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Design and production of a chemiluminescent
immunoassay for the early detection of HIV
infection

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Chapter 1

A chemiluminescent immunoassay for the detection of HIV infection

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

The first cases of acquired immunodeficiency syndrome (AIDS) were described in homosexual men in the US in 1981(1). Several years later, in 1983 and 1984, respectively, French and American scientists confirmed that the causative agent for AIDS was a retrovirus, the human immunodeficiency virus (HIV) (2-4).

HIV belongs to a class of viruses called retrovirus, and a subgroup of retroviruses known as lentiviruses or “slow viruses”. The course of infection with these viruses is characterized by a long interval between initial infection and the onset of serious symptoms (5).

Two genetically distinct viral types of HIV have been identified (13). HIV-1 is the most common and with the highest virulence: phylogenetic evidences suggest that it originated by zoonotic cross-species transmission of the simian lentivirus SIV_{cpz}(Simian Immunodeficiency Virus) from the chimpanzee subspecies *Pan troglodytes troglodytes* to humans; HIV-2 has been found mainly in infected individuals in western Africa and it is very similar to HIV-1 in that it has the same tropism for cells of the immune system and causes illness that results from immune deficiency.

HIV-1 is characterized by extensive genetic heterogeneity driven by several factors, such as the lack of proofreading ability of the reverse transcriptase (6, 7), the rapid turnover of HIV-1 in vivo (10), host selective immune pressures (11), and recombination events during replication (12).

Due to this variability, HIV-1 variants are classified into three major phylogenetic groups: group M (Main), group O (Outlier), and group N (non-M/non-O).

Group M, which is responsible for the majority of infections in the worldwide HIV-1 epidemic, can be further subdivided into 10 recognized phylogenetic subtypes, or clades (A to K), which are approximatively equidistant from one another (figure 1). Moreover, within a subtype, it is possible to identify groups of viral isolates forming genetically related sister clades, termed sub-subtypes, which appear to be phylogenetically more closely related to each other than to other subtypes. Furthermore, virus isolates have been identified, in which phylogenetic relations with different subtypes switch along their genomes. When an identical recombinant virus is identified in at least three epidemiologically unlinked people and is

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characterized by full-length genome sequencing, it can be designated as a circulating recombinant form (CRF) (14-16) (figure 1).

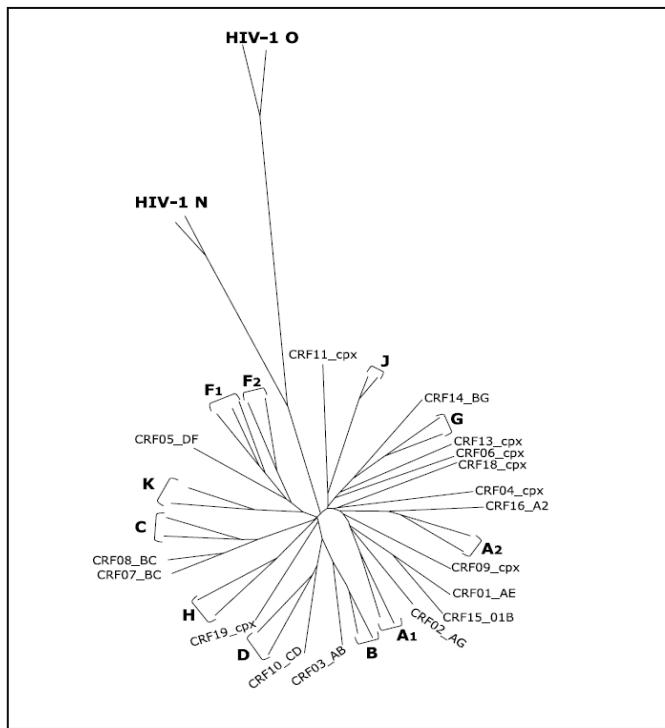


Figure 1 : Phylogenetic tree of HIV-1 (14).

Molecular epidemiological studies show that, with the exception of sub-Saharan Africa, where almost all subtypes, CRFs and several unique recombinant forms have been detected, there is a specific geographic distribution pattern for HIV-1 subtypes (17-18).

According to recent studies, on a global scale, the most prevalent HIV-1 genetic forms are subtypes A, B, and C, with subtype C accounting for almost 50% of all HIV-1 infections worldwide (figure 2). Subtype A viruses are predominant in areas of central and eastern Africa (Kenya, Uganda, Tanzania, and Rwanda), and in east European countries formerly constituting the Soviet Union. Subtype B is the main genetic form in western and central Europe, the Americas, and Australia, and is also common in

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several countries of Southeast Asia, northern Africa and Middle East. Subtype C viruses are predominant in those countries with >80% of all global HIV-1 infections, such as southern Africa and India (14, 19). The relevance of CRFs in the global HIV-1 pandemic is increasingly recognized, accounting for 18% of infections and representing the predominant local form in Southeast Asia and in West and West-Central Africa (14) (figure 2).

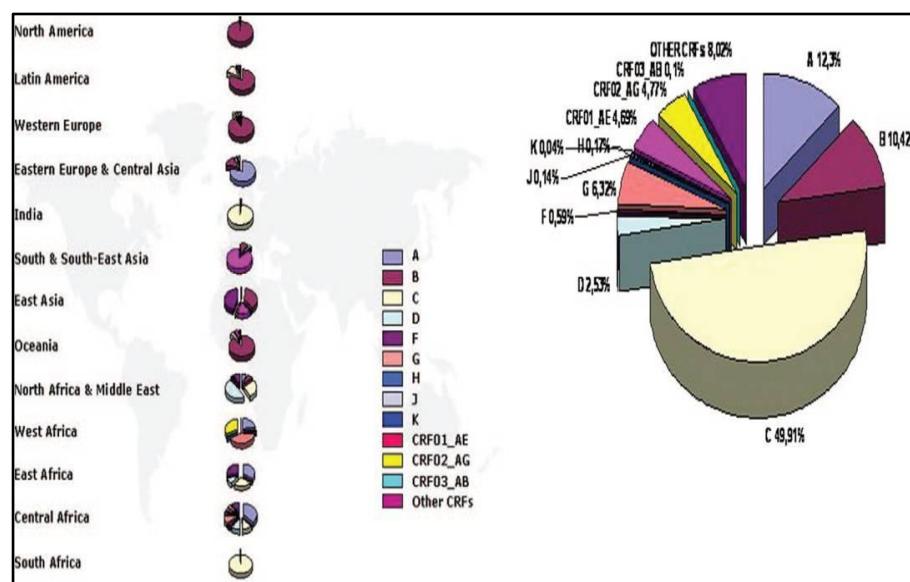


Figure 2: geographic distribution of subtypes and CRFs of HIV-1M (14)

The course of infection with these viruses is characterized by a long interval between initial infection and the onset of serious symptoms (5). Like all viruses, HIV can replicate only inside cells, commandeering the cell's machinery to reproduce.

HIV is different in structure from other retroviruses. It is roughly spherical with a diameter of about 120 nm, around 60 times smaller than a red blood cell, yet large for a virus. It is composed of two copies of positive single-stranded RNA that code for the virus's nine genes enclosed by a conical capsid composed of 2000 copies of the viral protein p24. The single-stranded RNA is tightly bound to nucleocapsid proteins, p7 and enzymes needed for the development of the virion such as reverse transcriptase, proteases, ribonuclease and integrase. A matrix composed of the viral protein p17

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surrounds the capsid ensuring the integrity of the virion particle. This is, in turn, surrounded by viral envelope which is composed of two layers of fatty molecules called phospholipids, taken from the membrane of a human cell when a newly formed virus particle buds from the cell. Embedded in the viral envelope are proteins from the host cell and about 70 copies of a complex HIV protein that protrudes through the surface of the virus particle. This protein, known as Env, consists of a cap made of three molecules called glycoprotein (gp) 120, and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope (61). This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the infectious cycle. Both these surface proteins, especially gp120, have been considered as targets of future treatments or vaccines against HIV. The RNA genome consists of nine genes (gag, pol, and env, tat, rev, nef, vif, vpr, vpu, and tev) encoding 19 proteins. Three of these genes, gag, pol, and env, contain information needed to make the structural proteins for new virus particles. Env codes for a protein called gp160 that is broken down by a viral enzyme to form gp120 and gp41. The six remaining genes, are regulatory genes for proteins that control the ability of HIV to infect cells, produce new copies of virus (replicate), or cause disease (8).

HIV enters macrophages and CD4⁺ T cells by the adsorption of glycoproteins on its surface to receptors on the target cell, followed by the fusion of the viral envelope with the cell membrane and the release of the HIV capsid into the cell (9).

Entry to the cell begins through interaction of the trimeric envelope complex (gp160 spike) and both CD4 and a chemokine receptor (generally either CCR5 or CXCR4, but others are known to interact) on the cell surface (9).

The first step in fusion involves the high-affinity attachment of the CD4 binding domains of gp120 to CD4. Once gp120 is bound with the CD4 protein, the envelope complex undergoes a structural change, exposing the chemokine binding domains of gp120 and allowing them to interact with the target chemokine receptor (20, 21). This allows for a more stable two-pronged attachment, which allows the N-terminal fusion peptide gp41 to penetrate the cell membrane. Repeated sequences in gp41, HR1 and HR2, then interact, causing the collapse of the extracellular portion of gp41 into a hairpin. This loop structure brings the virus and cell membranes close together, allowing fusion of the membranes and subsequent entry of the viral capsid. After HIV has bound to the target cell, the HIV RNA and various enzymes, including reverse transcriptase, integrase, ribonuclease, and

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protease, are injected into the cell. During the microtubule based transport to the nucleus, the viral single strand RNA genome is transcribed into double strand DNA, which is then integrated into a host chromosome.

The process of reverse transcription is extremely error-prone, and the resulting mutations may cause drug resistance or allow the virus to evade the body's immune system. The reverse transcriptase also has ribonuclease activity that degrades the viral RNA during the synthesis of cDNA, as well as DNA-dependent DNA polymerase activity that copies the sense cDNA strand into an antisense DNA. Together, the cDNA and its complement form a double-stranded viral DNA that is then transported into the cell nucleus. The integration of the viral DNA into the host cell's genome is carried out by another viral enzyme called integrase. This integrated viral DNA may then lie dormant, in the latent stage of HIV infection. To actively produce the virus, certain cellular transcription factors need to be present, the most important of which is NF- κ B, which is upregulated when T-cells become activated. This means that those cells most likely to be killed by HIV are those currently fighting infection.

During viral replication, the integrated DNA provirus is transcribed into mRNA, which is then spliced into smaller pieces. These small pieces are exported from the nucleus into the cytoplasm, where they are translated into the regulatory proteins Tat (which encourages new virus production) and Rev. As the newly produced Rev protein accumulates in the nucleus, it binds to viral mRNAs and allows unspliced RNAs to leave the nucleus, where they are otherwise retained until spliced. At this stage, the structural proteins Gag and Env are produced from the full-length mRNA. The full-length RNA is actually the virus genome; it binds to the Gag protein and is packaged into new virus particles.

