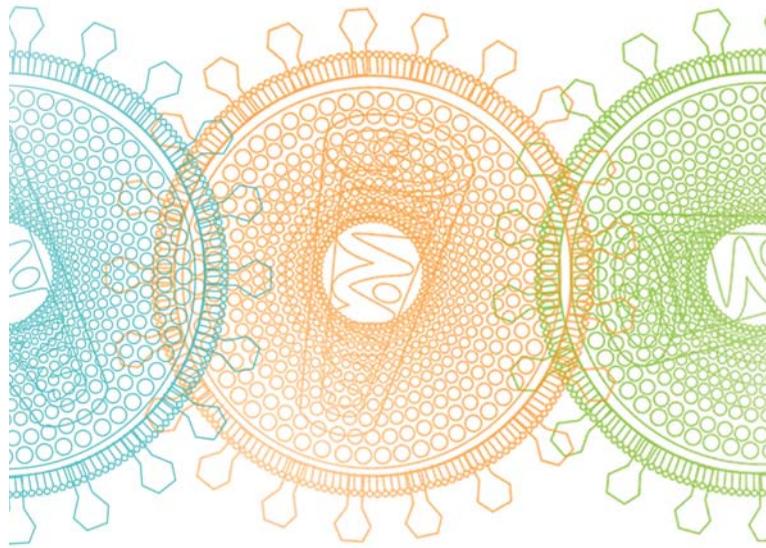


Diagnosis and Monitoring of HIV Infection





Introduction

An emerging disease identified in 1981 in the United States, AIDS (Acquired Immunodeficiency Syndrome) very quickly reached pandemic proportions. At the end of 2004, it was estimated that more than 25 million people had died of AIDS since the beginning of the epidemic, while approximately 40 million people worldwide were living with the virus. A little more than 20 years after the discovery of the virus, considerable progress has been made in the diagnosis, monitoring and treatment of the infection. Nevertheless, the epidemic continues to grow, with an estimated 4.3 million new infections in 2006, the equivalent of over 8 new infections each minute. There is an urgent need for information, screening and prevention ... and where education and prevention have failed, for patient management.

This booklet contains the basic information necessary to understanding the biology of the Human Immunodeficiency Virus (HIV) as well as the diagnosis and immunovirological monitoring of infection and treatment.

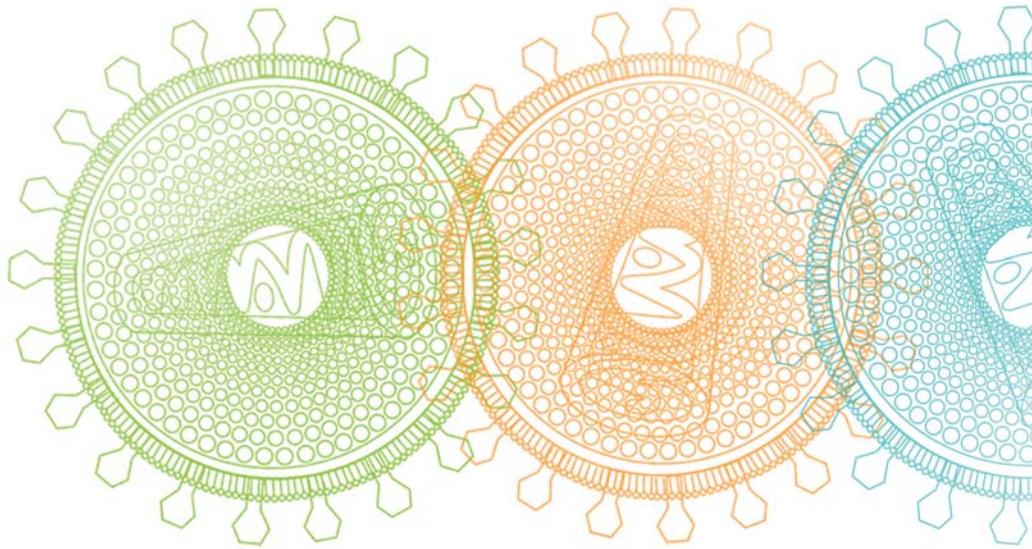
It is intended to provide a succinct and practical, although not exhaustive, guide for laboratory professionals and clinicians.

