum bank can be established where samples are neatly stored in order by accession number. A log book containing all pertinent information should be capable of identifying all stored samples. Samples should not be repeatedly thawed and refrozen, because this process can denature proteins and lead to inaccurate results.

When numbering storage vials, consider using typed or computer generated labels with one sticky surface rather than a handwritten system. Another alternative is to use waterproof marking pens. As samples are removed from the refrigerator and brought to room temperature, condensation may develop, and hand written numbers are easily smudged beyond recognition. If printed labels are unavailable, a piece of transparent tape over the number prevents smudging.

For specimen storage, we recommend 1ml plastic screw cap vials, as glass vials may eventually crack with long term frozen storage. Keep in mind that if the volume of the storage container is much greater than the volume of the serum, evaporation and concentration of the serum will also occur during long term frozen storage. For example, do not store 50 ul of serum in a 5 ml tube. Once a sample has been tested, it should be stored at -20°C in the appropriate vial.

An organized serum bank can serve many useful purposes. These include:

1. Providing a ready source of sera to assemble panels of known negative, positive and indeterminate samples for evaluation of new test methods as they become available.

2. Re-evaluating previously indeterminate classified samples as new tests become available.

3. Facilitating epidemiological surveys by accurate records that match properly cataloged sera.

4. Follow-up and monitoring individuals for seroprogression by

testing previously collected sera along with current sera.

5. Further testing of stored samples can be accomplished as new infectious agents are identified.

THE TESTING OF PREPARATIONS FOR INJECTION

Several countries have policies in effect stating any imported blood product intended for human injection must be tested for the presence of HIV antibody before it is released for public use. These products often include blood products such as Anti-Rh immune globulin, factor VIII and other pooled blood fractions. Although the intentions of such a policy are commendable, there are several reasons why it is ineffective in accomplishing the intended goal. The goal is to certify that a given preparation is incapable of transmitting HIV infection; however, the result may be a false sense of security or a waste of valuable products. Listed below are several points that should be considered when testing preparations other than normal human sera:

1. **Serologic tests for anti-HIV were designed to perform optimally on fresh normal human sera (NHS).** The validity of results on products other than sera are disputable because test performance may be altered due to the different concentrations of proteins

2. **The absence of antibody to HIV does not guarantee that a product is not infectious.** Antibody may not always be detectable even when the virus is present. **Likewise, the presence of antibody does not indicate that a sample is capable of transmitting HIV.**

3. **If the product is purchased from a reputable manufacturer, the process of preparation of these injectables virtually eliminates any chance of survival of viable virus.** All units used to contribute to the pool of "Anti-Rh immune globulin" for example, are individually screened prior to inclusion in the preparation. All seropositives are eliminated.

4. Approximately 500,000 doses of Rh_o-Ig are given annually to 350,000 women in the United States with **no evidence to implicate Anti-Rh immune globulin as a source of HIV infection.**

5. **The presence of antibody to HIV does not indicate that a**

sample is capable of transmitting HIV infection. This can only be confirmed by virus isolation.

6. **Pooled immune globulin and other preparations usually do not contain the same concentration of immunoglobulin as found in NHS.** Therefore, these preparations should not be evaluated using conventional screening methods.

7. When testing preparations, the limitations of such testing should be reported together with a disclaimer stating that test results may not be valid.

Dilution of concentrated products:

To determine what dilution to make of reconstituted preparation, first convert the given concentration to mg/dl. The concentration of immunoglobulin in NHS is approximately 900-2100 mg/dl, depending upon the population. Let us assume 1500 mg/dl for our purposes.

Example #1

A product is labelled 300 mcg/ml., find the concentration in mg./dl (1 dl. = 100 ml.)

NOTE: 1 mcg = 1 ug = 1×10^{-6}g.

$$\frac{300\text{ ug}}{\text{ml.}} \times \frac{1\text{ mg.}}{1000\text{ug.}} \times \frac{100\text{ ul}}{1\text{ dl.}} = \frac{300 \times 100}{1000} = \frac{300}{10} = 30\text{ mg/dl}$$

30 mg/dl is much more dilute than 1500 mg/dl and therefore the product is tested without making a prior dilution. Results are reported with a brief disclaimer citing limitations above.

Example #2

A product is labeled 750 mg./ml., find conc. in mg./dl.

$$\frac{750\text{ mg.}}{\text{ml.}} \times \frac{100\text{ ml.}}{1\text{ dl.}} = 75000\text{ mg./dl.} \quad (75\text{ gm./dl.})$$

This product will require a dilution before it approximates the concentration of immunoglobulin in NHS. Recall that we will use 1500 mg./dl..

$$\frac{75000 \text{ mg./dl.}}{1500 \text{ mg./dl.}} = 50$$

Therefore, this product should be diluted 1:50 before testing.

If your laboratory should happen to receive an enzyme preparation for HIV testing, note that enzymes are measured in units of activity (I.U. or m.I.U.) rather than concentration. These are not easily converted to mg./dl., therefore, test as is.

Remember that results of these preparations must be reported with a disclaimer mentioning test limitations on products other than NHS. The caveat often used in these circumstances is:

"Please note that specimens such as pooled human sera or enzyme preparations are not recommended for testing by commercial assays for HIV-antibody. Therefore, interpretation of the results is difficult, and results may not be valid. Also, since these assays detect only antibodies to HIV, a conclusion cannot be made concerning the presence of infective virus."

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LABORATORY TECHNIQUES

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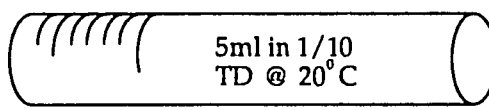
PIPETTING

Pipets are the basic instruments of any HIV laboratory. Obviously, the manner in which these instruments are handled has a direct effect on the accuracy of laboratory results. The three basic types of pipets routinely used in HIV testing are: **transfer (or pasteur pipets), serologic, and mechanical.** Proper technique is essential to accurately deliver volumes for all pipets.

1. **Glass pasteur pipets and plastic transfer pipets** are commonly used in many clinical laboratories for aspirating and transferring serum from clot tubes. While it is true that a greater volume of serum can be recovered using the glass pasteur pipet, safety must take priority over increased serum recovery; therefore, **plastic transfer pipets should always be substituted for glass pasteur pipets in HIV testing,** because the danger of puncture wounds due to broken glass represents a great risk.

2. **Serological pipets** are available in a variety of volumes from 0.2ml to 25 ml, and are routinely used when making dilutions or reconstituting reagents. Information about the pipet is usually printed at the aspirating end and indicates the total volume and units of delivery (see diagram below).



The pipet represented above will deliver a total volume of 5 ml. The information on the second line "TD @ 20°C" means: "To deliver at 20°C." Each large division is capable of delivering 1 ml. while each fine division will deliver 1/10 or 0.1 ml. Keep in mind that the accuracy of a 5 ml pipet will be +/- 0.1 ml. When selecting a serological pipet, choose a pipet where the finest units of measurements are one significant figure beyond the volume being measured. For example:

the above pipet is perfect for placing 1 ml volumes of diluent in test tubes for a serial dilution. However, this pipet is totally inappropriate for adding 200 µl (2/10 ml) of diluent to a 96 well microtiter assay, since the accuracy is +/- 1/10 ml (100 µl).

Consistency is critical for dispensing. The pipet should be viewed at eye level and the meniscus of the fluid observed in precisely the same manner from one volume setting to the next. The expected

accuracy can be obtained if pipetting is performed carefully, using the correct pipet.

There are a variety of mechanical and motorized aids for serologic pipets that increase the control and safety of pipetting. **Mouth pipetting is not acceptable,** due to the great risk of acquiring infections by mouth. Several different types of serological pipet aids are shown below.

Pi-pump (manual)

Pipet bulb (manual)

Mopet (motorized)

3. Manual mechanical pipets are the method of choice for pipetting small volumes. They are available in various ranges from 0.5µl to 5 ml. for the single channel variety. The multi-channel pipets (8 or 12 channel) are available in ranges from 5µl to 300µl. Most of the mechanical pipets operate on an air displacement principle. There are usually two or three stops on the plunger. From the release position to the first stop is the calibrated stroke that is determined by the volume setting (see figure 27). The area between the first and the second stop is used to blow-out residual sera that remains at the end of the pipet tip. Some pipets have the ability to eject the pipet tip if the plunger is pushed to a third stop.

- **Single channel mechanical pipets**

When using single channel mechanical pipets, it is important to avoid bubbles (which alter volume):

1. Depress the plunger to the first stop, (step 1, Figure 27) place the pipet tip just below the surface of the serum, then aspirate slowly (step 2, Figure 27).

2. If the tip is inserted too far below the surface, a small serum droplet can adhere to the outside of the tip. Carry-over of this excess serum will increase the actual volume delivered to the well, which may alter the test result. This error in the volume delivery is increased as your desired delivery volume decreases.

3. Always remember that it is important to aspirate **slowly** if correct volumes are to be obtained.

There are important specimen delivery steps to follow, depending upon the dispensing method:

When dispensing the volume with bead assays or tubes, place the tip against the side of the tube or well as close to the bottom as possible (step 3, Figure 27). Do not place the tip directly on the bottom of the well in a vertical position, since this affects delivery due to blockage of the tip orifice. Avoid delivering the volume at the top of the well or tube, since the entire volume may not sufficiently drip down the side of the tube and will not make sufficient contact with the reactants. Place the tip on the side very near the bottom and allow the liquid to flow to the bottom. (step 4, Figure 27).