HIV-1 and HIV-2 appear to package their RNA differently: HIV-1 will bind to any appropriate RNA whereas HIV-2 will preferentially bind to the mRNA which was used to create the Gag protein itself. This may mean that HIV-1 is better able to mutate (HIV-1 infection progresses to AIDS faster than HIV-2 infection and is responsible for the majority of global infections). The final step of the viral cycle, assembly of new HIV-1 virions, begins at the plasma membrane of the host cell. The Env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by protease and processed into the two HIV envelope glycoproteins gp41 and gp120. These proteins are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to

the membrane of the infected cell. The Gag (p55) and Gag-Pol (p160) polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. Maturation either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion. This cleavage step can be inhibited by protease inhibitors. The mature virus is then able to infect another cell (figure 3).

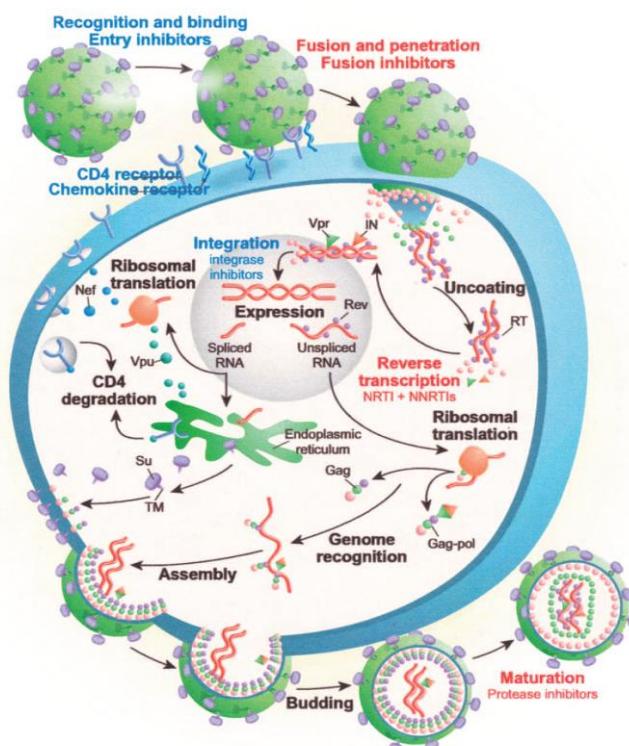


Figure 3: Life cycle of HIV (22).

DISEASE PROGRESSION

The typical progression of HIV-1 infection was first described in 1987, when viruses were isolated from patients with AIDS and related symptoms (23). Following infection with HIV-1, the rate of clinical disease progression varies between individuals. Factors such as host susceptibility, genetics and immune function, health care and co-infections, as well as viral genetic variability, may affect the rate of progression to AIDS. The clinical evolution results from a complex interaction between viral effects on host immunocompetent cells functions and host immunological response against viral determinants (27, 28).

HIV primarily infects vital cells in the human immune system such as helper T cells (specifically CD4⁺ T cells), macrophages, and dendritic cells. HIV infection leads to low levels of CD4⁺ T cells through three main mechanisms: first, direct viral killing of infected cells; second, increased rates of apoptosis in infected cells; and third, killing of infected CD4⁺ T cells by CD8 cytotoxic lymphocytes that recognize infected cells. When CD4⁺ T cell number declines below a critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections (24-26, 29).

Infection with HIV-1 is associated with a progressive decrease of the CD4⁺ T cell count and an increase in viral load. The stage of infection can be determined by measuring the patient's CD4⁺ T cell count, and the level of HIV in the blood (figure 4).

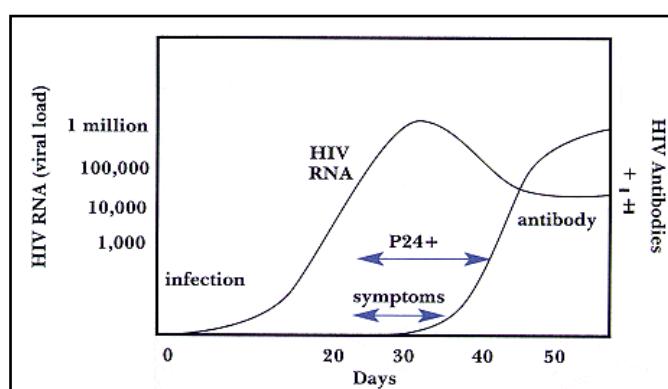


Figure 4: Blood markers during primary infection by HIV-1

HIV infection has basically four stages (24-29):

- ✓ incubation period: is asymptomatic and usually lasts between two and four weeks.
- ✓ Acute infection: generally occurs after transfer of body fluids from an infected person to an uninfected one. The first stage of infection, is a period of rapid viral replication that immediately follows the individual's exposure to HIV leading to an abundance of virus in the peripheral blood with levels of HIV commonly approaching several million viruses per ml. This response is accompanied by a marked drop in the numbers of circulating CD4⁺ T cells. This acute viraemia is associated in virtually all patients with the activation of CD8⁺ T cells, which kill HIV-infected cells, and subsequently with antibody production, or seroconversion. The CD8⁺ T cell response is thought to be important in controlling virus levels, which peak and then decline, as the CD4⁺ T cell counts rebound to around 800 cells per μ l (the normal blood value is 1200 cells per μ l). A good CD8⁺ T cell response has been linked to slower disease progression and a better prognosis, though it does not eliminate the virus (27). During this period (usually 2–4 weeks post-exposure) most individuals (80 to 90%) develop an influenza- or mononucleosis-like illness called acute HIV infection, the most common symptoms of which may include fever, lymphadenopathy, pharyngitis, rash, myalgia, malaise, mouth and esophageal sores, and may also include, but less commonly, headache, nausea and vomiting, enlarged liver/spleen, weight loss, thrush, and neurological symptoms. Infected individuals may experience all, some, or none of these symptoms. The duration of symptoms varies, averaging 28 days and usually lasting at least a week. Because of the nonspecific nature of these symptoms, they are often not recognized as signs of HIV infection.
- ✓ Latency stage: a strong immune defense reduces the number of viral particles in the blood stream, marking the start of the infection's clinical latency stage. Clinical latency can vary between two weeks and 20 years. During this early phase of infection, HIV is active within lymphoid organs, where large amounts of virus become trapped in the follicular dendritic cells (FDC) network. The surrounding tissues that are rich in CD4⁺ T cells may also become infected, and viral particles accumulate both in infected cells and as free virus.
- ✓ AIDS: when CD4⁺ T cell numbers decline below a critical level of 200 cells per μ l, cell-mediated immunity is lost, and infections with a variety

of opportunistic microbes appear. The first symptoms often include moderate and unexplained weight loss, recurring respiratory tract infections (such as sinusitis, bronchitis, otitis media, pharyngitis), prostatitis, skin rashes, and oral ulcerations. Common opportunistic infections and tumors, most of which are normally controlled by robust CD4⁺ T cell-mediated immunity then start to affect the patient..

A peculiar characteristic of HIV-1 infection is the great variability in disease progression between infected people. Three main groups of infected individuals have been described, depending on infection duration and kinetics of virological and immunological events (11):

- ✓ Typical progressors: about 80% of all infected individuals. The mean survival time is about ten years without antiretroviral therapy. HIV-1 infection shows the four typical stages.
- ✓ Rapid progressors: a small percentage of HIV-infected individuals rapidly progress to AIDS within four years after primary HIV-infection and are termed Rapid Progressors (RP). Indeed some individuals have been known to progress to AIDS and death within a year after primo-infection. Rapid progression was originally thought to be continent specific, as some studies reported that disease progression is more rapid in Africa (62-64), but others have contested this view (65, 66).
- ✓ Long term non-progressors: a subset of individuals (1-2% of all infected individuals) who are persistently infected with HIV-1, but show no signs of disease progression for over 12 years and remain asymptomatic are classified as Long Term Non-Progressors (LTNP). In these individuals, it seems that HIV-infection has been halted with regard to disease progression over an extended period of time (67-70). However, the term LTNP is a misnomer, as it must be noted that progression towards AIDS can occur even after 15 years of stable infection. LTNP are not a homogeneous group regarding both viral load and specific immune responses against HIV-1. Some LTNPs are infected with HIV that inefficiently replicates whilst others are infected with HIV that is virally fit and replicates normally, but the infected individual has had a strong and broad set of HIV-specific humoral and cell-mediated responses that seems to delay the progression to AIDS.

ANTIRETROVIRAL THERAPY (ART)

Without doubt, the most significant headway in the battle against HIV/AIDS has been the development of effective antiretroviral drugs that provide important treatment options for patients infected by HIV. These drugs now exist in a variety of categories based on the enzymatic and/or cellular targets (Table 1). The first drugs were targeted against viral reverse transcriptase but, in short order, we have seen the development of anti-HIV drugs that are targeted against protease, and most recently, integrase inhibitors. The development of combination therapies (HAART, *highly active antiretroviral therapy*) has been key in assuring prolongation of life, to an extent that HIV disease in almost all developed countries today can be considered a chronic manageable condition, unlike the death sentence that existed during the initial years of the epidemic through to the mid 1990s, when triple therapies first became available (30-32). Of course, it is also fair to state that treatments have improved over the years, not only because of the effectiveness of the drugs themselves but also because newer compounds developed to treat HIV disease are far less toxic than those initially used during the 1980s and 1990s. In addition, simplicity of dosing has become commonplace, such that patients can, in some cases, take only one pill per day, based on a co-formulation that involves two nucleoside/nucleotide reverse transcriptase inhibitors coupled with a non-nucleoside reverse transcriptase inhibitor. The ability to simplify dosing, coupled with current treatment protocols, make current therapies far less toxic than their predecessors, and this has also had important implications in another area, that is, today's treatment regimens are far less prone to the development of drug resistance than were the initially available combination therapies.

Antiretroviral drugs approved by the US FDA	
NRTI	Zidovudine, Didanosine, Zalcitabine, Stavudine, Lamivudine, Abacavir, Tenofovir, Emtricitabine
NNRTI	Nevirapine, Delavirdine, Efavirenz, Etravirine, Atazanavir, Fosamprenavir, Tipranavir
Protease inhibitors	Saquinavir, Ritonavir, Indinavir, Nelfinavir
Entry inhibitors	Marivoc
Integrase inhibitors	Raltegravir
fusion inhibitors	Enfuvirtide

Table 1: antiretroviral drugs approved by FDA.