This brochure was written with the collaboration of:
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For his contribution to the Q & A section, we would also like to thank:
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The virus



HIV observed under an electron microscope. Photograph used with the kind permission of P. Roingeard

■ HIV is an enveloped virus that measures 80-120 nm in diameter and has a tropism for CD4+ lymphocytes and monocytes. It belongs to the *Retroviridae* family (sub-family: *Lentivirinae*) and has three enzymes that are necessary for multiplication:

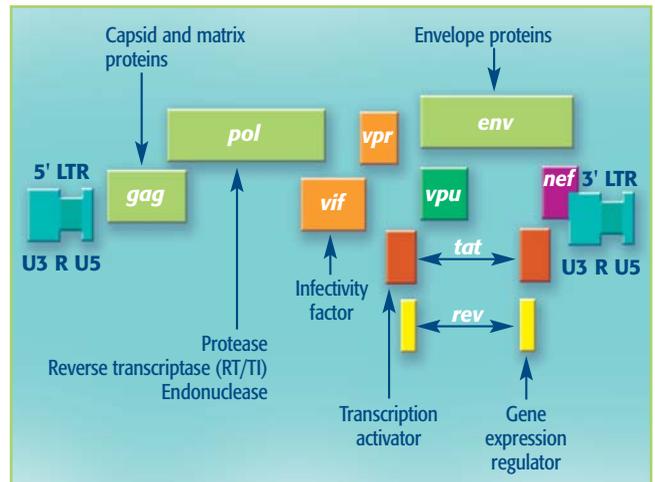
- 4 **reverse transcriptase** which, upon infection of the cell, enables the viral RNA to be transcribed into DNA
- 4 **endonuclease** which enables the DNA to be integrated into the host cell (the viral genome then becomes proviral DNA)
- 4 **protease**, which enables the virus to mature at a late stage in the cycle of intracellular multiplication

Structural organization of HIV and the main antigens used for diagnosis

Protein	HIV-1	HIV-2	Gene
GPSU	gp120	gp125	<i>env</i>
GPTM	gp41	gp36/41	
CA	p24	p26	<i>gag</i>
MA	p17	p16	
Reverse transcriptase	p66/51	p68/53	<i>pol</i>
Endonuclease	p32	p34	

■ Its **genome** comprises two RNA strands containing **approximately 9,200 nucleotides**. From the 5' extremity to the 3' extremity are located the three genes that characterize retroviruses: *gag-pol-env*. The *gag* gene codes for the internal structural proteins, the *pol* gene for the three viral enzymes, and the *env* gene for the envelope glycoproteins. LTR (Long Terminal Repeat) sequences are found at each extremity of the genome, containing the signals for the regulation of expression of the viral genes. The genome also has six additional genes called "accessory" genes: *vif*, *nef*, *vpr*, *tat*, *rev* and *vpu* (HIV-1) or *vpx* (HIV-2).

HIV Genome





The Diversity of HIV

■ The genetic diversity of HIV is vast. Current **classification** systems distinguish between two types, HIV-1 and HIV-2, which are further divided into groups and/or subtypes. The pandemic is caused by **HIV-1 group M**. The global distribution of the different viral strains reflects the development of sub-epidemics. This classification is constantly evolving due to the continued diversification of the virus, especially in connection with viral recombination.

Type	Group	Subtype, remarks
HIV-1	M	- 9 pure subtype: A, B, C, D, F, G, H, J, K - More than 15 CRF, the most prevalent being CRF01-AE (Asia) and CRF02-AG (Western Africa)
	O	Rare, limited to Equatorial West Africa (Cameroon)
	N	Rare, limited to Equatorial West Africa (Cameroon)
HIV-2		Essentially limited to Western Africa

Certain **recombinant HIV** strains are known as CRF (Circulating Recombinant Forms) due to their epidemiological importance. Although **HIV-2** is also associated with AIDS, it is not transmitted as readily and, generally speaking, progression toward immunodeficiency is much slower in individuals with an HIV-2 infection.

■ The strains found in France for example belong primarily to subtype B, which is the case in most industrialized countries. A steady increase in the prevalence of non-B strains has, however, been observed over the past few years (30% of strains identified during primary infections in 2000-2002). HIV-2 represents 2 to 3% of strains and HIV-1 group O represents 0.3%.

Geographical distribution of different HIV strains

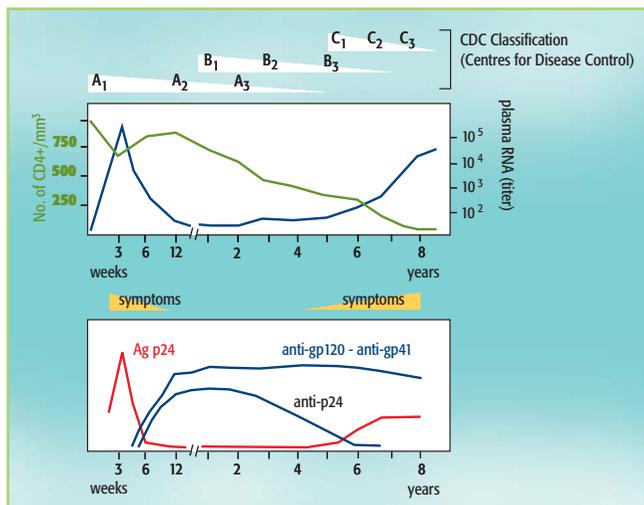




Physiopathology of the Infection

■ HIV-1 is responsible for a **chronic infection** that gradually develops and causes the destruction of the body's CD4+ T lymphocytes. If no anti-retroviral treatment is given, the continuous replication of the virus in the lymph organs leads to the daily production of 10^9 - 10^{10} virions in the body for years, even among patients whose plasma viral load is low or undetectable.