On a microtiter plate, when dispensing a volume of diluent or conjugate into a well, it is important to get as close to the bottom of the well as possible without actually touching the bottom. Touching the well can cause scratches in the antigen or antibody coat, which will alter the reaction.

Never add serum directly to Ag or Ab coated wells. Add diluent first, followed by addition of serum.

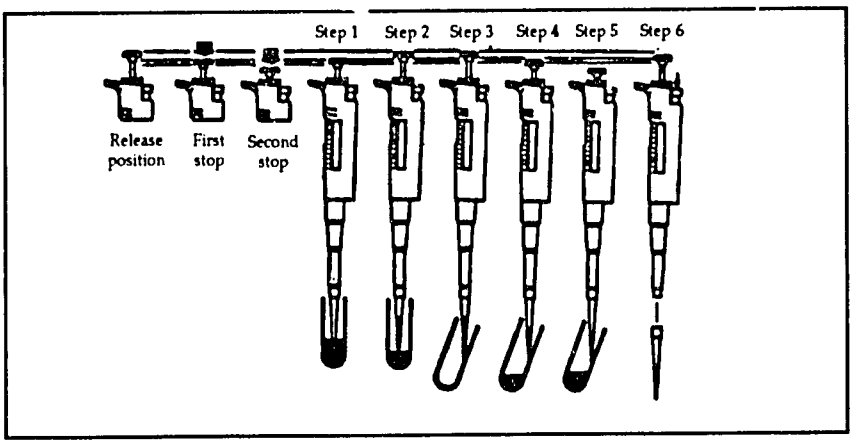


Figure 27 Single channel mechanical pipets

- **Multi-channel mechanical pipets**

Adding diluent or reagents to many consecutive wells with a single channel pipet may introduce slight variations in volume, regardless of the level of technical skill. Consequently, uneven reaction conditions due to pipetting variability will occur, possibly affecting assay performance. **Multi-channel pipets**

help in overcoming these conditions by making repeated additions of the same volume of diluent or reagent to many wells an easier and more accurate procedure. When adding reagents or diluent, never touch the wells of a 96 well plate with the pipet tips; otherwise, well to well cross- contamination or scratching of the absorbent may occur.

Bubbles are often an annoying problem with multi-channel pipets. To avoid bubbles:

1. Make sure the reagent is placed in sufficient volume (in a reagent boat or trough) to fill the entire plate.

2. Press the plunger to the first stop before aspirating the diluent or reagent.

3. Place the pipet tips below the surface of the reagent in the reagent boat.

4. Raise the plunger slowly to the release position, keeping the pipet tip below the surface of the liquid.

5. Remove the pipet and dispense the liquid by slowly depressing the plunger to the first stop (do not release plunger).

6. Proceed to depress the plunger to the second stop (blow out position).

7. Next, keep the plunger at the blow out stop while removing the tip from the well. At this point the pipet plunger can be released to the first stop, and the pipet used to aspirate additional reagent and the process continued.

However, if a pipet is used to transfer reagent to consecutive wells without changing tips, there must not be any residual reagent remaining in the tips. If this occurs, it is recommended that new tips be placed on the pipet before addition of reagent to other wells. The most accurate method to ensure precision is to change pipet tips for each row.

There is some debate about whether a pipet tip should be "primed" or "wetted" before use. These terms refer to the aspirating of the liquid several times before proceeding. **Some may argue that this "priming" practice increases volume precision:**

For the multi-channel pipet this argument may be valid since there will be a slight difference in volume between the first and second row of the microtiter tray if the multi-channel pipet tip is not wetted before adding diluent to the first row.

However, for the single channel pipet, the most important consideration when adding serum is consistency. Since the tip is changed for each specimen, it is much more important that the reagents be pipetted in a consistent manner in every well.

This point can be clearly demonstrated when adding controls: suppose you have an assay requiring three negative and three positive controls. To save tips you use the same tip for all three negatives, then with a single new pipet tip you add positive control to all three wells. If the tip was not wetted or primed before adding control to the first well, well number one will have slightly less volume than wells two and three. This may produce a sufficient enough drop in O.D. to cause a low reject. To avoid this problem **always use a separate pipet tip for each well,** even if adding the same control to three different wells. Also, **if a particular tip is damaged or manufactured incorrectly, the use of different tips will prevent all controls from being inaccurately delivered.** By pipetting in this manner, consistency is insured from start to finish between controls and specimens.

When reconstituting reagents, note that glassware such as volumetric flasks will have a "TC" designation such as: TC 250 ml @ 20°C. A volumetric flask with these designations will "contain" 250 ml at the prescribed temperature, yet will not "deliver" exactly 250 ml as the flask is emptied, because residual amounts will adhere to the walls of the container. **It is important to take note of designations on pipets and glassware and select the appropriate vessel or serologic pipet.** Some glassware such as beakers and Erlenmeyer flasks will not have a TC or TD designation since the volumes indicated do not require the precision necessary for this designation.

Most importantly, it is necessary to:

thoroughly read all information provided by the manufacturers to ensure that these pipets and glassware are being used in accordance with recommendations; periodically inspect, clean, and lubricate devices as necessary.

Assay results cannot be expected to be accurate if the tools of the laboratory are not in good working condition, or if they are used incorrectly.

PREPARING DILUTIONS

Basic laboratory investigation requires that laboratorians be well educated in preparing solutions and preparing samples prior to the actual testing. Obviously, if any test is to perform optimally and yield accurate results, the reagents and samples must be prepared correctly. Although most commercial kits for HIV testing contain reagents that require very little preparation, some prior reconstitution or mixing is usually necessary. In addition, serum samples are almost always diluted prior to testing. Usually the package insert will indicate the correct volumes of reconstitution fluid or diluents to add; these steps must be performed carefully and accurately.

With certain serologic assays, samples must be diluted prior to addition to the test system. A notable example is the IFA test which usually requires a pre-dilution of 1:20 in phosphate buffered saline (PBS). The aim of the examples below is to make the concept of dilutions more understandable by showing:

how dilutions can be manipulated to save time and reagents;

how to eliminate unnecessary steps affecting accuracy.

When a serum sample is collected from a patient, many times the HIV test is not the only laboratory test requested; the sample must be shared with other laboratory departments for additional testing. With a small sample volume, it becomes critical to conserve as much serum as possible for repeat and confirmatory HIV testing. **When performing HIV or any other serological assays, never use less volume than is required for an assay as this will produce invalid results.** When a sample with insufficient volume is submitted to the laboratory for testing, notify the submitter and indicate on the request form "Quantity Not Sufficient" (QNS) for testing. However, if dilutions are required, the general principle is to use the minimum practical volume of serum possible when making all dilutions.

To prepare a dilution, mix a specified volume of the specimen with a certain volume of a buffer (or diluent). In a simple case, one part of serum and one equal part of diluent will yield a 1:2 dilution. The expression "1:2" represents the ratio of the number of parts of

serum to the total number of parts, or one part serum and one part diluent for 2 total parts. Likewise a 1.100 dilution can be prepared by mixing 1 part of serum with 99 parts of diluent; for example 1 ml + 99 ml (100 total parts), or similarly, 50 µl. and 4950 µl (4.95 ml) can also be used.

When making a 1:100 dilution, it is readily apparent that 1 ml is an excessive volume of serum to use for a dilution. A 50 µl volume is a more conservative amount of serum to use, but the volume of diluent required may not be practical with a heavy workload. In these and similar situations serial dilutions are the practical choice for preparing dilutions.

Serial dilutions involve mixing and transferring a constant volume of serum into successive tubes containing diluent. When working with dilutions it is helpful to express all dilutions in terms of fractions. This makes solving a serial dilution problem a matter of multiplying fractions. For example, a 1:2 dilution may also be expressed as the fraction:

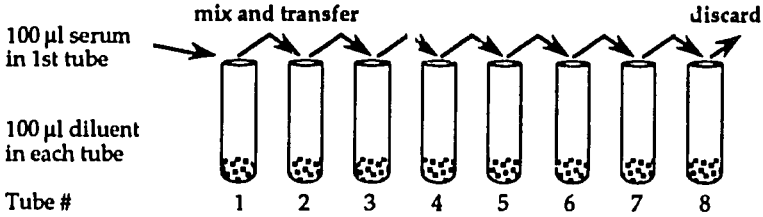
$$\frac{1}{2} = \frac{\text{Parts of serum}}{\text{total parts (serum + diluent)}}$$

A two-fold serial dilution is a dilution where a fixed volume of serum is mixed and transferred into successive tubes containing the identical volume of diluent. This dilution fold can be expressed as:

$$\frac{1}{\text{dilution fold}} = \frac{\text{parts transferred}}{\text{total parts}}$$

Example #1
a two-fold dilution:

This can be prepared by adding 100 µl of serum to the first tube which contains 100 µl of diluent. Mix sufficiently by moving pipet plunger up and down three to four times, then transfer 100 µl of this volume to the next tube and do the same until reaching the final tube. The last volume can either be discarded or left in the tube. The pipette tip should be changed after each transfer step.