REVERSE TRANSCRIPTASE INHIBITORS

NRTI

Nucleoside drugs act as competitive inhibitors of viral reverse transcriptase, blocking viral RNA replication (38). Selectivity of action derives from the much higher affinity for RT than human polymerase. These compounds have an hydrogen in position 3' of ribose: in this way they can compete with cellular desoxynucleotides for incorporation in the newly forming DNA molecule, and act as chain terminators (figure 5).

When used alone, *in vivo*, are not sufficient in restoring immune homeostasis, and lead to the generation of drug-resistant viral variants. Nevertheless, in an HAART regimen, combined with NNRTI give satisfactory results.

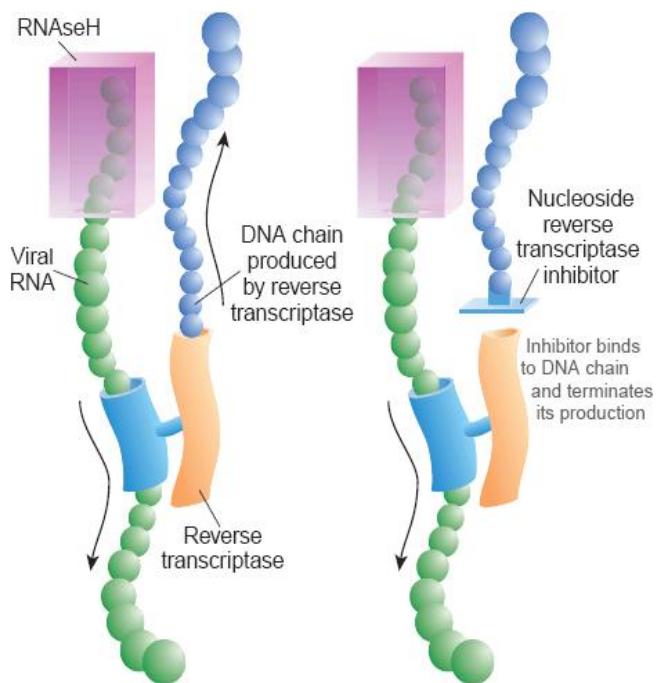


Figure 5: mechanisms of action of NRTI

NNRTI

These compounds are derived from benzodiazepines, and inhibit directly viral reverse transcriptase (35, 37): they interact near the catalytic site of RT (figure 6), and have high antiretroviral activity *in vitro*.

In vivo, they should be combined with protease inhibitors in order to give satisfactory results.

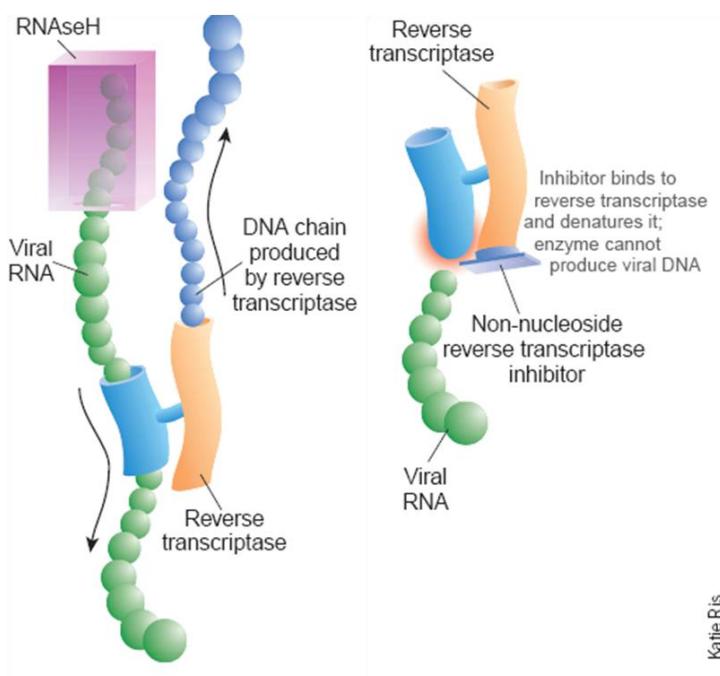


Figure 6: mechanisms of action of NNRTI

PROTEASE INHIBITORS (PI)

HIV-1 protease is an aspartyl-protease that catalyzes proteolytic cleavage of polypeptidic precursors, translated from *gag* and *pol* genes, that will originate core structural proteins and viral-associated enzymes. Thus, protease inhibitors are able to reduce viral replication, also in chronically infected cells, such as macrophages (36). Irreversible inhibition of protease with covalent binding to the active site, leads to the formation of immature and non-infective viral particles, blocking the replicative cycle of HIV-1.

HAART AND SIDE EFFECTS

The main purpose of antiretroviral therapy is the suppression of HIV replication: if the virus does not replicate, the immune system (residual or regenerated) can indefinitely protect host from opportunistic infections induced by HIV (32).

Currently, the best results, such as viraemia reduction (<50copies/ml) and CD4 lymphocytes recovery, are achieved with a combined regimen.

Highly Active AntiRetroviral Therapy (HAART) can reduce morbidity and mortality of HIV-1 infection. Generally this type of therapy combines two NRTI and one NNRTI or one PI.

The decision of when to begin ART should be made on an individual basis. For those individuals whose CD4⁺ count and clinical health assessment do not indicate a high risk of disease progression, initiating treatment may cause more harm than good because of the clinically significant rate of toxicities and side effects.

Side effects include immune reconstitution inflammatory syndrome, which results from the rebound in immune responses to a variety of occult infections and includes high fever, abdominal pain, and inflammatory masses. A fulminate flair of hepatitis B virus infection may occur as well as severe hypersensitivity drug reactions, cardiovascular complications, and exacerbation of underlying liver disease. An example of a common side effect is the development of lipodystrophy syndrome, characterized by fat redistribution dominated by peripheral fat loss and complex metabolic alterations including dyslipidemia and insulin resistance. According to current recommendations from the Panel on Antiretroviral Guidelines for Adults and Adolescents from the Department of Health and Human Services, patients who have not previously been treated (naïve patients) may be treated if their CD4⁺ count falls <350 cells/mm³. Patients should be treated if they present with an AIDS-defining illness, or if the CD4⁺ count is <200/mm³ because they are at a greater risk of developing complications due to HIV infection. Treatment should be started with pregnant women, people with HIV-associated nephropathy, and with individuals coinfected with hepatitis B regardless of their CD4⁺ count. Psychosocial factors, which may create a barrier to patient adherence, should be addressed before the initiation of treatment. Adoption of treatment strategies recommended by the current National Institutes of Health guidelines has resulted in substantial reductions

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in HIV-related morbidity and mortality (71). The principles of therapy of HIV infection are based on our understanding of the immunologic damage caused by ongoing viral mutation from early in the infection process through late stages of the disease. Because the virus is highly mutable, every effort should be made to shut down viral replication completely. The goals of treatment are to suppress plasma viraemia for as long as possible, to delay the selection of drug resistance mutations, and to preserve immune function. Studies show that HAART protects from death, and the data suggest that this occurs by increasing immune subset counts and decreasing viral load. Although HAART “maintains” immunocompetence, this may occur in conjunction with viral suppression itself having a direct beneficial effect on adaptive and innate parameters. The degree of improvement of the total CD4 count is, in part, a function of the initial degree of immune destruction. High baseline CD4⁺ T-cell counts predicted a better virological outcome of HAART (71).

DIAGNOSIS

Early HIV screening and detection are imperative for the long-term survival of infected patients and prevention of further transmission of the virus.

Laboratory tests to identify HIV infections detect virus presence in blood, saliva and urine. Depending on the test, it is possible to detect anti-HIV antibodies, viral antigens or RNA (39-41).

Antibody tests

HIV antibody tests are specifically designed for routine diagnostic testing of adults; these tests are inexpensive and extremely accurate.

Antibody tests may give false negative (no antibodies were detected despite HIV being present) results during the window period, an interval of three weeks to six months between the time of HIV infection and the production of measurable antibodies to HIV seroconversion. Most people develop detectable antibodies approximately 30 days after infection, although some seroconvert later. The vast majority of people (99%) have detectable antibodies by three months after HIV infection; a six-month window is extremely rare with modern antibody testing. During the window period, an infected person can transmit HIV to others although their HIV infection may not be detectable with an antibody test. Antiretroviral therapy during the window period can delay the formation of antibodies and extend the window period beyond 12 months.

The enzyme-linked immunosorbent assay (ELISA), was the first screening test commonly employed for HIV. It has a high sensitivity.

In an ELISA test, a person's serum is diluted and applied to a plate to which HIV antigens have been attached. If antibodies to HIV are present in the serum, they may bind to these HIV antigens. The plate is then washed to remove all other components of the serum. A specially prepared secondary antibody is then applied to the plate, followed by another wash. This secondary antibody is chemically linked in advance to an enzyme. Thus the plate will contain enzyme in proportion to the amount of secondary antibody bound to the plate. A substrate for the enzyme is applied, and catalysis by the enzyme leads to a change in color or fluorescence. ELISA results are reported as a number. The most controversial aspect of this test is determining the "cut-off" point between a positive and negative result.

In case of positive result, a confirmatory test is needed: usually Western blot is used. In the Western blot procedure, HIV-infected cells are opened and the proteins within are placed into a slab of gel, to which an electrical current is applied. Different proteins will move with different velocities in this field, depending on their size, while their electrical charge is leveled by a surfactant called sodium lauryl sulfate. Once the proteins are well-separated, they are transferred to a membrane and the procedure continues similar to an ELISA: the person's diluted serum is applied to the membrane and antibodies in the serum may attach to some of the HIV proteins. Antibodies which do not attach are washed away, and enzyme-linked antibodies with the capability to attach to the person's antibodies determine to which HIV proteins the person has antibodies.

Rapid Antibody Tests have been also described: they are qualitative immunoassays intended for use as a point-of-care test to aid in the diagnosis of HIV infection. These tests should be used in conjunction with the clinical status, history, and risk factors of the person being tested. The specificity of Rapid Antibody Tests in low-risk populations has not been evaluated. These tests should be used in appropriate multi-test algorithms designed for statistical validation of rapid HIV test results. A rapid antibody test gives the result in 20 minutes.

Antigen tests

The antigen tests detect the presence of the p24 protein of HIV (also known as CA), the capsid protein of the virus. Monoclonal antibodies specific to the p24 protein are mixed with the person's blood. Any p24 protein in the person's serum will stick to the monoclonal antibody and enzyme-linked antibody to the monoclonal antibodies to p24 causes a color change if p24 was present in the sample. The p24 antigen test is not useful for general diagnostics, as it has very low sensitivity and only works during a certain time period after infection before the body produces antibodies to the p24 protein. The advantage of this test is the reduction of window period.