Development in immunovirological markers



■ **Primary infection** is asymptomatic in more than 50% of cases. In the remaining cases, symptoms appear two to three weeks after infection and clinical signs usually resemble those of flu-like or mononucleosis syndromes.

The main clinical signs during primary HIV infection :

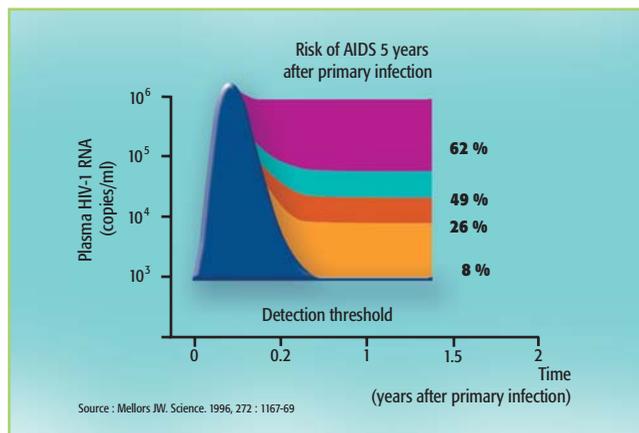
(ref. P. Vanhems *et al.*, Clin Infect Dis 1997, 24 : 965-970)

- Fever ($\geq 38^\circ\text{C}$)
- Asthenia
- Adenopathy
- Skin rash and eruptions
- Myalgia, arthralgia
- Cephalgia
- Pharyngitis

■ These symptoms disappear rapidly and spontaneously as the infected individual enters the clinically asymptomatic carrier phase. After a variable length of time, different symptoms may appear, indicating clinical deterioration: chronic fever, weight loss, diarrhea, oral candidiasis, herpes zoster infection. At the same time, biological signs will reveal immunosuppression, the main sign being CD4 lymphopenia ($< 200/\text{mm}^3$). The development of **opportunistic infections** (pneumocystosis, toxoplasmosis, mycobacteria infections, severe cytomegalovirus infections, etc.) and cellular proliferation (Kaposi's Sarcoma, B-cell lymphoma, cervical cancer, etc.) mark the progression to full-blown AIDS. If no anti-retroviral treatment is given, the average incubation period for AIDS is estimated to be 8 years.

■ **Plasma viremia** levels are generally high ($\geq 10^6$ copies of viral genome/ml) during primary infection. They drop very rapidly and stabilize after two to three months at a variable level, which depends on the intensity of the immune response. The viral load level is a predictive factor of the progression of the disease; the higher the viral load, the faster the disease progresses.

Predictive value of viral load





Epidemiology

How HIV is transmitted

■ Sexual transmission,

■ Through the **blood** when IV drug users share needles or during accidents involving exposure to blood (this last risk is quantified at 0.3% according to A. Tarentola *et coll.* Am. J. Infect. Control 2003; 31: 357-63). Since the implementation of highly effective screening methods for blood donors (serological screening and molecular screening in industrialized countries), the residual risk of contamination due to blood transfusions is estimated to be 0.3/10⁶.

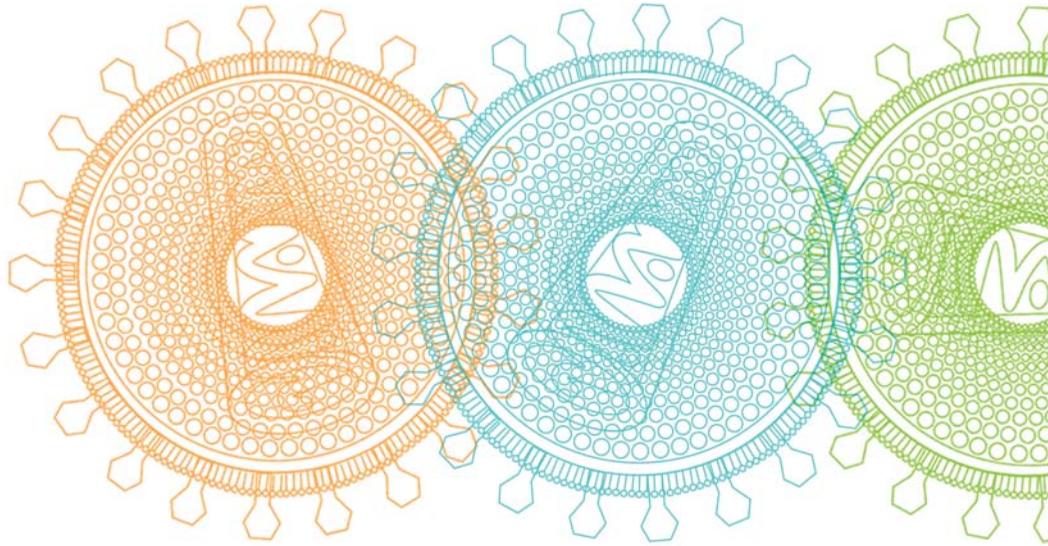
■ **Mother to child transmission (MCT):** HIV can be transmitted from a mother to her infant during three periods: prenatal (rare), peri-natal (the most frequent), and post-natal (through breastfeeding). Due to protocols to prevent MCT using drugs and recommendations to give artificial milk, the frequency of viral transmission from seropositive mothers to their infants has been lowered to reach approximately 1-2% (compared to 15-30% when no preventive measures are taken).