Each tube is a 1:2 dilution of the previous tube and the final dulution of each tube is a product of the fractions.

$$\frac{1}{2} \times \frac{1}{2} \times \frac{1}{2} \times \frac{1}{2} \times \frac{1}{2} \times \frac{1}{2} \times \frac{1}{2} \times \frac{1}{2} = \frac{1}{256}$$

Multiply the fractions to find the final dilution of any tube

$$\frac{1}{2} \times \frac{1}{4} \times \frac{1}{8} \times \frac{1}{16} \times \frac{1}{32} \times \frac{1}{64} \times \frac{1}{128} \times \frac{1}{256}$$

For each tube above:

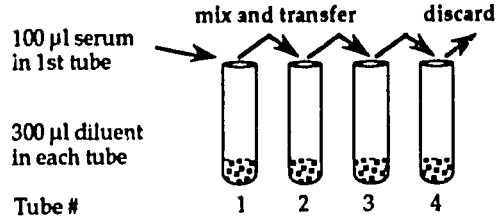
$$\frac{\text{Parts transferred}}{\text{total parts}} = \frac{100\ \mu l}{200\ \mu l} = \frac{1}{\text{dilution fold}} = \frac{1}{2}$$

Thus, a two fold dilution.

The two-fold dilution shown above yielded a final dilution of 1:256 and involved the use of 8 dilution tubes. Using a constant 100 µl volume to mix and transfer, the final dilution of 1:256 can also be made by several different manipulations of diluent volumes.

Example #2
a four-fold dilution:

Set up four tubes with 300 µl of diluent in each tube, mix and transfer 100 µl again. The total volume of 400 µl. in each tube makes a 1:4 dilution in each tube.



$$\frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} = \frac{1}{256}$$

final dilution of each tube : $\frac{1}{4} \times \frac{1}{16} \times \frac{1}{64} \times \frac{1}{256}$

Example #3
A 16 fold dilution:

This dilution can be made by placing 1500 µl (1.5 ml) of diluent in two dilution tubes. Then mix and transfer 100 µl of serum as before.

$$\frac{1}{16} \times \frac{1}{16} = \frac{1}{256}$$

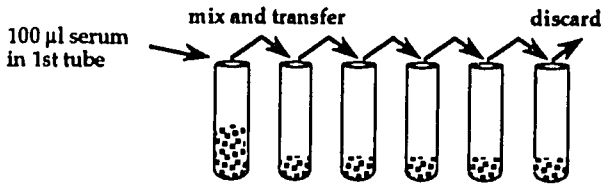
Example #4
Varying the diluent volume:

In this example, it is necessary to quantitate a positive HIV IFA result. On the previous day, 3+ fluorescence was observed at a 1:20 dilution. What is the most appropriate range of dilutions to use to find the endpoint (1+ fluorescence)? What is the most efficient way to make these dilutions?

Ordinarily, when positive samples are quantitated by fluorescence the brightness of the apple-green fluorescence is graded on a scale of 1+ to 4+, with 4+ being the strongest. The titer can be estimated by knowing that for each two- fold increase in dilution the degree of

fluorescence will diminish by approximately one unit of brightness (observation by the authors). In this case the estimation is that the endpoint dilution would be approximately a 1:80 dilution. The most efficient way to approach this situation is to insure that the least amount of wells on the HIV IFA slides are used, and to select the most appropriate range (as to avoid the necessity of repeating the test). Therefore test a 1:40, a 1:80, and a 1:160 dilution of the positive sample. However, these are general guidelines and further dilutions may be necessary before an endpoint reaction can be obtained.

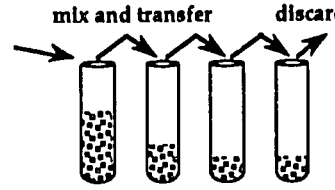
One dilution alternative would be to mix and transfer 100 µl. of serum into tubes as follows:



Tube #	1	2	3	4	5	6
volume of diluent:	400	100	100	100	100	100
tube dilution:	$\frac{1}{5}$	x $\frac{1}{2}$	x $\frac{1}{2}$	x $\frac{1}{2}$	x $\frac{1}{2}$	x $\frac{1}{2}$
final dilution:	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$

In this alternative, six tubes were necessary to get the desired dilutions. It should be noted that any slight error in the dilution process is exponentially increased with each additional tube. Therefore, it is always best to use the minimum number of tubes to obtain the desired dilutions.

A more efficient way of obtaining the same dilutions and only using four dilution tubes is represented below:



Tube #	1	2	3	4
volume of diluent:	900	300	100	100
tube dilution:	$\frac{1}{10}$ X	$\frac{1}{4}$ X	$\frac{1}{2}$ X	$\frac{1}{2}$
final dilution:	$\frac{1}{10}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$

In summary, when making serial dilutions:

- Remember the volume of serum used to mix and transfer remains constant, while the diluent volume is easily changed to yield the most appropriate dilution.

- To conserve serum, always use the smallest possible volume to dilute.

- While using the smallest possible volume to dilute, remember that it is not recommended to use any lower than 50µl (final volumes) for serial dilutions, because a volume less than this would be difficult to control accurately.

Suggestion: many errors occur when making dilutions because of losing track of the dilution sequence. This can be avoided by repositioning each tube in the rack following the addition of fluid. When using microtiter plates, a cover can be used, or a marking on the bench and the plate moved after each addition.

SPECIMEN COLLECTION

When collecting blood for testing, safety first must be practiced, followed by proper procedure in order to collect a specimen that is adequate for testing. Practicing universal precautions includes wearing gloves and protective clothing while drawing blood. When drawing blood from individuals for HIV testing, the most important

consideration is to prevent infection being passed from the individual to the laboratory worker, and from laboratory worker to the individual. When handling many individuals, always wash hands and put on fresh examination gloves between blood drawings.

When drawing blood, hemolysis can be avoided by not leaving the tourniquet on the arm more than 1 minute before drawing the blood. If it is difficult to find the vein and it takes longer than one minute, remove the tourniquet and allow the circulation to return a minute or two before repeating.

Hemolysis can also be caused by transferring blood forcefully through a needle to a tube. Some phlebotomists use a vacuum tube system to draw blood in order to avoid hemolysis. Do not insert the needle directly into the stopper of the vacuum tube when drawing blood with a syringe and transferring to a vacuum tube. The proper procedure to avoid injury and hemolysis is to place the plastic cap of the needle on the bedside or countertop, then insert the needle into the cap using one hand only. Next, remove the cap and needle from the syringe. Afterwards, remove the rubber cap from the vacutainer tube and inject the blood gently and directly into the tube. Replace the rubber cap on the tube for transport to the laboratory.

SPECIMEN PROCESSING

It is extremely important to establish proper identification of the patient or tested individual to avoid incorrect reporting of HIV status. All tubes must be fully labeled before leaving the phlebotomy site. Double check that all information matches on the tube and laboratory request slip. A clerical error can cause a person to be falsely classified.

Serum or plasma are acceptable specimens for most HIV test procedures. If blood is collected without anticoagulant, allow original tube to sit at room temperature for at least 30 minutes to allow clotting. The blood is then centrifuged at approximately 3000 RPM for 15 mintues to separate serum from cells. Alternatively, blood can be left undisturbed for a longer period of time and natural separation will occur. Blood may be collected and mixed with anticoagulants such as Heparin, EDTA or citrate. Plasma can be separated from cells by centrifugation or by gravity.

Antibody titer is not greatly affected in samples left at room temperature for up to one week; however, the problem is that a

sample can become grossly contaminated with bacteria even at refrigerated temperatures if unsterile containers are used and sterile technique is not practiced. Therefore, specimens should:

- not remain at room temperature for any longer than necessary (hours only);
- be refrigerated as soon as possible; and
- be frozen if not tested within 3-5 days.

The Use of Filter Paper as a Carrier for Blood Specimens

Collecting and processing blood specimens in remote laboratories or under field conditions is often troublesome. It may be difficult to separate the serum or to refrigerate the specimens during storage or transport. A technique has been developed for collecting whole blood and it is particularly useful for large serosurveys and for pediatric specimens. A drop of whole blood is obtained from a finger prick and is absorbed onto a specially designed and standardized piece of filter paper (Schleicher and Schuel #903, Keene, New Hampshire, USA). The blood is allowed to dry thoroughly and can be transported to the laboratory or stored for up to 3 months at room temperature without detectable loss of antibody. The filter paper specimens can also be refrigerated or frozen but should always be kept from elevated humidity. This can be accomplished by storing the specimens in sealed plastic bags with desiccant.

For antibody testing, a prescribed circle is punched out of the blood spot and placed in an elution buffer or diluent. A 6.3mm disk that is completely saturated with blood and dried is estimated to contain 10µl of blood or approximately 5µl of serum. The disk is placed in a blank microtiter well and a specified diluent is added to elute the antibody from the filter paper, resulting in a dilution of serum. Eluates at proper dilution can be used in modified ELISA and WB procedures.

Many of the commercial suppliers of ELISA and WB tests have adapted their protocols for use with whole blood specimens collected on filter paper. The manufacturer's recommendations should be followed precisely in order to assure that final dilutions of the specimen are correct. Only those tests that have been adopted for filter paper methods and evaluated should be used.

As with any laboratory method, careful quality control measures are critical. Control filter paper disks can be prepared with HIV positive and HIV negative blood. These controls should be included in each test run. If possible, a set should be stored with specimens as a control for storage conditions.

Pooling of Specimens for HIV testing

The cost of HIV testing is prohibitive for many countries. One approach that has been suggested for cutting costs of large scale HIV screening is to pool 3 to 10 serum specimens and test them as one. For surveillance, the prevalence can be directly calculated from the number of pools that test reactive; however, if individual test results are required, only the reactive pools need be retested individually. This technique is most cost effective for large scale testing where the prevalence of HIV is low (<5%). The technique of pooling should only be used with tests that require a diluted sample since the pooling itself results in a dilution. This method would not be appropriate for use with competitive ELISA.