NAT (nucleic acid tests)

Nucleic-acid-based tests amplify and detect a 142-base target sequence located in a highly conserved region of the HIV *gag* gene. Since 2001, donated blood in the United States has been screened with nucleic-acid-based tests, shortening the window period between infection and detectability of disease to about 12 days. Since these tests are relatively expensive, the blood is screened by first pooling some 8-24 samples and testing these together; if the pool tests positive, each sample is retested individually. A different version of this test is intended for use in conjunction with clinical presentation and other laboratory markers of disease progress for the management of HIV-1-infected patients.

In the RT-PCR test, viral RNA is extracted from the patient's plasma and is treated with reverse transcriptase (RT) to convert the viral RNA into cDNA. The polymerase chain reaction (PCR) process is then applied, using two primers unique to the virus's genome. After PCR amplification is complete, the resulting DNA products are hybridized to specific oligonucleotides bound to the vessel wall, and are then made visible with a probe bound to an enzyme. The amount of virus in the sample can be quantified with sufficient accuracy to detect ten-fold changes.

Other tests

The CD4 T-cell count is not properly an HIV test, but rather a procedure where the number of CD4 T-cells in the blood is determined.

It is used to monitor immune system function in HIV-positive people. Declining CD4 T-cell counts are considered to be a marker of progression of HIV infection. In HIV-positive people, AIDS is officially diagnosed when the count drops below 200 cells/ μ l or when certain opportunistic infections occur. Low CD4 T-cell counts are associated with a variety of conditions, including many viral infections, bacterial infections, parasitic infections, sepsis, tuberculosis, coccidioidomycosis, burns, trauma, intravenous injections of foreign proteins, malnutrition, over-exercising, pregnancy, normal daily variation, psychological stress, and social isolation.

Duo/combined tests are also available which combine antigen and antibody testing, thereby making earlier detection possible.

LIAISON® SYSTEM

The Liaison® system is an instrument designed to perform immunometric analyses of biological fluid samples (such as serum or plasma) in a completely automated way. Up to 15 different tests can be performed at once on up to 144 samples in a sequential or random access mode. The output of the analysis is generated through the formation of an immune complex, followed by a chemiluminescent reaction that produces an emission of light. The instrument is composed of two modules, designed to be both allocated on a single workbench (figure 7); the first module is a personal computer with touch screen, that hosts the user interface software and all the system data (assay protocols, reagent cartridges database, output of analyses, calibration history, network controls etc.). The second module is the actual analyzer, that performs the analysis from sample loading all the way to the final output for the user. Key components of the analyzer include:

- ✓ Cuvette loader and stacker: two conveyor belts allow continuous loading of the reaction modules, that are stored on a multilevel rack (7 levels).
- ✓ Sample rack slots: in the left-hand part of the instrument, a storage area can hold up to 12 sample racks, each carrying up to 12 samples. A barcode reader allows error-free catalogation of samples.
- ✓ Reagent slots: in the right-hand part of the instrument, another storage area can hold up to 15 different reagent cartridge simultaneously. This area is kept at a constant temperature of 15°C for optimal conservation of the reagents, while a stirring device keeps the microbeads always in homogenous suspension.
- ✓ Barcode reader for cartridge identification.
- ✓ Robot dispense arms: two robotic arms each carrying a dispensing needle. One arm is usually dedicated to dispensing samples, another to dispensing reagents. Each one has a separate washing well to clean the needle after each pipetting.
- ✓ Incubator: this area hosts the reaction cuvettes during incubation times, at a constant temperature of 37°C.
- ✓ Washing station: through the application of a magnetic field, this part allows retention of the paramagnetic microbeads and removal of the reaction liquids. Any number of washing steps with the desired washing buffer can be set.

- ✓ Read area: contains the injection devices of trigger reagents and the photomultiplier tube

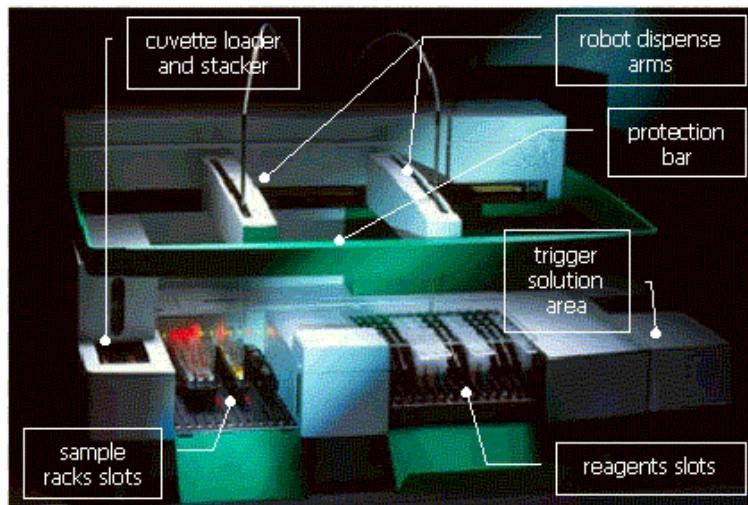


Figure 7: Liaison® system.

The Liaison® system is based on two key features: the use of paramagnetic microbeads as the solid phase (figure 8) and the generation of signal by means of chemiluminescence.

The adoption of microbeads as the solid phase instead of the classic immunoassay supports, as the ELISA microwells gives a clear edge in terms of available reaction surface, which in turn increases the kinetic rates of the antigen-antibody complex formation. Moreover, diffusion of both the analyte and the solid phase in the reaction volume is allowed, while in ELISA system only the analyte can diffuse, decreasing the possibility of the immune complex formation. The microbeads adopted in the Liaison® system are colloidal particles composed of a ferric oxide core covered by a polystyrene layer formed by spontaneous coalescence of polystyrene linear chains. This structure is in turn coated with another layer composed of polyurethane activated with tosyl- groups. The tosyl- group (4-toluenesulfonyl chloride) can undergo nucleophilic attack, allowing the beads to covalently bind proteins through their available aminic groups (ϵ -amino groups of lysines, N-terminal end). The paramagnetic properties of

these microbeads allow easy manipulation through the application of a magnetic field. The particles respond to a magnet but are not magnetic themselves and retain no residual magnetism after removal of the magnet.

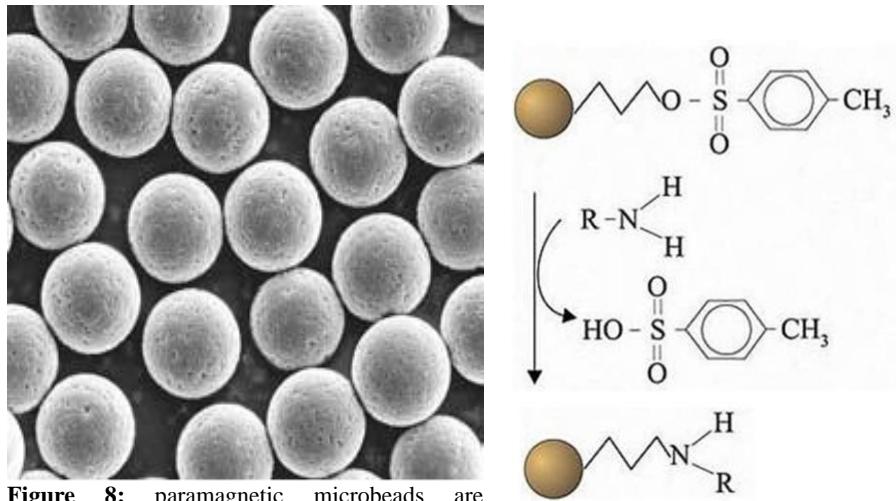


Figure 8: paramagnetic microbeads are functionalized with a tosyl-group, to allow binding on amino-groups of proteins.

The tracer molecule is an antigen or antibody conjugated to a signal generating compound. Chemiluminescent tracers are formed by conjugating the antibody (or antigen) to a molecule that can generate a photon emission upon addition of certain reagents. The entity of this photon emission is measured with a luminometer, usually equipped with a photomultiplier tube. The chemiluminescent molecule used in the Liaison® system is the luminol derivate ABEI (N-(4-Amino-Butyl)-N-Ethyl-Isoluminol), which is converted to its activated ester to allow conjugation with the antibody or antigen (figure 9). In presence of H₂O₂ and a microperoxidase (deuteroferriheme), ABEI achieves an excited state in consequence of a chemical reaction. This excited level decays to the ground level generating energy in form of light. The emission of light is recorded by the photomultiplier tube for an interval of just 3 seconds (“flash” chemiluminescence) and the signal is integrated over this interval. The final result is expressed in RLU (Relative Light Units).

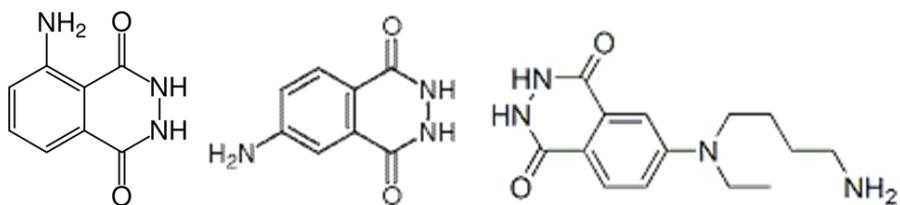


Figure 9: luminol, isoluminol and ABEI.

Using chemiluminescence is a great improvement over enzymatic signal generation of classic ELISA format assays. Sensitivity is highly increased and a greater dynamic range can be achieved. Lower molecular weight and steric hindrance of ABEI compared to horseradish peroxidase allow conjugation of more signal generating molecules per tracer molecule. Moreover, generation and recording of signal is completed in a very short time (3 seconds), with a sensible throughput increase.

MARKETING EVALUATION

Fourth-generation HIV screening assays that make combined HIV antigen and antibody detection possible in one are available since 1997 in Europe (90). These combined assays offer the advantage of early detection of HIV infection via p24 Ag detection and financial cost slightly higher than those for HIV antibody testing using third generation EIAs (Enzyme ImmunoAssay).

The diagnostic window is reduced by 4 to 5 days compared with that for antibody detection alone (third generation assay) (91).

Several commercially available fourth generation assays are currently used worldwide and are rapidly replacing third generation assays.

Despite their increased sensitivity, numerous reports indicate that the specificity of HIV Ag/Ab assays vary significantly. The risk of interference is potentially higher with the HIV combined assays than with single-Ag or -antibody EIAs, since both serological markers are determined in one test batch. The higher rate of false positive results of HIV combined assays has a particularly negative effect in cases of high sample throughput and low prevalence as in blood donor screening .

In addition, combined assays exhibit a relatively high detection limit for p24 antigen. The detection limit of fourth-generation assays (20 to >100pg of p24 antigen per ml) is higher than that of antigen assays (3.5-10 pg of p24 antigen per ml) and for this reason they cannot replace single antigen determination .

Highly sensitive antigen assays detect primary infection on average 1 to 2 days earlier than fourth generation tests.