Estimated number of adults and children living with HIV/AIDS.

Source: WHO, December 2006.



Viral Diagnosis



■ The viral diagnosis of an HIV infection is foremost an **immunological diagnosis** based on the identification of **HIV antibodies** using immuno-enzymatic (ELISA) tests or other immunological techniques of equivalent sensitivity. Such detection methods are effective because HIV antibodies are produced continuously and are detectable as early as several weeks after infection (on average 22 days), and because serological testing is practical to perform. Most antibody assays detect both HIV-1 and HIV-2 antibodies. The rapid, single-use tests that require subjective interpretation (visual observation) are slightly less sensitive and specific than ELISA tests. However, as these tests are easy-to-use and do not require sophisticated equipment, they lend themselves well to use in emergency situations and in areas where testing using sophisticated techniques is not feasible.

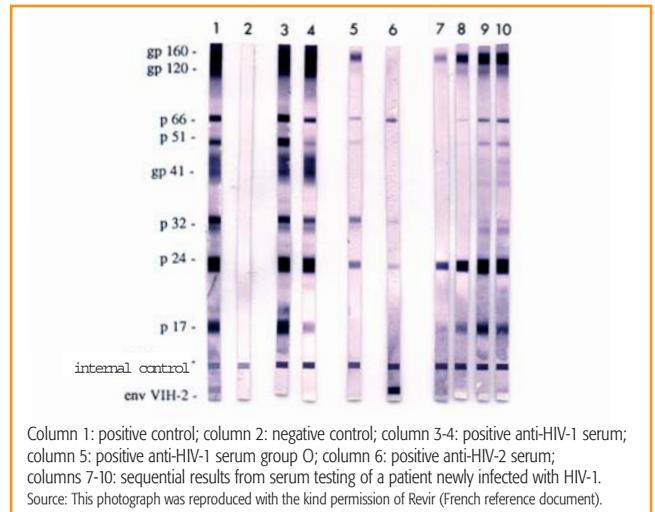
Examples of HIV tests



■ Because of the consequences associated with an HIV positive diagnosis and the possibility of false positives, **it is absolutely necessary for a confirmation test to be performed before a positive diagnosis is made.** See the interpretation **algorithm** of 4th generation ELISA tests (antigen + antibody) and the advanced 4th generation tests that, in addition,

differentiate between the separate antibody and antigen signals with a single test. A confirmation test (Western Blot or immunoblot) is performed if screening results give a positive signal, which makes it possible to visualize with precision the presence of specific HIV protein antibodies. It is strongly recommended to use a confirmation test that distinguishes between HIV-1 and HIV-2, as the viral load progression and therapeutic choices may be different depending on the virus.

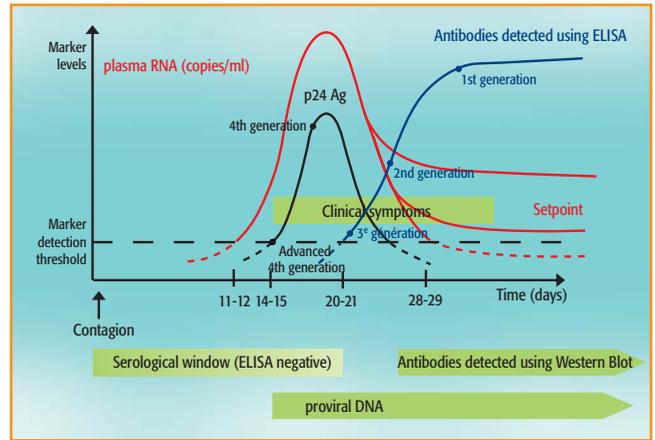
Typical Western Blot results (HIV blot 2.2. Genelabs)



- During **primary infection**, antibodies may not be detectable because they are either absent or present in quantities that are too small to detect. Diagnostic testing should therefore include a detection of both antibodies and **p24 antigenemia** (ELISA test). A positive p24 antigen result should be checked using a neutralization assay.