An ELISA or agglutination test must be carefully modified for pooling and thoroughly evaluated before being adopted by a laboratory. The following points should be considered:

- What is the largest number of samples that can be pooled without compromising accuracy due to specimen handling and record keeping?

- How many specimens can be pooled without sacrificing sensitivity?

- How many specimens can be pooled without decreasing specificity due to high background O.D. readings?

- Will a weakly reactive specimen be detected following dilution with other specimens that are negative?

PART 8
HOW TO EVALUATE DIAGNOSTIC TEST KITS

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INTRODUCTION

There are a large number and variety of HIV diagnostic assays commercially available, many of which offer characteristics that are attractive as well as essential. Literature and package inserts state that these tests have extremely high performance levels. In Parts 3 and 4 we discussed the advantages and disadvantages of some of these tests, and indicated the different reasons why a particular test may be more useful in certain diagnostic situations; Part 5 included a discussion on the various parameters that are used to determine how accurate, reliable, and useful a test is and how the test indicators are calculated. Considering these points, it is reasonable that a particular laboratory will want to use this information in considering which test to adopt.

We stressed that tests do indeed perform differently in various testing situations (conditions in the laboratory, technical expertise, etc.). **It is essential that when evaluations of test kits are to be conducted, they must be performed under the same conditions where the test will eventually be performed.** In addition, certain basic guidelines must be followed to ensure that the test is evaluated properly. This will prevent incorrect information about a test from being reported, and will result in a fair and unbiased opinion concerning a manufacturer's product. It will also provide information to the manufacturers so that they have an opportunity to subsequently modify their products to improve the ability of the tests to achieve optimal performance in all testing situations. We have devoted this part of the Guide to discussing the necessary guidelines for the proper evaluation of HIV test kits.

REFERENCE TESTS

Obviously, if a new test is to be compared to another existing test, there must be a means to determine which test is actually producing the most desirable results. Just because a test has been used by many laboratories, or used for a long time, is no assurance that it is producing adequate results. New and improved tests are always being developed. Therefore, an apparently false positive result by a new test may actually be a true positive result, and the existing test may actually be producing a false negative result. If two tests are being compared, there must always be a "tie breaker." However, even in this case, a third test may also be producing an incorrect result. The point here is that **the quantities of tests used do not necessarily indicate the quality of results.**

A reference test is one that has been accepted by the medical community as a highly accurate and dependable test. It must possess a reputation as being highly accurate and its results must be sufficiently correlated with the actual status of the patient.

Only then can other tests be compared to it and evaluated by it. The reference test in HIV testing is currently considered to be the WB. However, even this test may yield inconclusive results and other tests are required to elicit the true status of the patient. These other tests include synthetic peptide based tests, PCR, and viral culture. In conclusion, **the WB usually can be considered as the reference test, but often must be used in combination with more sophisticated technologies.**

SPECIMENS

To evaluate serologic tests, panels of well characteried specimens must be used. The five following guidelines are presented as part of an evaluation process:

1. **Panels of sera should include reactive, non-reactive, weakly reactive, and indeterminate sera.** If HIV-2 infections occur in the area (or are suspected) then a panel of HIV-2 reactive sera should also be included.

2. **At least 30 specimens should be included in each panel.** Commonly, many more are used, especially negatives. If these numbers of sera are not available, use as many as possible. Note that the greater the number of sera used, the more valid the comparison.

3. **Specimens for testing should be from the same geographic location, and from essentially the same populations that will be subsequently tested.**

4. **If specimens are not freshly collected, they should be stored frozen in aliquots.** The use of aliquots is necessary to ensure that equivalent samples are tested by each method (not after repeated freeze-thaw cycles).

5. **All specimens must be in good condition for testing** (non hemolyzed, non lipemic, etc.).

TESTING CONDITIONS

Each test being evaluated must be performed under identical conditions, including being tested:

- within the same laboratory;
- using the same equipment (pipets, instruments, etc.);
- on the same day (if possible);
- by the same technician;
- using kits that have not expired and have been stored properly.
- in addition, the individual performing the tests must not know results from other tests (i.e. the specimens are tested blindly).

DISCREPANCIES

If discrepant results between the tests occur, there are certain measures that must be performed:

- **All specimens yielding discrepant results must be repeated by both tests.** This will ensure that technical error did not occur. This repeat testing should be performed under the conditions stated above.
- **If specimens still exhibit divergent results, those specimens should be retested again by both tests, but this time by another technician.** This second individual must not know the results from before (i.e. the specimens are tested blindly).
- **If the tests being compared do not include a reference test, all discrepancies must be tested using such a test.**

PART 9
SAFETY IN THE LABORATORY

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INTRODUCTION

Common sense safety practices are the principal means by which laboratory personnel can avoid infection from biohazards. These practices must include prudent measures for personal hygiene, for cleanliness in the work space, and for proper handling of biohazardous materials. An understanding of these necessities and an awareness while performing lab duties will help to prevent accidents, injury, and infection.

OCCUPATIONAL RISKS

HIV has been isolated from blood, semen, saliva, tears, breast milk, vomitus, cerebrospinal fluid, urine, cervical secretions, amniotic fluid, and tissue from infected persons. Practicing "universal precautions" is perhaps the most effective and the most efficient way of preventing exposure to HIV and other pathogens in the laboratory. Universal precautions are used in the care of all individuals since medical history and examination cannot reliably identify those individuals who are infected with HIV. All blood and body fluids are handled as if infectious and all practical precautions are observed.

The primary dangers are parenteral exposure through accidental needle sticks, cuts from contaminated equipment, exposure of mucous membranes to aerosolized droplets, and exposure of broken skin, wounds and scratches to contaminated specimens. **Treat all equipment and pipets in the laboratory as if contaminated,** including writing utensils (avoid such common behavior as putting pens and pencils in the mouth.)

The overall risk of acquiring a work-related HIV infection in the laboratory is actually low; however, a second more ubiquitous virus to be considered as an occupational hazard is Hepatitis B Virus (HBV). Hepatitis B virus, like HIV, is present in blood and body fluids and is transmitted through similar exposure routes as HIV. Neither virus has been found to be transmitted by aerosols alone. **HBV is the most common cause of work related laboratory infections. Therefore, it is recommended that all laboratory workers receive a vaccine series against HBV to prevent occupationally acquired infection.**

To provide a safe working environment in the laboratory, a comprehensive and continually upgraded policy regarding the containment of infectious material must be systematically enforced

to ensure maximum safety to all workers. Observing the following practices while working in the laboratory will minimize exposure to primary and other opportunistic pathogens associated with blood and body fluids.

HYGIENIC PRACTICES

The following practices must always be employed:

1. Always practice universal precautions with all specimens at all times (i.e. treat all specimens as if they were infected).

2. Lab coats must always be worn in the work area.

3. Lab coats should not be removed from the laboratory area except for laundering.

4. Keep lab coats away from dining or food areas.

5. Cuts and scratches on hands and arms should be well covered and protected.

6. Always wash hands after removing gloves, and before leaving the laboratory.

7. Eating, drinking, and smoking in the work areas is a serious health risk and must not be practiced.

8. Long hair should be pinned up or covered.

9. Always wear eye protection when generating droplets or aerosols.

10. Avoid activities such as applying make-up and combing hair in the laboratory.

11. Protective shoes that completely cover feet should be worn, (no open-toed shoes or sandles).

12. Always wear gloves when handling blood or bodily fluids

13. If gloves are in short supply, gloved hands can be washed frequently and wiped with disinfectant throughout the day.

WORK SPACE

A clean, organized work space is a safer work space:

- Reagents should be well-labelled, in proper containers, and stored properly (example: store acids and bases in a separate cabinet below bench-top level.)
- Keep bench top free from clutter (non-essentials).
- Do not place materials near the edges of counters or shelves.
- Clean countertops with disinfectant before and after each work day using one of the following:

Suggested Disinfectants
Sodium hypochlorite, 0.1% - 0.5% available chlorine; Ethanol, 70%; Isopropyl alcohol, 70%; Formaldehyde, 4%; Glutaraldehyde, 2%; Hydrogen peroxide, 6%; Polyvidone iodine, 2.5%.

- Restrict entry to authorized personnel only, receive visitors outside of laboratory area.
- Immediately contain spills and wipe contaminated surface with one of the disinfectants listed above.
- Avoid use of sharp instruments whenever possible (scapels, needles, scissors).
- Pest control prevents known and unknown vectors which carry disease.
- Breakable materials (glass) should be used only when absolutely necessary and should be stored safely.
- Periodically clean doors, handles, telephones with disinfectant.
- Each lab should have a first-aid kit available.
- Report accidents immediately to the supervisor.

- An eye wash station should be available for accidental exposure from splashes.

BIOHAZARDOUS MATERIAL

Proper handling prevents infection to yourself and others:

- Biohazards warning signs must be plainly posted.

- All laboratory waste must be properly decontaminated before disposal. Autoclave at 121°C at one atmosphere for 20 minutes or incinerate.

- Do not bury infectious waste.

- Use a separate puncture resistant container for glass waste and needles.

- Never recap a needle with two hands. Place the cap on the counter and insert needle into the cap with one hand only, or place entire needle/syringe into puncture resistant container for disposal.