The antigen detection module of fourth generation tests shows a variable sensitivity for detection of different HIV type 1 non-B subtypes, including group O and HIV-2. Since the genetic diversity of HIV is rapidly increasing worldwide, fourth generation assays need to accurately detect all HIV-1 subtypes and HIV-2.

Fourth generation assay demand a special algorithm for the analysis of reactive samples. For the anti-HIV part of the assay, confirmation of reactivity should be done first with an assay that lacks the p24 antigen detection module, and when reactivity persists, immunoblot should be used. For the p24 antigen part, confirmation of reactivity should be analyzed in an

assay that lacks the anti-HIV detection part and when reactivity persists, a nucleic acid-based assay should be used.

Confirmation of this part of reactivity is hampered by the fact that actually none of the commercially available nucleic acid based assays is able to detect HIV-1 group O and the HIV-2 genome.

Currently some advanced system are already commercially available, and have been used to confirm and validate the results in this work. The accepted “gold-standard” for a close system is, at the moment, Architect Ag/Ab Combo (Abbott): it is a chemiluminescent immunoassay, based on magnetic microparticles for the detection of anti-HIV1 and 2 antibodies and antigen p24 in human serum or plasma. Acridinium ester is used for the signal generation.

Other systems are currently available:

- ✓ AxSym HIV Ag/Ab combo automated assay (Abbott) (93): this assay is a microparticle enzyme immunoassay (MEIA), performed on the AxSym instrument and utilizes a blend of microparticles coated with 3 HIV recombinant antigens (HIV1 group M gp41, HIV1 group O gp41, HIV2 gp36) for the capture of antibodies and microparticles coated with HIV1 p24 specific monoclonal antibodies for the capture of HIV antigen in a test sample. Two conjugates are used in the assay: the first contains biotin-labelled HIV1/2 antigens and biotin-labelled p24 MoAb. The second contains anti-biotin alkaline phosphatase conjugate (which reacts with the added substrate to create a fluorescent product that can be measured). Time to completion is approximatively 26 minutes, with a throughput of 90 tests/2 hours.
- ✓ VIDAS HIV Duo Ultra assay (92): the assay is based on enzyme linked fluorescence assay technology (ELFA). The sample is incubated simultaneously with an anti-p24 polyclonal antibody within the entire solid-phase receptacle. Antibodies against HIV binds to the antigens (gp160 of HIV1, immunodominant region peptides of HIV2 and HIV1 group O) coated in the lower part of the cone. The p24 antigen in the sample binds to the monoclonal anti-p24 antibody coated in the upper part of the cone and is recognized by biotinilated anti-p24 poyclonal antibody. After a wash step to remove the unbound material, biotinilated antigens (the same used in the solid phase) are incubated in the lower part of the cone to detect the presence of antibodies. Following a wash step, streptavidin coupled to alkaline phosphatase is incubated in the entire cone, and binds to the biotin. After a washing step, the substrate is

Introduction

incubated first in the lower part of the cone and then an initial measurement of fluorescence is performed to reveal the presence or the absence of HIV antibodies. Then the substrate is incubated in the entire cone, and a second measurement of fluorescence is performed, which detects presence or absence of HIV p24 antigen.

- ✓ anti-HIV Traelisa (Biotest) (94): the assay is a highly sensitive sandwich enzyme immunoassay of third generation, for the determination of HIV-1(group M and O) and HIV-2 specific antibodies in blood or plasma. 4 antigen components are used for the detection of HIV antibodies

The availability of the HIV combo assay will complete the already wide menu of the Infectious Disease line on the LIAISON platform , allowing the penetration of the HIV testing market segment. Therefore the HIV assay is a strategic focus for company growth, especially for countries (Nordic, UK and export) were the TORCH market segment is too small to allow the introduction of the LIAISON® instrument in the labs.

AIM OF THE PROJECT

Aim of the project

Combined, simultaneous detection of anti-human immunodeficiency virus (anti-HIV) immunoglobulin and HIV core protein distinguishes fourth-generation (combination assay) HIV screening and diagnostic immunoassays from third-generation (double-antigen sandwich) antibody detection immunoassays. Prior to the introduction of fourth-generation assays, commercial immunoassays for blood screening and diagnosis of HIV infection were based either on detection of HIV core (p24) protein or on detection of HIV-specific antibodies, notably those antibodies directed against HIV transmembrane proteins (tmp). Antibodies against these proteins consistently appear during seroconversion of HIV-infected individuals and remain throughout the course of infection. Fourth-generation immunoassays have targeted reduction of the seronegative window period to achieve a continued decrease in the residual risk of transfusion-transmitted HIV infection. Combining antibody and antigen detection in a single immunoassay format achieves a reduction in the seroconversion window because HIV core protein (p24) appears transiently in the blood and has been used as a marker of antigenemia prior to a detectable humoral immune response to HIV infection. Antigen (p24) testing in a combined (fourth-generation) format has been estimated to reduce the seroconversion window by a few days to as much as about 2 weeks in comparison with third-generation single-format antibody detection assays. Despite enhanced seroconversion sensitivity, fourth-generation assays will be most valuable only if the specificity and sensitivity of individual antibody and antigen detection formats are not compromised when they are combined into a single immunoassay. Sensitive combination assays will need to detect antigen at levels equivalent to those of single-format antigen assays in order to be recommended as replacements for current antigen tests. This prerequisite, however, presents a considerable technical challenge not met by all combination assays. In addition, not all combined formats have achieved the low (non confirmed) repeat-reactive rates expected of blood donor screening and diagnostic assays that result in high specificities such as those typically displayed by third-generation single-format antibody assays.

The aim of this project is the production of a fourth generation chemiluminescent immunoassay for the detection of HIV-1 and HIV-2 infections, achieving an antigen assay sensitivity approaching that of single-format antigen tests, a specificity equivalent to that of a third-generation single-format antibody assay, and a high degree of total precision.

MATERIALS AND METHODS

CLONING AND PROTEIN EXPRESSION

Synthetic genes, encoding for HIV proteins were purchased by Geneart AG (Regensburg, Germany) and subcloned into pET vectors (Novagen, Merck Chemicals Ltd, Beeston, UK).

For cloning and protein expression, different *Escherichia coli* strains were used:

- XL1-Blue (Stratagene, CA) for cloning
- BL21(DE3) (Novagen) for protein expression

Bacterial cells, grown in LB with antibiotics (ampicillin 50 μ g/ml or kanamycin 30 μ g/ml, depending on the used vector), were induced with 1mM IPTG at OD_{600nm}=0.6.

PLASMID STABILITY ASSAY

Serial dilution of a culture at OD_{600nm}=0.6 were plated on:

- LB agar
- LB agar with antibiotic
- LB agar IPTG 1mM
- LB agar antibiotic IPTG 1mM

Plasmid is stable if (95):

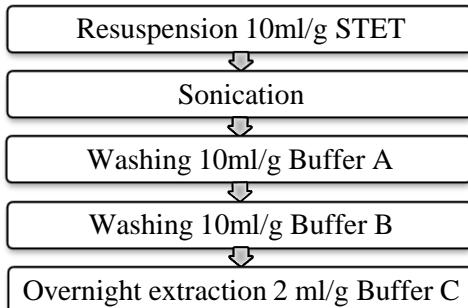
- CFU in LB = CFU in LB+antibiotic ($\pm 10\%$)
- CFU in LB+IPTG < 2% CFU in LB
- CFU in LB+IPTG+antibiotic < 0.01% CFU in LB

PROTEIN PURIFICATION:

gp41-O solid phase

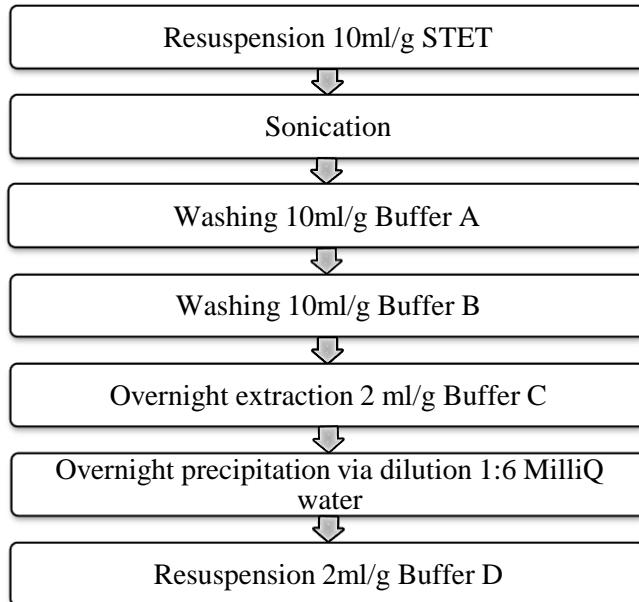
- Extraction with urea, without chromatographic steps

Materials and methods



Buffer	Composition
STET	50 mM TRIS, 50 mM EDTA, 8% Sucrose, 5% TRITON X-100 pH 8,00
Buffer A	50 mM TRIS pH 8,00
Buffer B	50 mM TRIS, 8 M Urea, NaCl pH 8,00
Buffer C	TRIS, 1% SDS, 5mM TCEP, 0,5M Urea, 0,3 M NaCl pH 8,50

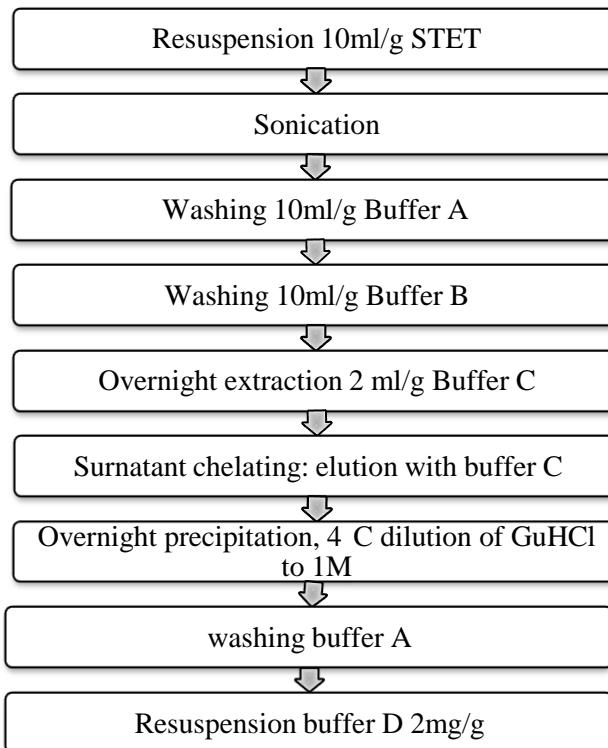
- Extraction with guanidinium HCl without chromatographic steps