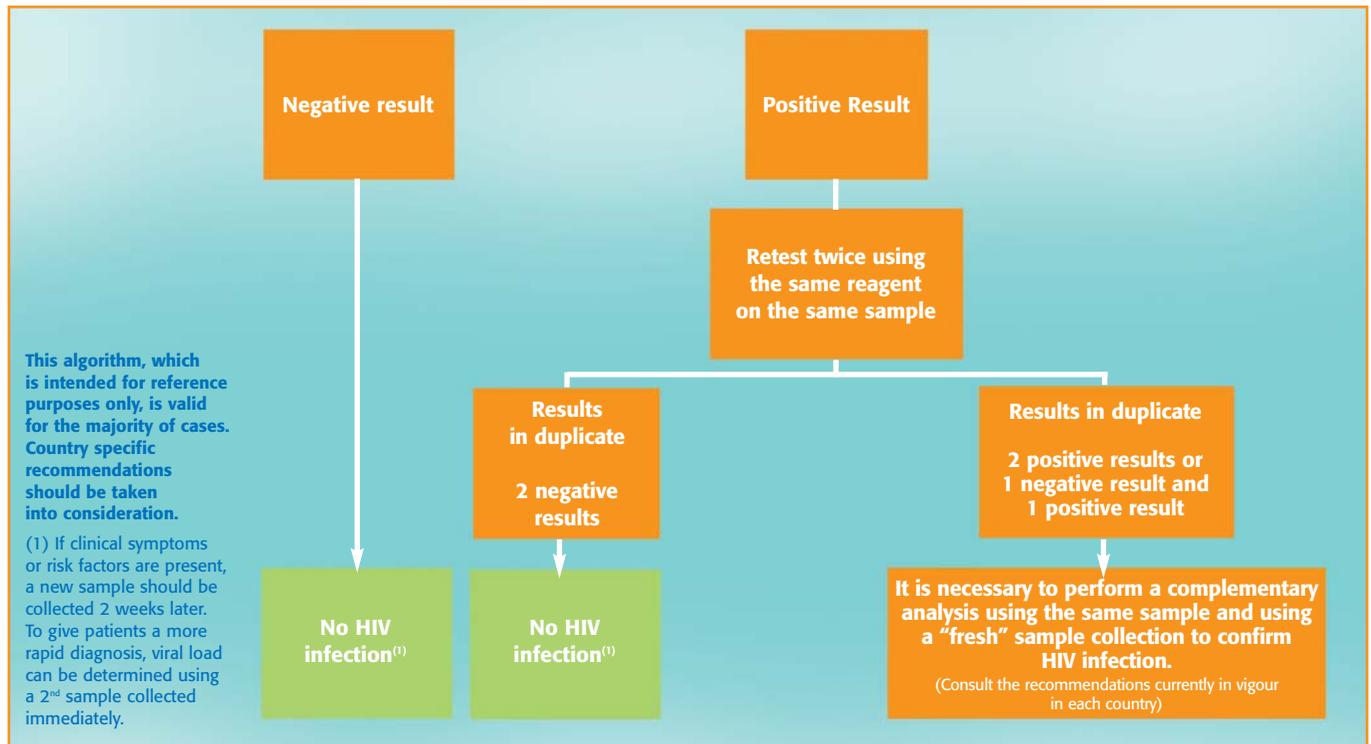
- The use of **combined ELISA tests** called “4th generation” tests, which simultaneously detect **HIV antibodies and the HIV1 p24 antigen**, enables for more effective early detection of infections which are very often asymptomatic. In most cases, 4th generation tests used in the absence of treatment can **reduce the serological window** for the detection of HIV-1 by approximately one week when compared to 3rd generation tests (antibodies alone).

Kinetics of viral markers during the early stages of infection



Interpretation algorithm for 4th generation format serological tests (orientation of positivity origin with differentiated antibody/antigen signals)

(antigen/ antibody combined detection) or “Advanced” 4th generation tests





Immunological Monitoring

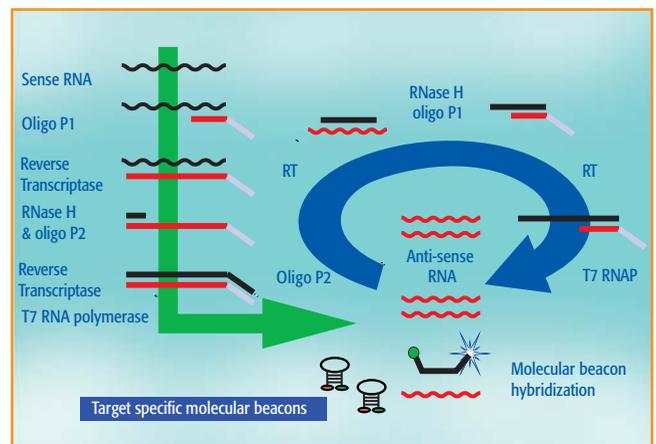
When to begin anti-retroviral treatment

■ Biological monitoring of HIV infection is essentially based on counting the number of **CD4+ lymphocytes** and measuring **viral load** (quantification of plasma viral RNA). These tests are performed every 6 months if the CD4 count is $> 500/\text{mm}^3$ and every 3 to 4 months if the CD4 count is between 200 and $500/\text{mm}^3$. Given that the hepatitis B (HBV) and C (HCV) viruses are important co-morbidity factors, screening for possible co-infection with one of these two viruses should also be carried out.