- Spills must be immediately wiped up with one of the disinfectants listed under "work space."

- Liquid wastes should be decontaminated by autoclaving or by adding sufficient sodium hypochlorite before discarding.

CLOSING REMARKS

HIV testing presents a major challenge to the laboratory worker. Without error, the specimens must be handled, tested, and results must be interpreted and reported. One false positive or false negative result due to negligence or technical error is unacceptable and can have dramatic consequences for the tested individual, the patient, the transfusion recipient, or the patient's contacts. In addition to the normal care and expertise required to perform laboratory tests, the correct diagnosis of HIV infection requires a systematic and thorough quality assurance program.

In this manual we have provided the laboratorian with basic information about the HIV virus, the tests currently used to reveal infection, the interpretation of results, and the necessary quality assurance measures needed to ensure that test results are obtained and reported as accurately and correctly as possible. With a thorough educational background in these principles, the laboratorian will have the skills necessary to produce the best quality results. This will be the laboratorian's contribution toward the prevention and control of HIV infection and AIDS.

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GLOSSARY

Agglutination: The clumping of cells or particles due to a lattice formation, as occurs when antigen complexes with antibody.

Agglutination assay: An immunoassay in which antibody reacts specifically with antigen coated particles or cells to form a lattice network resulting in the clumping or agglutination of the particles.

Amino acids: The chemical building blocks of proteins.

Antibody: Proteins produced by the body in response to the presence of a foreign substance, an antigen, or immunogen.

Antibody titer: The concentration of antibody in the serum expressed as the reciprocal of the dilution. (e.g. when a sample can be diluted 1:160 and still be reactive, but further dilutions result in a non reactive result, the titer is 160).

Antigen: Foreign substances that usually induce the immune system to produce antibodies. These antibodies can specifically react with the specific antigen.

Antigenemia: The presence of viral components or antigens in the blood.

Antigen capture assay: An immuno assay to detect antibody in a serum sample. A monoclonal antibody is used to capture the viral antigen and attach it to the solid support. The remaining part of the assay can be a competitive or an indirect ELISA for antibody detection.

Antigen detection ELISA: An enzyme immunoassay that has specific HIV antibody bound to the solid support phase. HIV antigen present in the serum or tissue culture fluid sample is bound to the antibody and detected by a conjugate-substrate system.

ARC: AIDS related complex. A stage of HIV infection in which the patient exhibits some signs of disease but does not meet the criteria for a diagnosis of AIDS.

Autoradiography: A technique in which film exposed to radioisotopically labeled substances is used to visualize the labeled substance.

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B cells: A class of human lymphocytes that mature in the bone marrow and are important in humoral immunity.

Biologic false negative: A falsely negative test result due to parameters related to the specimen or patient, not to the testing process.

Borderline control: An external control that reacts just above (indirect ELISA) or just below (competitive ELISA) the cutoff point. A borderline control will detect minor changes that may greatly affect test results.

Borderline reactor: An ELISA OD reading that is just above or just below the cutoff point. The borderline reactor specimen is usually retested.

CD4: Receptor molecule that binds the HIV. Found on T helper lymphocytes as well as some other cells, it can be used as a marker to identify these cells.

Coefficient of variation: The degree to which the numerical data tend to disperse about the mean; an indicator of reproducibility of values.

Competitive ELISA: An enzyme immunoassay in which the unknown antibody in the sample competes with the conjugate for reactive sites on the bound antigen. The resulting color change is inversely proportional to the amount of antibody in the serum sample.

Confidence interval: A statistical estimate of the precision of some parameter.

Confirmatory testing: Tests performed following reactive screening tests to assure that the screening tests were correct. Also called supplemental testing.

Conjugate: As used in the ELISA, an enzyme coupled or linked to an antibody. As used in the IFA test, a fluorescent dye linked to an antibody.

Conserved region: Sequences within the viral genome that do not vary from virus to virus.

Core: The internal region of the virus, bound by a protein coat, containing the RNA genome and viral enzymes such as reverse transcriptase, integrase, and protease.

Criteria: Guidelines for determining if a test result fulfills the requirements to be labeled as positive, negative, or indeterminate.

Cutoff: A reference point for determining whether the optical density reading obtained from an ELISA test represents a reactive or non reactive result. The cutoff point is usually calculated based on the mean of the negative control values.

Cytotoxic cells: T lymphocytes that recognize specific antigens on infected or malignant cells, and kill those cells.

Dilution: Mixing of one part of a substance with one or more parts of another substance thus reducing the concentration of the first substance.

Dot blot assays: Immuno assays using paper or nitrocellulose as the solid support incorporating a dot of viral antigen blotted on the support.

EIA: Enzyme Immuno Assay (See ELISA.)

Electrophoresis: A technique for separating proteins, usually based on their different electrical charges and molecular weights.

ELISA: Enzyme Linked Immunosorbent Assay. An assay in which antigen or antibody is bound to a solid support. Unknown antibody or antigen in the specimen is then specifically bound and detected by an enzyme-substrate system.

ENV: Refers to the gene that directs the production of the envelope glycoproteins of HIV-1 including gp160, gp120, and gp41.

Envelope: The outer shell of the virus, composed of glycoproteins and lipids.

Enzyme: An organic catalyst. In the ELISA test the enzyme acts upon a specific substrate to create a colored end product that is measured and reflects the amount of enzyme specifically bound in the reaction.

External control: Control sera with known values derived from a source other than the test kit. These controls are included in test runs to monitor consistent performance, lot to lot variation, etc.

Fluorochrome: A substance such as fluorescein isothiocyanate, which fluoresces when exposed to ultraviolet light.

GAG: The group associated antigen gene that encodes the core proteins p55, p24, p17 and p15 of HIV-1.

Gene products: Proteins that make up the virus on instruction from the viral genome.

Genome: The sum of all the genes of an organism.

Glycoproteins: Proteins coupled to carbohydrates.

Gray zone: The test results falling within a defined zone immediately below the cutoff value. Results falling within this zone often warrant further testing.

Helper T lymphocytes: A type of T cell that stimulates immune responses. Helper T lymphocytes cooperate with B-cells and other T-cells in initiating antibody-mediated responses. Also referred to as T-4 cells because they contain the CD4 marker.

HIV-1: The initial human immunodeficiency virus type 1 associated with AIDS. Initially called HTLV-III or LAV-1.

HIV-2: A second human immunodeficiency virus type 2 associated with AIDS sharing 40 to 45% homology with HIV-1; is also associated with AIDS.

HTLV-I: Human T lymphotropic virus type I, associated with adult T cell leukemia, HTLV-I associated myelopathy, and tropical spastic paraparesis.

HTLV-II: Human T lymphotropic virus type II has been linked with "Hairy Cell" leukemia.

Immunogenic: That which generates an immune response.

In-house controls: Control sera prepared in the investigator's own laboratory. In-house controls are often prepared by pooling several specimens together, determining the test result and aliquoting for

routine use as a control. External controls are not included in the test kit.

In Situ Hybridization: A technique in which radiolabelled or enzyme labeled HIV RNA is used to probe mononuclear cells for the presence of HIV DNA.

Indeterminate: A result that cannot be classified as positive or negative based on the test results.

Indirect Fluorescent Antibody Assay (IFA): An assay that uses a fluorochrome-labeled antibody to indirectly detect antibody or antigen.

Indirect ELISA: An enzyme immuno assay for detection of antibody in which the antigen is attached to the solid phase, specific antibody in the serum is allowed to bind to the antigen, and an anti human conjugate is allowed to react with the bound specific antibody. A substrate is degraded by the conjugated enzyme, resulting in a colored product in direct proportion to the amount of specific antibody in the serum.

Inducer cell: T-4 lymphocytes that induce other immune cells to respond.

Infection: The stage at which virus has attached, penetrated and been incorporated into the host cell.

Inter-run reproducibility: A measure of repeatability between test runs.

Internal control: The positive and negative control sera included in a test kit and tested with each run.

Intra-run reproducibility: A measure of repeatability within one test run.

Lymphocyte: A mononucleated white blood cell.

Lymphocyte phenotyping: Test procedure describing the physical characteristics of a lymphocyte as opposed to its genetic make-up.

Lymphokines: Chemical effector substances produced and secreted by stimulated lymphocytes. Lymphokines serve to direct and regulate functions of the lymphocytes and other white blood cells.

Macrophages: Large cells of the mononuclear phagocyte system that devour microbes, present antigen, and secrete important immune system effector substances.

Mean: In HIV testing, the average value of a set of O.D. values or O.D./cutoff ratios.

Monoclonal antibodies: Very specific antibodies produced by a single cell, or the identical progeny of that cell against a specific epitope of an antigen.

NK cells: Natural killer cells. Lymphocytes that are non-specific killers of certain tumor and virus-infected cells.

Negative: The reported result of a confirmatory or supplemental test indicating that the test did not detect evidence of HIV infection.

Negative predictive value: A statement of the probability that a negative test result truly represents non-infection.

Non-reactive: The reported result of a screening test indicating that the test did not detect evidence of HIV infection.

Non-specific antibodies: Antibodies that may be bound in an immunoassay, but are not directed specifically to the viral antigen. In tests for HIV, non-specific antibodies usually represent antibodies to non-viral proteins.

O.D. / cut off ratio: The test optical density reading divided by the cutoff value.