Materials and methods

Buffer	Composition
STET	50 mM TRIS, 50 mM EDTA, 8% Sucrose, 5% TRITON X-100 pH 8,00
Buffer A	50 mM Sodium Phosphate pH 6,80
Buffer B	50 mM Sodium Phosphate, 3 M Urea, 0,3 M NaCl pH 6,80
Buffer C	50 mM Borate, 6 M Guanidinium HCl, 5 mM TCEP, 0,3 M NaCl pH 8,50
Buffer D	50 mM Borate, 1% SDS, 5mM TCEP, 0,5 M Urea, 0,3 M NaCl pH 8,50

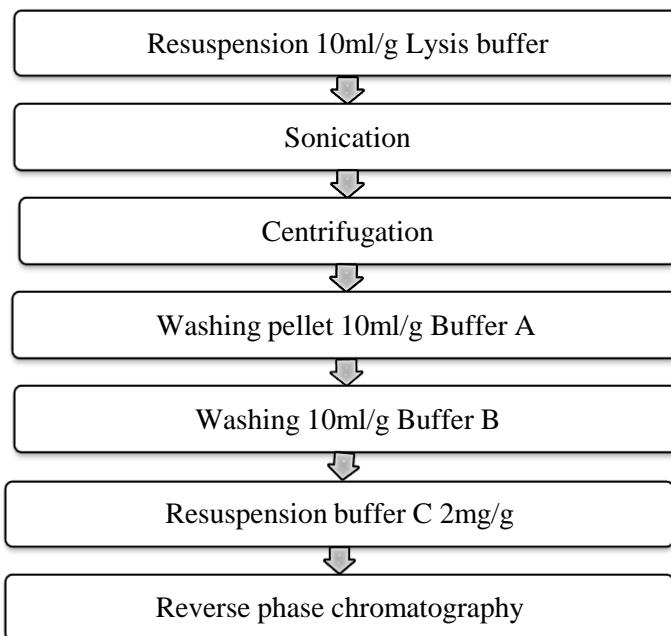
- Extraction with Guanidinium HCl and chelating



Materials and methods

Buffer	Composition
STET	50 mM TRIS, 50 mM EDTA, 8% Sucrose, 5% TRITON X-100 pH 8,00
Buffer A	20 mM Tris pH8.5
Buffer B	20 mM Tris pH8.5
Buffer C	50 mM Tris, 6 M Guanidinium HCl,
Buffer D	50 mM Borate, 1% SDS, 5mM TCEP,

gp41-M and gp35 solid phase:

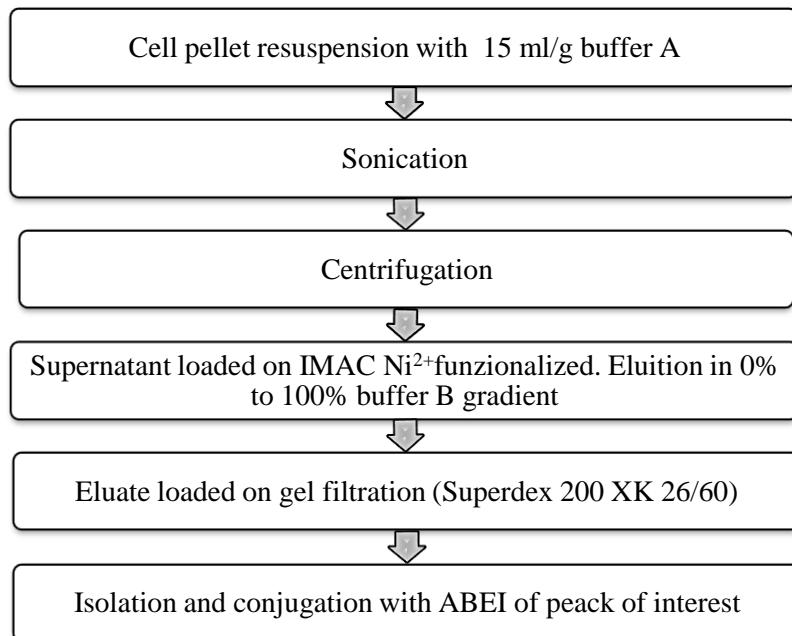


Buffer	Composition
Lysis Buffer	50 mM NaH ₂ PO ₄ *H ₂ O pH 8,00
Buffer A	50 mM NaH ₂ PO ₄ *H ₂ O , Triton X-100 0.2% (v/v) pH 8.0
Buffer B	50 mM NaH ₂ PO ₄ *H ₂ O , Triton X-100 0.2% (v/v), Urea 3M pH 8.0
Buffer C	50 mM NaH ₂ PO ₄ *H ₂ O, 500mM NaCl, 7 M Guanidinium HCl, 5mM TCEP, pH 8.0

Materials and methods

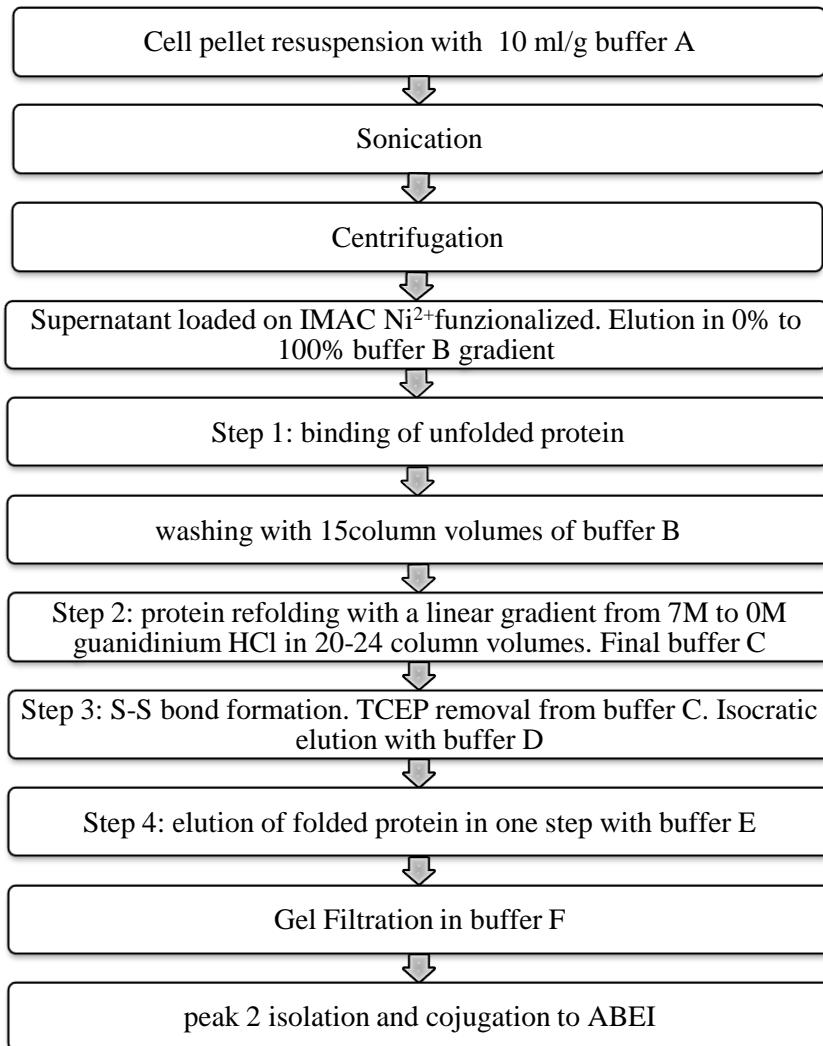
Pool of selected fractions from RPC is further purified: protein is precipitated by modifying pH (dilution with 1.7V of PBS, pH 7.4). After an overnight incubation (4°C) and washing, the purified protein is solubilized in storage buffer (H_3BO_3 100 mM, urea 0.5 M, SDS 1% (v/v), glycerol 10% (v/v), TCEP 5 mM, pH 9.0), and conserved at -30°C.

FH1_gp41-O:



Buffer	Composition
Buffer A	50 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 10 mM Imidazole, 5 mM β -mercaptoethanol, 100 mM NaCl, 1 M Urea, pH 7.8
Buffer B	50 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 500 mM Imidazole, 5 mM β -mercaptoethanol, 100 mM NaCl, 1 M Urea, pH 7.8

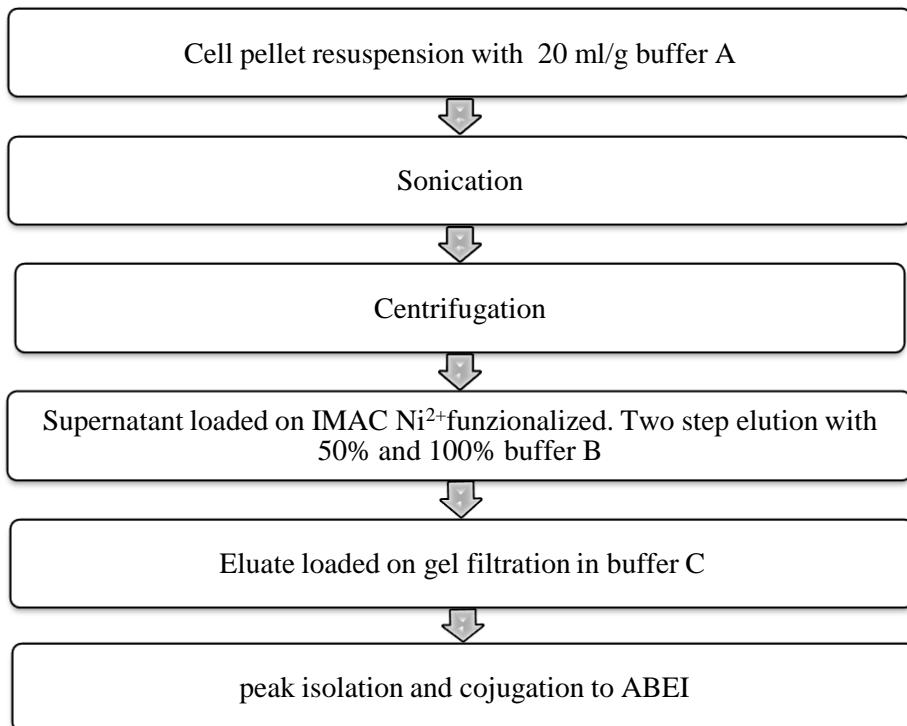
FH2_gp1-M:



Materials and methods

Buffer	Composition
Buffer A	50 mM NaH ₂ PO ₄ *H ₂ O, 7M Guanidinium HCl, 5 mM Imidazole pH 8
Buffer B	50 mM NaH ₂ PO ₄ *H ₂ O, 7M Guanidinium HCl, 5 mM Imidazole, 5mM TCEP pH 8
Buffe C	50 mM NaH ₂ PO ₄ *H ₂ O, 100mM NaCl, 5 mM Imidazole, 5mM TCEP pH 7.8
Buffe D	50 mM NaH ₂ PO ₄ *H ₂ O, 100mM NaCl, 5 mM Imidazole, pH 7.8
Buffer E	50 mM NaH ₂ PO ₄ *H ₂ O, 100mM NaCl, 250 mM Imidazole, pH 7.8
Buffer F	50 mM NaH ₂ PO ₄ *H ₂ O, 100mM NaCl, 2mM EDTANa ₂ * H ₂ O, pH 7.8

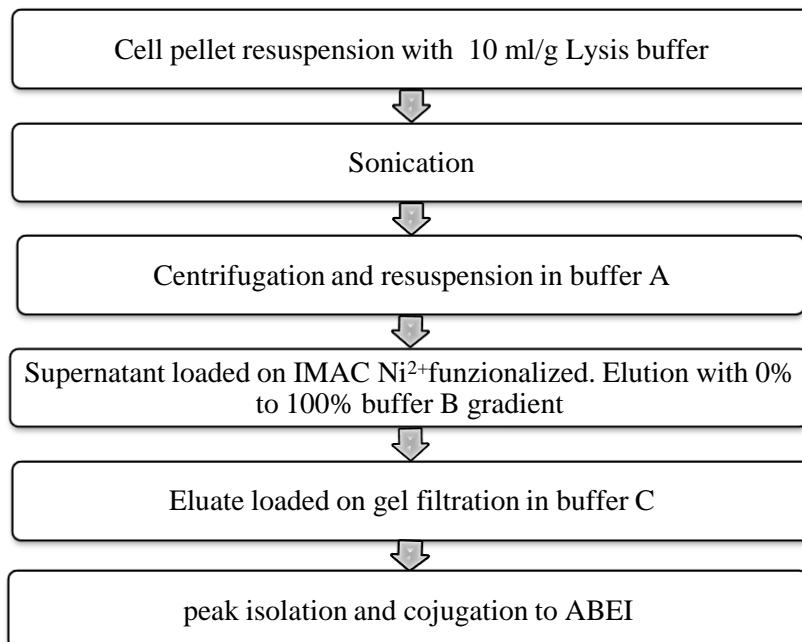
FH2_gp35:



Materials and methods

Buffer	Composition
Buffer A	50 mM Tris, 5 mM β -mercaptoethanol, 100 mM NaCl, 1 M Urea, pH 8
Buffer B	50 mM Tris, 500 mM Imidazole, 5 mM β -mercaptoethanol, 100 mM NaCl, pH 8
Buffer C	50 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 100 mM NaCl, 1mM EDTA pH 7.5

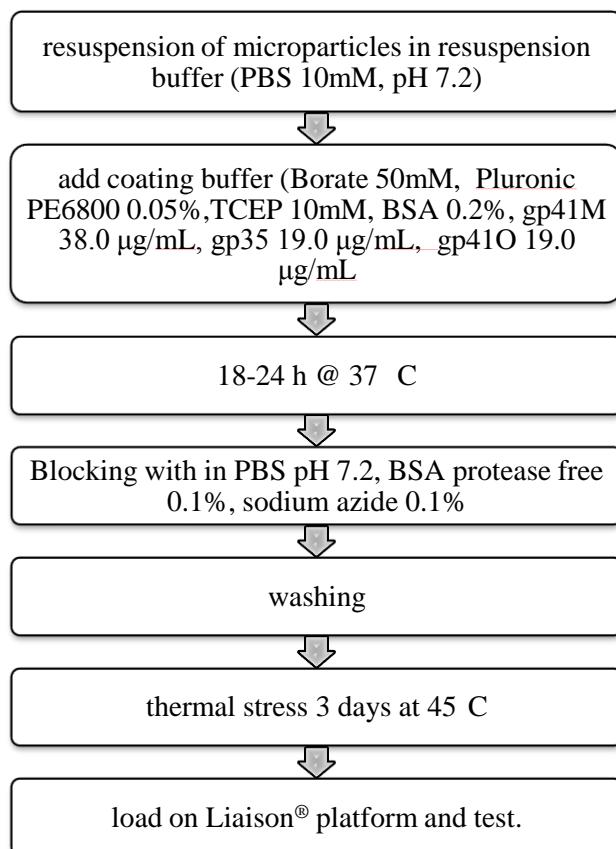
p24 antigen



Buffer	Composition
Lysis buffer	20 mM NaH_2PO_4 , 10 mM Imidazole, 100 mM NaCl, 1 M Urea, pH 8
Buffer A	30 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 20 mM Imidazole, 300 mM NaCl, 1M Urea pH 7.8
Buffer B	30 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 500 mM Imidazole 300 mM NaCl, 1mM Urea pH 7.8
Buffer C	50 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 100 mM NaCl, 1mM EDTA pH 7.5

LIAISON® ASSAY

Microparticles coating for antibody detection:



Microparticles for antigen detection are streptavidinated. Monoclonal anti-p24 antibodies are added together with the sample. One of the monoclonal antibodies is biotinilated and the other is conjugated to ABEI: the immunocomplex is then captured on the microparticles. In presence of H₂O₂ and a microperoxidase (deuteroferriheme), ABEI achieves an excited state in consequence of a chemical reaction. This excited level decays to the ground level generating energy in form of light. The emission of light is recorded by the photomultiplier tube for an interval of just 3 seconds and the signal is integrated over this interval. The final result is expressed in RLUs (Relative Light Units).

Materials and methods

Standard curves are memorized on the instrument, to facilitate the interpretation of the final result. Two points recalibration algorithm in Liaison[®] allows a working curve being recalculated from a stored master curve from the actual RLU scored by two calibrators.

The RLU obtained for each calibrator in a specific run is matched with the signal expected for the same calibrator if it was on the master curve. The percentage deviations obtained for both calibrators between values found and expected are then used to define a straight line, describing percentage deviation as a function of the logarithm of the concentration over the full range the master curve spans over.

Expected signal for each master curve virtual calibrator in master curve is then corrected by the corresponding percentage deviation in order to obtain a full set of modified signals.

The two series of pairs of modified RLUs and master curve virtual calibrators concentrations are then used to define the working curve of the specific run.

RESULTS

ANTIGEN SELECTION

The 2008 European Guideline on HIV testing (96) provides practical guidance to clinicians in these settings who undertake HIV testing, and suggests appropriate standards for the audit of service provision. To be more precise:

- ✓ Venous blood (or serum) is the preferred specimen for HIV testing
- ✓ Fourth generation screening assays that simultaneously test for anti-HIV-1 antibodies and HIV-1 p24 antigen as well as anti-HIV-2 antibodies are recommended to be used as HIV screening tests in European STI clinics (97, 98). If available, fourth generation assays which also detect infection with HIV-1 group O should be used. According to the common technical specifications, these assays must have sensitivities and specificities at least of 99.7% and 99.4% respectively (99).
- ✓ Nucleic acid amplification tests (HIV-1 viral load assays) are not recommended as a diagnostic screening assay because of an only marginal advantage over fourth generation screening assays for detecting primary HIV infection and the possibility of false-positive results (100-102).

During primary HIV infection, lot of antibodies are generated, directed against glycoproteins of viral envelope. Most of these antibodies are non neutralizing, or isolate-specific (42-43).

Only few highly reactive antibodies have been identified, and six of them are monoclonal (44-50). Among them, three are directed against glycoprotein gp41: these antibodies are of great interest for the development of a vaccine candidate, because they are highly cross-reactive with other HIV subtypes (50-54).

These evidences lead to the conclusion that gp41 could be one of the best antigens for the development of diagnostic assays for anti-HIV antibody detection.

Therefore we chose three different variants of glycoprotein gp41, the most conserved antigen of HIV:

- ✓ gp41-M, for HIV-1 group M
- ✓ gp41-O, for HIV-1 group O
- ✓ gp35 (analogue of gp41), for HIV-2

Results

To detect HIV infection at early stage, the best system is the detection of p24, a core protein, which is the first detectable marker, after viral RNA. Liaison® system allows the generation of a sandwich immunoassay, with the same antigen used as solid phase and tracer, so increasing the specificity. Thus, Liaison® HIV immunoassay will combine (figure 10):

- ✓ Antibodies detection: solid phase is made of gp41-M, gp41-O and gp35 coated on tosyl-activated paramagnetic microparticles. The same antigens will be used as soluble tracers, conjugated with ABEI.
- ✓ Antigen detection: anti-p24 monoclonal antibodies will be captured by the beads and antibodies with high affinity for a different p24 epitope will be conjugated to ABEI and used as tracer to complete the “sandwich”.

The chemiluminescent signal will be detected by a photomultiplier and interpolated on a standard curve: this curve is memorized on the software, and adjusted every time with two calibrators, which are a standard positive serum (for antibodies detection section) and a recombinant p24 (for the antigen detection section). Thanks to this standard curve there is no need to interpret the results, which will be just “antibodies and/or antigen positive/negative”.

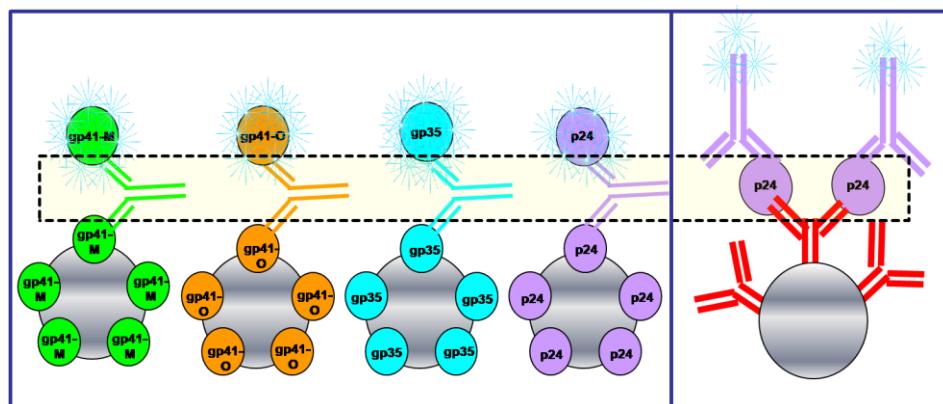


Figure 10: assay structure. HIV antigens or anti-24 monoclonal antibodies are coated on tosyl-activated paramagnetic microparticles. If the patient's serum is HIV positive, specific anti-HIV antibodies (or p24 antigens, depending on the stage of infection) are captured. Signal is generated by tracers conjugated to ABEI and detected by a photomultiplier.

**Phase 1:
Detection of anti-HIV-1M, HIV-
1O and HIV-2 antibodies**

SOLID PHASE ANTIGENS: gp41-O

Glycoprotein gp41 comprises three domains:

- ✓ An extracellular domain (ectodomain)
- ✓ A transmembrane domain
- ✓ A cytoplasmatic tail

Ectodomain of gp41 has a key role in the fusion process: this domain contains a highly hydrophobic N-terminal tail (*fusion peptide*) and two heptad repeat motifs, referred to as the N-helix and the C-helix, linked together by a closed loop with a disulfide bridge.