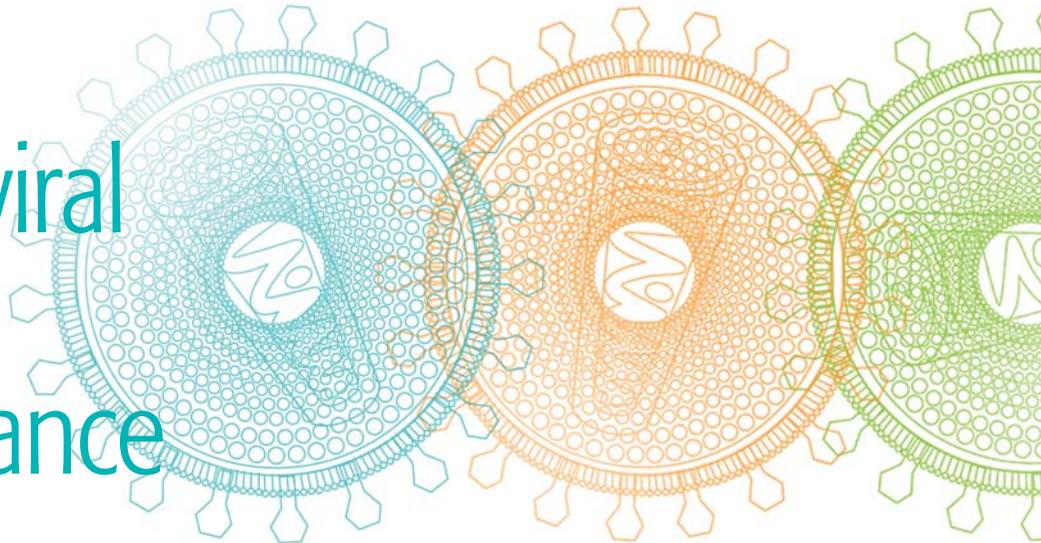
■ Plasma **viral load** is measured using quantitative tests based on molecular tools: gene amplification (PCR-polymerase chain reaction, LCR-ligase chain reaction, TMA-transcription mediated amplification, NASBA-nucleic acid sequence based amplification) or hybridization followed by signal amplification (bDNA-branched DNA). Most tests have sensitivity of the order of 50-100 copies/ml. In order to facilitate comparisons, the results are usually expressed in \log_{10} (examples: 100 copies/ml = $2 \log_{10}$, 10,000 copies/ml = $4 \log_{10}$). Due to biological fluctuations and the reproducibility of these techniques, only a **variation $0.5 \log_{10}$ is significant**. The vast genetic diversity of HIV very often accounts for discordant results when two different quantification techniques are used. Consequently, a patient should be consistently monitored using the same technique, insofar as possible.

■ Treatment should be started imperatively for any patient who has less than 200 CD4 lymphocytes/ mm^3 . In most situations, treatment is started for patients who have fewer than 350 CD4 lymphocytes/ mm^3 since currently available data suggest that there is no apparent benefit to initiating antiretroviral treatment when the number of CD4 lymphocytes is above 350.

Real-time NASBA Detection



Anti-retroviral therapy and resistance



- Currently available **drugs** belong to 4 therapeutic classes: reverse transcriptase nucleoside or nucleotide inhibitors (RTNI), non-nucleoside reverse transcriptase inhibitors (nNRTI), protease inhibitors (PI), fusion inhibitors (FI).

Class	Name	Brand	Laboratory
RTNI	Abacavir (ABC)	Ziagen*	GSK
	Didanosine (ddI)	Videx*	BMS
	Emtricitabine (FTC)	Emtriva*	Gilead
	Lamivudine (3TC)	EpiVir*	GSK
	Stavudine (d4T)	Zerit*	BMS
	Tenofovir (TDF)	Viread*	Gilead
	Zalcitabine (ddC)	Hivid*	Roche
	Zidovudine (ZDV or AZT)	Retrovir*	GSK
	AZT + 3TC	Combivir*	GSK
	AZT + 3TC + ABC	Trizivir*	GSK
nNRTI	Delavirdine (DLV)	Rescriptor*	Agouron
	Efavirenz (EFV)	Sustiva*	BMS
	Névirapine (NVP)	Viramune*	Boehringer
PI	Amprénavir (APV)	Agenerase*	GSK
	Atazanavir (ATV)	Reyataz*	BMS
	Fosamprenavir (fosAPV)	Telzir*	GSK
	Indinavir (IDV)	Crixivan*	MSD
	Nelfinavir (NFV)	Viracept*	Roche
	Ritonavir (RTV)	Norvir*	Abbott
	Saquinavir (SQV)	Fortovase*	Roche
	Tipranavir (TPV)	Tipranavir*	Boehringer
	Lopinavir (LPV) + RTV	Kaletra*	Abbott
	FI	Enfuvirtide (T20)	Fuzeon*