Opportunistic infections: Infections occurring in an immunocompromised host that would normally not occur in a host with an intact immune system.

Optical density: The units expressed by an ELISA reader representing the light adsorbed by the colored end product of an ELISA reaction.

PCR: Polymerase Chain Reaction. A test amplifying specific nucleic acids so that the sequence can be detected.

Plasma cells: A fully differentiated, activated B-lymphocyte that produces antibody specific for a particular antigen.

PMN: Polymorphonuclear leukocytes. While blood cells that are granulocytic and phagocytic.

POL: The polymerase gene or polymerase proteins including p66, p51 and p31.

Positive: The reported result of a confirmatory or supplemental test indicating that an individual has been infected by HIV.

Positive predictive value: A statement of probability that a positive test result truly represents an infection.

Precursor molecule: A molecule produced early in infection and later cleaved to produce other proteins.

Predictive values: Description of the value of tests taking into account the actual prevalence of infection in the population being tested.

Primer: A specific sequence of amino acids that can be annealed to a matching region on the HIV genome for extension during the PCR assay.

Profile: The pattern of bands apparent on a Western blot test strip representing the array of specific antibodies present in an individual at a specific time.

Provirus: Viral genome that has been integrated into host cell DNA, and directs the synthesis of specific viral components.

Prozone: Inhibition of agglutination when excess antibody is present resulting in a falsely negative test result at very high antibody concentrations.

Quality assessment: The means to determine the quality of results. Usually an external system to check laboratory performance.

Quality assurance (QA): The overall program ensuring that the final results reported by the laboratory are as accurate as possible.

Quality control (QC): Those measures that must be included during each assay to verify that the test is working properly.

Parallel testing: A comparison of performance of new lots of kits with the previous lots via a common control material.

Passive agglutination: An agglutination assay in which the antigens are bound passively to the carrier, without specific attachment procedures.

Proficiency panel: A set of well characterized sera sent to laboratories for testing in a routine manner. The laboratory test results are compared to the known values to determine the proficiency of the laboratory.

PHA: Passive hemagglutination assay. An agglutination assay that uses red blood cells as the carrier system.

Random error: Error due to chance, not necessarily that of the assay or the technician.

Reactive: The reported test result from a screening test which indicates that HIV infection was detected.

Receptor molecule: The molecule on the host cell to which the virus specifically attaches.

Recombinant antigen: Antigens produced when a portion of the viral genome is inserted into a biological vehicle, resulting in the production of the gene product, which can be easily replicated in the host.

Reference Test: A test that other tests are compared against: the "gold standard".

Retroviruses: Viruses that use a copying enzyme to take an RNA genome and make a DNA complementary strand.

Reverse transcriptase (RT): The copying enzyme used by retroviruses.

Run: A group of specimens tested together in one batch.

Sensitivity: The ability of a test to detect very small amounts of analyte, or the ability of a test to detect infected individuals.

Serial dilutions: Mixing and transferring a constant volume of serum into successive tubes containing diluent.

Seroconversion: The point at which specific antibody can first be detected in an infected individual who was previously negative for antibody.

Seroprogression: Evolution of antibody response; an increase in antibody over time.

Sero-reversion: The point at which antibody can no longer be detected in an infected individual.

Shift: Rapid changes in control sera values noted on a quality control graph indicating that a major change has occurred.

Significant error: Error that is not random but occurred due to a technical error, or errors associated with the test.

Specificity: The ability of the test to identify all negatives correctly.

Spectrophotometer: The instrument used to measure absorbance of light at specific wavelengths. An ELISA reader is a spectrophotometer.

Standard deviation: A measure of variation defining the expected range of a value in relation to the mean value.

Standard Operating Procedure: A book containing protocols for all procedures performed within the laboratory.

Substrate: A specific chemical that is degraded by an enzyme to form a colored product. Also used to describe the fixed cell layer in the IFA test.

Supplemental Testing: Tests performed after screening tests to confirm the results of the screening assays (See confirmatory testing).

Suppressor cells: T lymphocytes that inhibit or suppress the immune response of other cells of the immune system. Suppressor cells contain the CD8 molecule and therefore are also called T-8 cells.

Syncytia: Fusion of cells as a response to viral infection.

Synthetic peptide: Peptides created in the laboratory by combining amino acids in a known sequence to form the desired peptide.

T cells: Cells that derive in the bone marrow and migrate to the thymus where they multiply and mature into lymphocytes that are capable of an immune response. They play a significant role in cell mediated immunity.

T-4 lymphocyte: A helper/inducer lymphocyte carrying the CD4 receptor molecule. Also called T-4 cells.

Test efficiency: The overall ability of a test to correctly identify positives and negatives. The combination of the sensitivity and the specificity of an assay gives an idea of the total effectiveness of the assay.

Transmembrane: Gp41, the glycoprotein antigen which is located between the inner and outer membrane of the envelope in HIV-1.

Trend: Subtle changes in the same direction on a quality control graph, indicating a gradual change has occurred.

Troubleshooting: Identifying problems and solving them.

Two-fold serial dilution: A dilution where a fixed volume of serum is mixed and transferred into successive tubes containing diluent.

Universal precautions: Precautions taken by health care workers to avoid being infected. Since medical history and examination cannot reliably identify all of those who are infected with HIV, all blood and body fluids are handled as if they were infectious.

Variable region: Sequence within the viral genome that may vary between virus strains.

Vigilance: Watchfulness over laboratory procedures and record keeping.

Viral lysate: Disrupted whole virions, often used as the antigen in viral immuno assays.

Western blot (WB): An enzyme immuno assay using antigens previously separated by electrophoresis to detect antibody to specific viral proteins. Considered as a confirmatory or supplemental test for antibodies to HIV.

Window period: The period after infection has occurred but before antibody can be detected.

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APPENDIX
WORKSHEETS

Bead assay
Microtiter plate assay
IFA
Western blot
Quality control graph - simple
Quality control graph - OD / cutoff
WESTERN BLOT TROUBLESHOOTING CHART
APPLICATION FORM FOR PERFORMANCE EVALUATION

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HIV SCREENING TEST WORKSHEET
(Bead Assay)

Sheet No. _____ Date _____

	Specimen	Result
A1		
A2		
A3		
A4		
A5		
B1		
B2		
B3		
B4		
B5		
C1		
C2		
C3		
C4		
C5		

	Specimen	Result
E1		
E2		
E3		
E4		
E5		
F1		
F2		
F3		
F4		
F5		
G1		
G2		
G3		
G4		
G5		

	Specimen	Result
J1		
J2		
J3		
J4		
J5		
K1		
K2		
K3		
K4		
K5		
L1		
L2		
L3		
L4		
L5		

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HIV SCREENING TEST WORKSHEET
(Bead Assay)

Sheet No. _____ Date _____

	Specimen	Result
D1		
D2		
D3		
D4		
D5		

	Specimen	Result
H1		
H2		
H3		
H4		
H5		

	Specimen	Result
M1		
M2		
M3		
M4		
M5		

Source: _____ Lot. No. _____

Exp. Date: _____ Technician _____

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HIV SCREENING TEST WORKSHEET
(Microtiter Plate)

Sheet No. _____ Date _____

	Specimen	Result		Specimen	Result		Specimen	Result		Specimen	Result
A1			A4			A7			A10		
B1			B4			B7			B10		
C1			C4			C7			C10		
D1			D4			D7			D10		
E1			E4			E7			E10		
F1			F4			F7			F10		
G1			G4			G7			G10		
H1			H4			H7			H10		
A2			A5			A8			A11		
B2			B5			B8			B11		
C2			C5			C8			C11		
D2			D5			D8			D11		
E2			E5			E8			E11		
F2			F5			F8			F11		
G2			G5			G8			G11		

HIV SCREENING TEST WORKSHEET
(Microtiter Plate)

Sheet No. _____ Date _____

	Specimen	Result		Specimen	Result		Specimen	Result		Specimen	Result
H2			H5			H8			H11		
A3			A6			A9			A12		
B3			B6			B9			B12		
C3			C6			C9			C12		
D3			D6			D9			D12		
E3			E6			E9			E12		
F3			F6			F9			F12		
G3			G6			G9			G12		
H3			H6			H9			H12		

Source: _____ Lot. No. _____

Exp. Date: _____ Technician _____

HIV CONFIRMATORY TEST WORKSHEET
(Indirect Fluorescence)

Date: _____

Technician: _____

Source: _____

Lot #: _____

Expiration Date: _____

Slide Number:		Results			
Well No.	Serum No.	HIV	H9	Interp.	Comment
1					
2					
3					
4					
5					
6					
7					
8					

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HIV CONFIRMATORY TEST
WORKSHEET
(Indirect Fluorescence)

Date: _____

Technician: _____

Source: _____

Lot #: _____

Expiration Date: _____

Slide Number:		Results			
Well No	Serum No.	HIV	H9	Interp.	Comment
1					
2					
3					
4					
5					
6					
7					
8					

HIV CONFIRMATORY TEST WORKSHEET
(Western blot - HIV-1)

Date: _____ Source: _____

Technician: _____ Lot #: _____

Strip No.	Sample No.	p15	p17	p24	p31	gp41	p51	p55	p66	gp120	gp160	Comments

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HIV CONFIRMATORY TEST
WORKSHEET
(Western blot - HIV-1)

Date: _____ Source: _____

Technician: _____ Lot #: _____

Strip No.	Sample No.	p15	p17	p24	p31	gp41	p51	p55	p66	gp120	gp160	Comments

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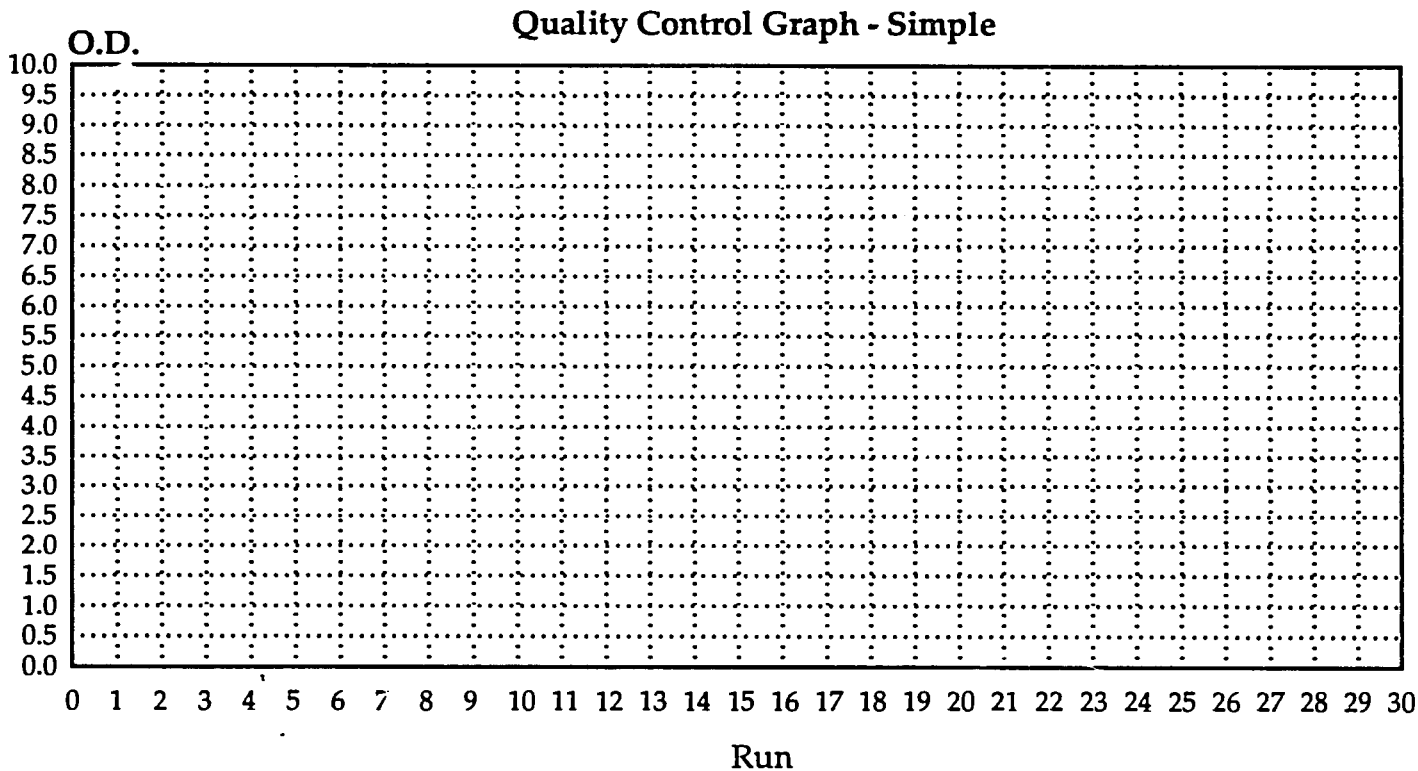
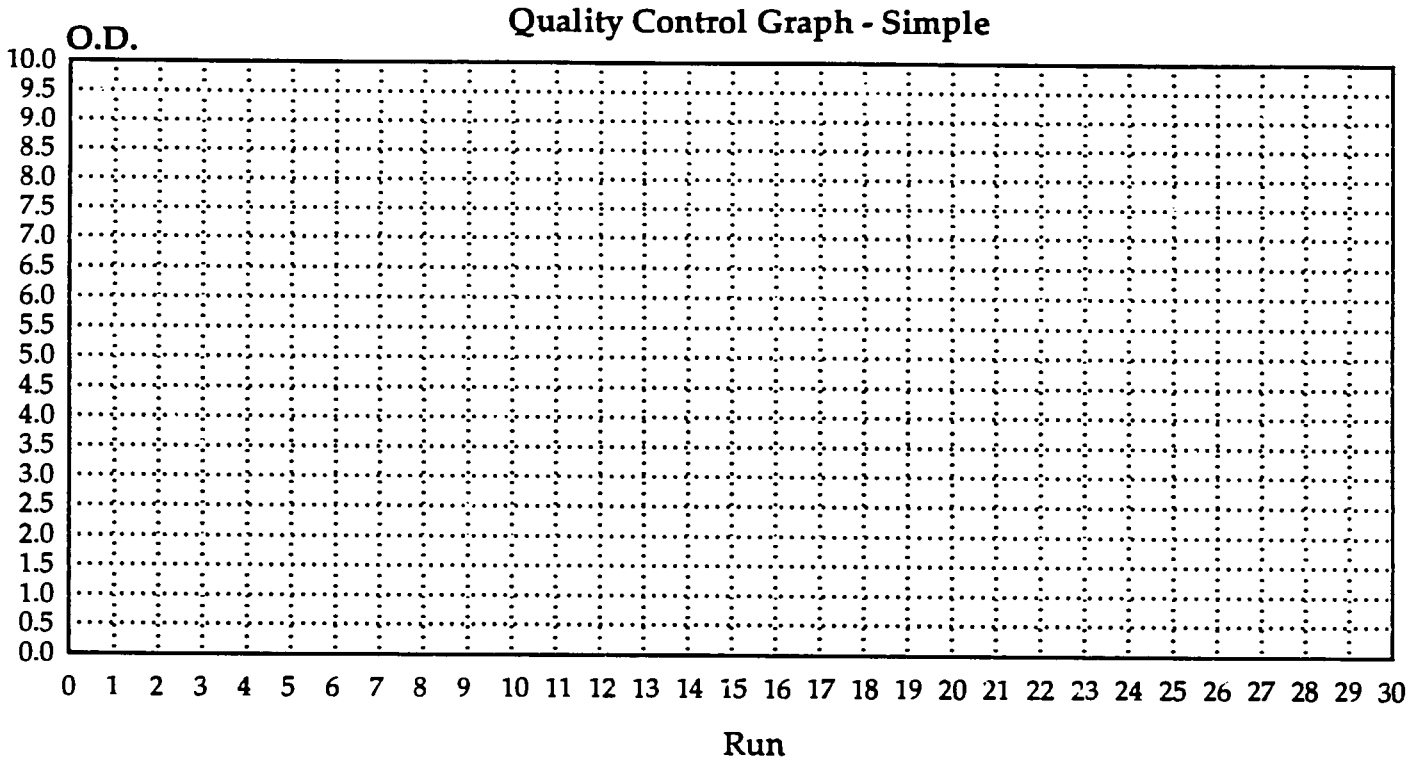

Quality Control Graph - Simple
O.D.
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Run


Quality Control Graph - Simple
O.D.
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Run

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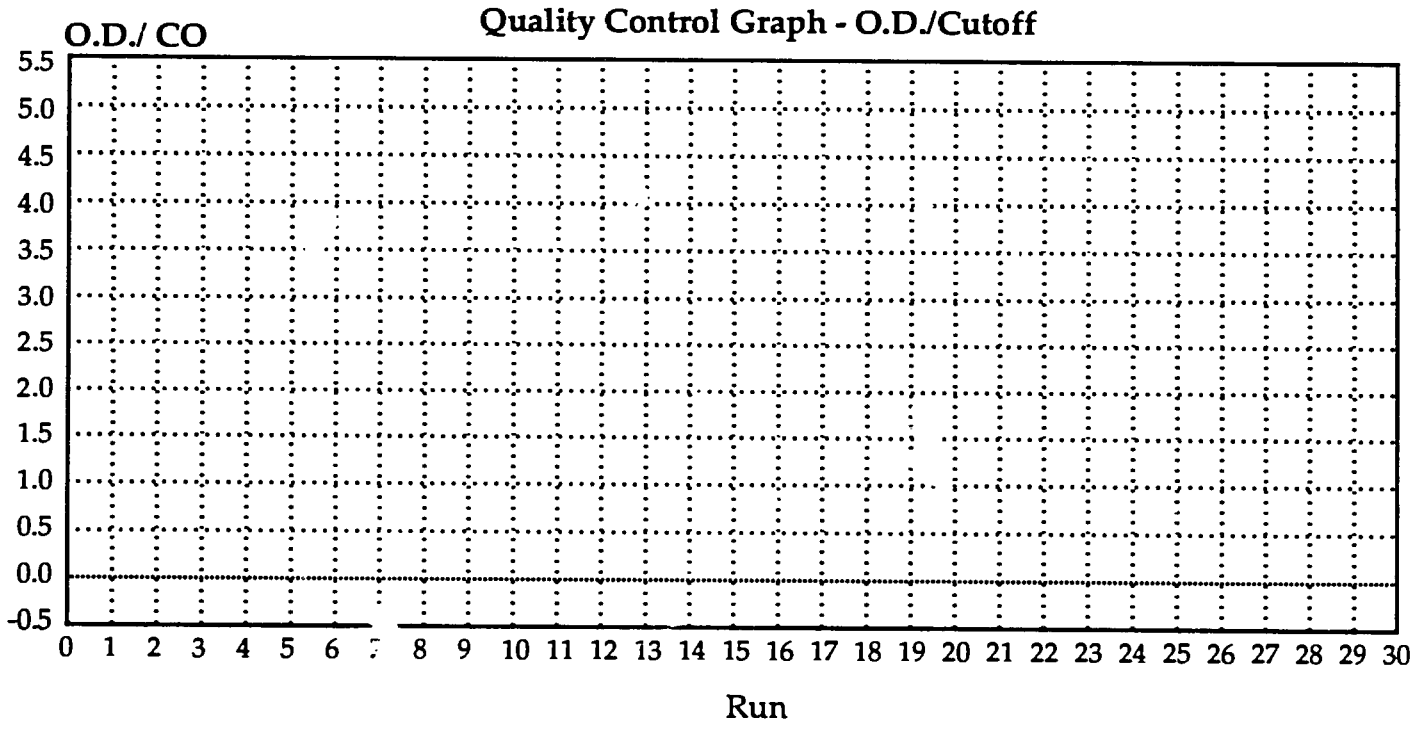
Quality Control Graph - O.D./Cutoff
O.D./ CO
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Run