- Monotherapies and bitherapies are not sufficiently efficient and should not be used. In order to optimize the immunovirological response, especially during the initial 6 months of first-line treatment, a powerful therapeutic approach should be implemented by combining three drugs (**triple therapy**). The recommended combinations for first line treatment make use of [2 RTNI + 1 nNRTI or 1 PI]. For pharmacological reasons, most PI are themselves "boosted" by being administered with Ritonavir.

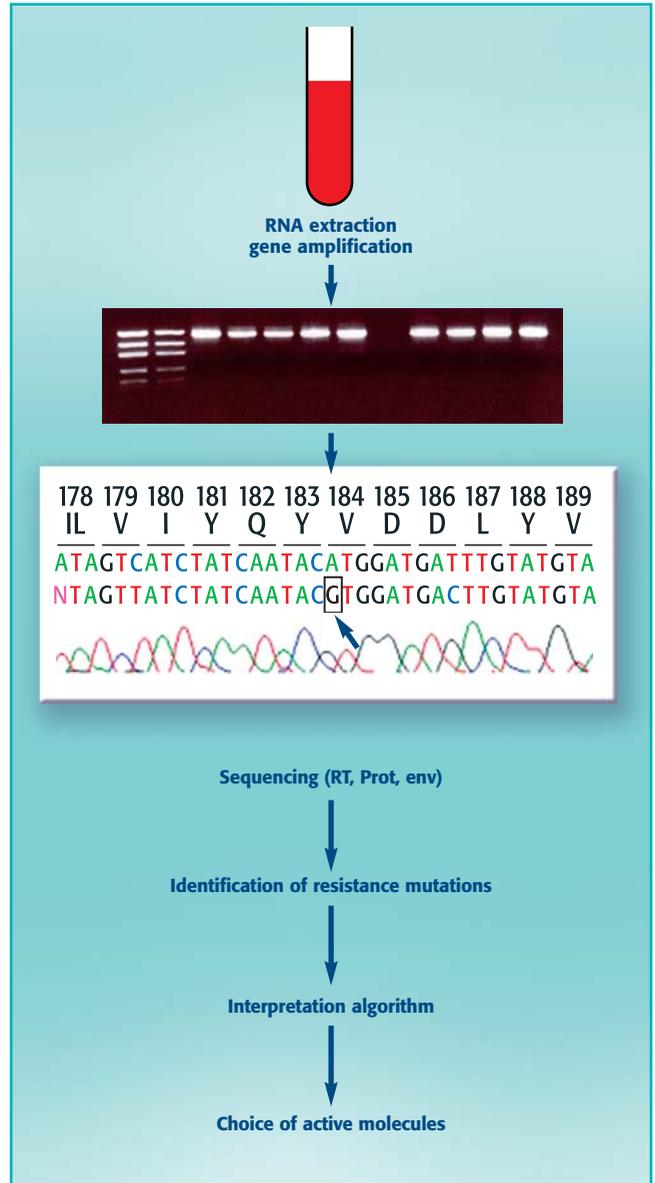
- HIV-1 Group O and HIV-2 are naturally resistant to nNRTIs.

- **One of the treatment objectives is to lower plasma viral load so that it can no longer be detected.** Regular **clinical and biological monitoring** are usually recommended (CD4, viral load, safety and metabolic monitoring) one month after beginning treatment and subsequently every 3 to 4 months. Viral load usually becomes undetectable after 3 to 6 months. Effective antiretroviral treatment also leads to a gradual increase in CD4 lymphocyte levels. In the event that viral load reappears or increases to over 1000 copies/ml, a control using a new sample is advisable to confirm viral escape. **Viral escape** may be due to poor compliance with the treatment regimen, metabolic problems or the selection of resistant mutants.

■ **Resistance testing** plays an important role in supporting therapeutic decision making. Genotype resistance testing is recommended in the event of therapeutic failure and for primary infection. Such tests detect mutations that are known to confer resistance to this or that drug, in the reverse transcriptase (RTNI and nNRTI), protease (PI), or envelope (E) genes. The reference technique currently in use is nucleotide sequencing after gene amplification (resistance genotyping). In the future, this indication may represent an important application for DNA chip technology. Interpretation is based on algorithms updated regularly by groups of experts and posted on internet sites. The result then serves to guide therapeutic decisions.

Resistance phenotyping tests (which analyse viral strains' sensitivity *in vitro* when put in the presence of each drug) are time-consuming and costly, and their superiority has not been proven when it comes to predicting a patient's response to treatment.

Principle of resistance genotyping by sequencing



Questions & Answers



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What are the differences between 3rd and 4th generation reagents?

F. Barin : A 3rd generation assay is an ELISA assay, which has excellent sensitivity and detects only the antibodies directed against HIV. A 4th generation reagent does two things simultaneously: it detects HIV antibodies as well as the HIV p24 antigen itself, thus enabling earlier detection of primary infection, in particular asymptomatic primary infection which, by definition, will not be suspected clinically. However, these 4th generation reagents cannot replace the p24 antigen detection reagents, which are more sensitive and, consequently, are the only reagents indicated for the screening of primary infection. In certain situations, a negative result with a 3rd generation test and a positive result with a 4th generation test can be obtained. If this happens, a primary infection should be suspected and a test to detect p24 antigen performed, in addition to the mandatory Western Blot.

Can measuring HIV viral load help diagnose primary infection?

F. Barin : The reagents used to determine HIV viral load are registered only for monitoring HIV seropositive patients and not for diagnosing primary infection, due to their low specificity. In this case, it is necessary to use the reagents that screen for p24 antigenemia. It is estimated that, with these reagents, a reaction will be positive for a viral load of around 10,000 copies (4 log) per ml. It should be noted that during a primary infection, HIV viral load is often very high. It is also estimated that the p24 antigen makes it possible to speed up detection, which can be obtained one week earlier when compared to screening for antibodies alone.

Is it absolutely necessary to perform a differential diagnosis for HIV-2?

F. Lucht and F. Barin : Yes, of course, because the physiopathology of the infection is not the same, and, more importantly, biological monitoring and treatment will be specifically adapted. Clinical progression of the disease is slower and mother-to-child transmission is less likely with HIV-2 than with HIV-1 (maternal-fetal transmission < 2% in the absence of treatment). From a biological viewpoint, monitoring is more difficult because there are no tools on the market to measure viral load with HIV-2. From a therapeutic viewpoint, non-nucleoside reverse transcriptase inhibitors are not effective against HIV-2.

How is HIV viral load interpreted from a clinical perspective?

F. Lucht : Among untreated patients, clinical interpretation has changed fundamentally. In the past, the CD4 and HIV viral load biological parameters were used in an equivalent way as technological tools, and treatment would be started for an untreated patient with a high HIV viral load ($> 30,000$ cp/ml). Today, a CD4 level of < 350 /ml is the first biological parameter taken into account, with viral load as a backup. Viral load is no longer the only criterion for deciding whether to start treatment, except when it is higher than 100,000 copies/ml or if there are significant clinical signs of a poor prognosis and disease progression.

In terms of monitoring patients, what are the main limitations of viral load techniques?

F. Lucht and F. Barin : Limitations are connected to variations in the physiological viral load detected, for example, during immune stimulation, with intercurrent infections such as the flu, and with vaccination. In these situations, one often sees temporary and at times significant increases in HIV plasma viral load. Second, there are also technical variations: variations of the order of a factor of 3 may be observed for the same sample tested using the same reagent, without this being significant. With the same reagent, a variation in viral load of more than 0.5 log is considered significant. When different reagents are used, it is possible to observe variations of more than 1 log for the same sample, which justifies monitoring the viral load with the same reagent insofar as possible. If there is a noteworthy increase in HIV viral load between two samples, it is not enough to look at a single value, and it is essential to confirm this value by taking an additional sample a few weeks later, before beginning or adjusting treatment.

What are the indications for resistance genotyping?

F. Lucht and F. Barin : Resistance genotyping, i.e., looking for mutations identified as being associated with resistance to the active molecules in certain drugs, is indicated in two cases:

- for primary infection, to put in the patient's medical file for treatment at a later date;
- in the case of therapeutic escape, to adjust the treatment.

■ Selection of original articles

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<http://www.medscape.com>

<http://www.hivandhepatitis.com>

<http://hivdb.stanford.edu>

<http://www.iasusa.org>

<http://www.hivfrenchresistance.org>

■ bioMérieux products

3rd generation screening tests

Vironostika® HIV Uni-form II plus O

VIKIA® HIV

4th generation screening tests

Vironostika® HIV Uni-form II Ag/Ab

VIDAS® HIV DUO QUICK

VIDIA™ HIV DUO*

“Advanced” 4th generation

screening tests

VIDAS® HIV DUO ULTRA

* under development

Determination of p24 antigen

VIDAS® HIV P24

VIDAS® HIV P24 CONFIRMATION

Vironostika® HIV 1Ag

Vironostika® HIV 1Ag Confirmation

Determination of HIV viral load

NucliSens® EasyQ HIV-1

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