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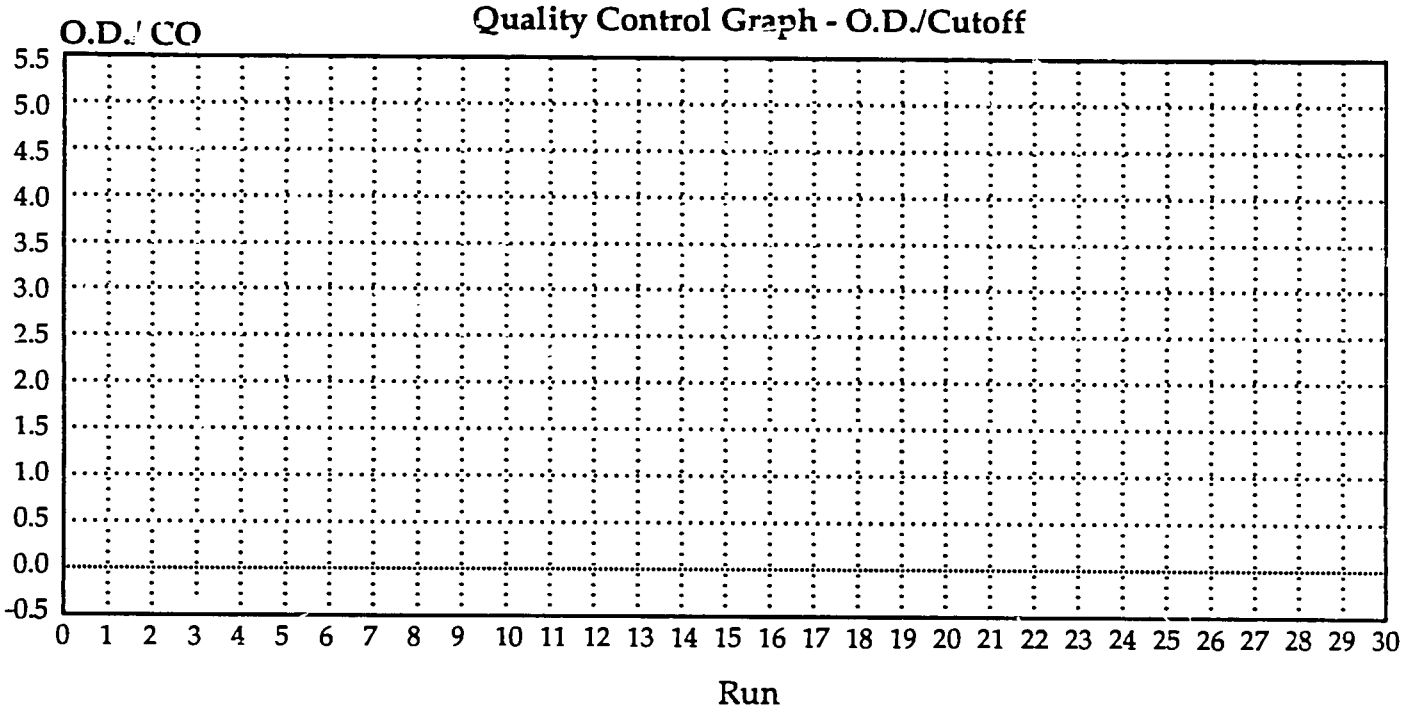
Quality Control Graph - O.D./Cutoff
O.D./CO
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Run

WESTERN BLOT ASSAY TROUBLESHOOTING CART

Dark spots develop on strips

The problem is probably caused by:

1. Contamination of test samples by bacteria. Samples of questionable origin should be run in the presence of .02% NaAzide for sample incubation but not in conjugate
2. Immune complexes in aged test samples are precipitating out (see instructions at the bottom of page 4 in the directional insert)

Bands don't develop or show up weak.

Check positive control

Positive control weak

The problem is probably caused by the reagents:

1. If substrate A shows color upon opening, then it has become oxidized
2. If the tray was bleached, then substrate A may have become oxidized during the assay run
3. Reagents may not have been mixed correctly

Positive control OK

The problem is probably caused by test sample:

1. Sample may have been diluted too much
2. Sample may have been inactivated through improper handling and/or storage
3. Sample wells in incubation tray may have been contaminated with oxidizing chemicals
4. Test sample may be an ELISA "false" positive

Unspecific bands develop

The problem is probably caused by the test sample:

1. Sample is so strong that it reacts with trace amounts of intermediates especially gag ie 22,29, 32 39, 45
2. Sample reacts with H-9 protein present in viral preparation (e.g. HLA, ABC, (45) DR (55, 33, 29) or p70-80) other possible cellular proteins p64, p53
3. If env gp 160 is strong there could be a multimer of gp41 (ie dimer, trimer (runs close to actual gp 120 band))

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WESTERN BLOT ASSAY TROUBLESHOOTING CART

Strong background develops on strip in the absence or presence of positive bands

↓

The problem is probably caused by:

↓

1. Overdeveloped strips (stop reaction sooner)
2. Incomplete washing steps especially after conjugate 2

Bands develop on negative control

↓

Tray wells or assay reagent may have been cross contaminated

Strips are defective if:

↓

1. They are cracked
2. They contain air bubbles which cause the appearance of white spots in reactive zones big enough to prevent band detection

Caution:

A The strips have been fine tuned to work best with the reagents provided

B Incubate strips with the reactive (numbered) side up. Follow assay instructions to the letter

C. Do not cut strips lenghtwise. You are sverely increasing the chances for misinterpretation; strips may flip over in the incubation tray and, more importantly, possible artifacts in reactive zones may be mistaken as positive bands or may prevent recognition of positive bands. **There are four good reasons for making the stiprs 4mm wide**

D. All strips should be used immediately upon initial opening of the strip tube; if this is not possible, then tubes should be stored tightly reasealed in a cool, dark, dry place: Biotech disavows any responsibility for stripcondition if not stored exactly as described above after opning of tube

BIOTECH RESEARCH LABORATORIES, INC.

1600 E. GUDE DR
Rockville, MD 20850
USA
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Fax 301-762-1327

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OMB No.: _____
Exp. Date: _____

Centers for Disease Control
Model Performance Evaluation Program
Human Immunodeficiency Virus Type 1 (HIV-1) and Human T-Lymphotropic Virus Type I and II (HTLV-I/II)
Laboratory Enrollment Form

1. Name of Laboratory: _____

2. Mailing Address: _____

3. City: _____ State: _____ ZIP Code: _____
   [ ] Please check if this address differs from that on the mailing label.

4. Telephone Number: (___) _____ - _ Ext.: _____

5. Laboratory Director's Name: _____

6. Laboratory Director's Title: _____

7. Laboratory Supervisor's Name: _____

8. Laboratory Supervisor's Title: _____

9. Please indicate whether your laboratory would like to participate in the CDC Model Performance Evaluation Program for HIV-1, HTLV-I/II, or both.

HIV-1	HTLV-I/II
[ ] Yes	[ ] Yes
[ ] No	[ ] No

If No, please check he appropriate box below:
[ ] Our laboratory does not perform HIV-1 testing.
[ ] Our laboratory does not perform HTLV-I/II testing.
[ ] Other reasons, please specify (optional) _____

10. Please read the following and sign the form.

We understand that as participants in the program, we will be asked to send the following to CDC: (1) results of our testing of samples provided by CDC; (2) information on methodology used with the samples; and (3) demographic and other information related directly or indirectly to the testing process.

Director's Signature _____

11. Please mail the completed enrollment form to:

MPEP Survey Coordinator
Program Resources, Inc.
Science and Technology Center
P.O. Box 12794
Research Triangle Park, NC 27709

If you have any questions about this program, or if you know of other facilities of your organization doing HIV-1 or HTLV-I/II testing, please call toll free 1-800-322-4383.

Public reporting burden for this collection of information is estimated to average 1 minute per response. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to PHS Reports Clearance Officer, ATTN: PRA, Hubert H. Humphrey Building, Rm 721-B, 200 Independence Ave., S.W., Washington, DC 20201, and to the Office of Management and Budget, Paperwork Project (0920-XXXX), Washington, DC 20503.

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