These two helical sequences, in the context of a gp41 ectodomain trimer, pack in an antiparallel fashion to generate a six-helix bundle.

Interaction with CD4 and coreceptors leads to conformational rearrangements of ectodomain, which adopts an extended conformation: this allows the insertion of fusion peptide in the target cell lipid bilayer (figure 11). The N- and C- helices, as shown by structural analysis of gp41 trimeric form, change their conformation, folding into a highly stable six-helix bundle, bringing the membranes in apposition and allowing membrane fusion to occur (figure 11).

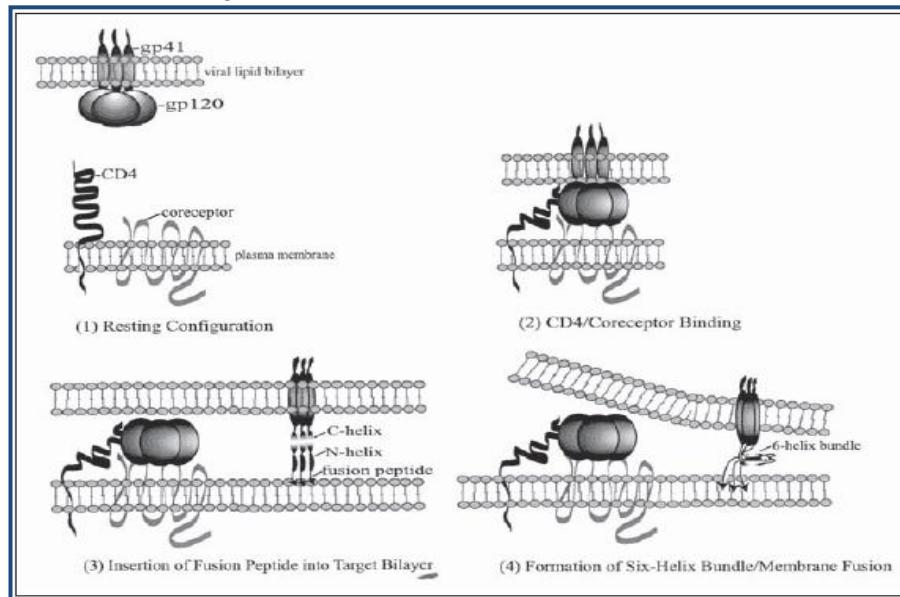


Figure 11: gp41-mediated fusion between virus and target cell membrane allows the entry of the virus in the host cell cytoplasm.

Results

The large structural rearrangements of gp41 during membrane fusion probably require substantial conformational flexibility; together with its high hydrophobicity, this dynamic character renders gp41 a very difficult protein for recombinant production in a functional form.

A multiple alignment on gp41 from different HIV-1 O strains was performed in order to identify the most conserved domains: we found a sequence of 143 aminoacids in the ectodomain as a good candidate for the solid phase (figure 12).

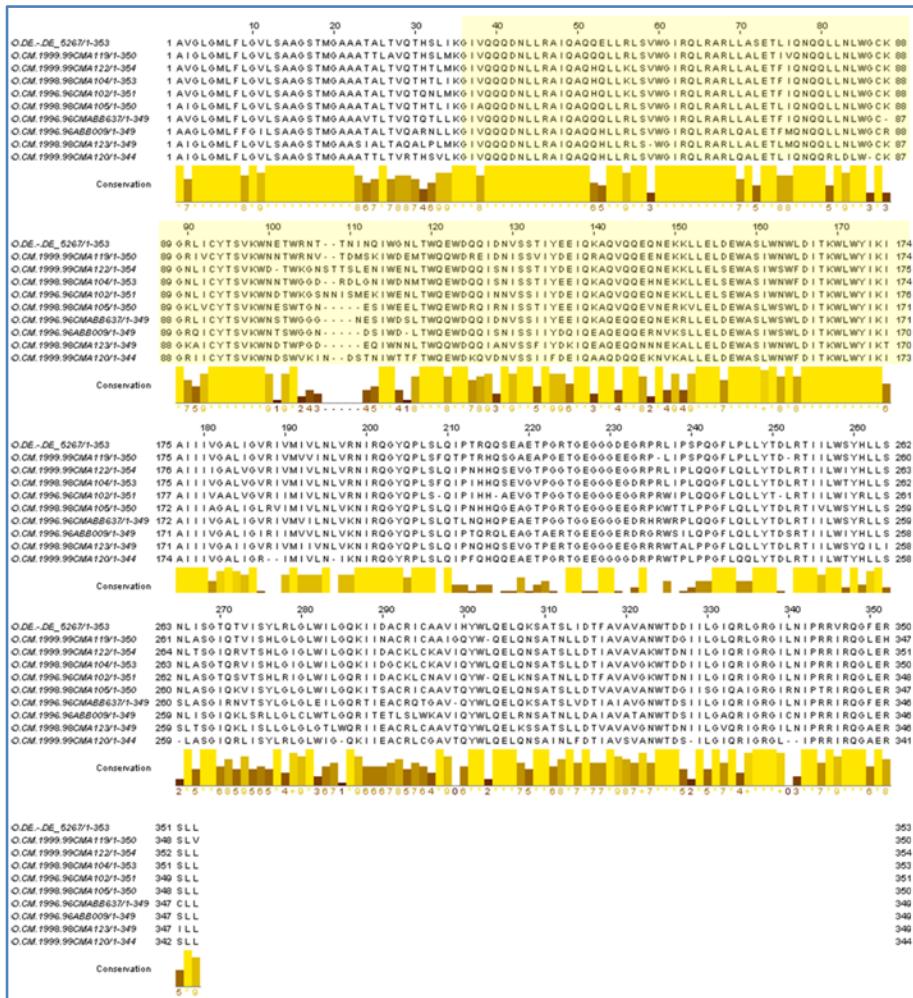


Figure 12: multiple alignment of different gp41-O. Evidenced sequence shows the most conserved part of ectodomain.

Results

Synthetic gene encoding for gp41-O ectodomain portion was subcloned into pET30 vector between BamHI and HindIII restriction sites. This allowed the fusion of gp41-O with a N-terminal 6-histidine tag, that facilitated purification process. pET plasmids are relatively stable, and are maintained in host cells even in a non-selective medium. Nevertheless, problems of instability can occur if the subcloned gene encodes for a protein that is toxic for the host cell. Since plasmid stability during cell growth is important in order to optimize the productive process we kept the cells in selective media, using antibiotics that forced bacterial cells to maintain the vector carrying a resistance. Moreover we made a plasmid stability test on *E.coli* BL21(DE3) cells carrying pET30gp41-O (table 2). Immediately before induction, cells were plated on four different plates (95, table 2). In the presence of IPTG, cells carrying a protein production plasmid do not grow because have dedicated all their resources to the production of the recombinant protein instead of cell maintenance. In a typical culture useful for producing target protein, almost all cells will form colonies both on the LB plate and on the LB plate + antibiotic; less than 2% of the cells will form a colony on the LB plate + IPTG; and less than 0.01% will form a colony on the LB plate + antibiotic + IPTG.

Growth medium	Cells that grow	
LB	All viable cells	
LB+KAN 30µg/ml	Cells that still carry the plasmid	
LB+IPTG 1mM	Cells that have lost the plasmid or mutants that have lost the ability to express the target gene	
LB+KAN 30µg/ml +IPTG 1mM	Only mutants that retain the plasmid but have lost the ability to express the target gene	
Growth medium	#CFU (mean of three replicates)	Dilution factor
LB	250	10^{-6}
LB+KAN 30µg/ml	230	10^{-6}
LB+IPTG 1mM	240	10^{-4}
LB+KAN 30µg/ml +IPTG 1mM	2	10^{-3}

Table 2: plasmid stability assay of pET30-gp41O. In the upper part of the table cells that are able to grow on each plate are showed. In the lower part experimental colony count for each plate are shown.

Results

Since criteria suggested by Novagen (95) are respected, we considered vector pET30-gp41O as stable.

Batch culture was performed at 37°C and protein production was induced by IPTG 1mM. gp41-O was produced in large amount; it was insoluble and accumulated in inclusion bodies.

Three different approaches were tested for the purification, to identify the method that combines high yield, high purity, low cost and no time wasting:

- ✓ Denaturation and purification of inclusion bodies using urea (a well known method, used for ELISA antigen purification)
- ✓ Denaturation and purification of inclusion bodies using Guanidinium HCl
- ✓ Denaturing chelating with GuHCl

Denaturation and purification of inclusion bodies using urea:

The first method of purification is similar to the purification of other insoluble antigens produced by Diasorin S.p.A.: inclusion bodies were washed and resuspended with high urea concentration (8M). This kind of purification, however, allowed the recovery of only 50% of total protein (figure 13).

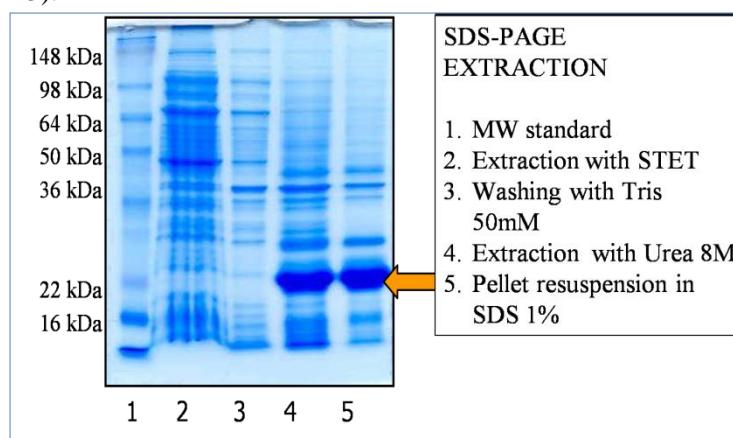


Figure 13: purification of gp41-O. After sonication and washing with saline buffers, gp41-O was resuspended with urea 8M. 50% of total protein was still insoluble even after urea solubilization.

Results

Denaturation and purification of inclusion bodies with GuHCl:

In order to increase purification yield, we modified washing and resuspension buffers, adding GuHCl (56).

Purification procedure was the same: washing steps with saline buffers with increasing extraction potential, and resuspension with urea.

Adding GuHCl in the purification steps allowed a strong increase in protein yield, but there was a reduction in purity (figure 14).

To enhance purity, without chromatographic steps, we decided to dilute GuHCl from 6 to 1M in an overnight precipitation process: in this way, many of the contaminants were solubilized, and gp41-O reached a purity of about 70% (figure 15).

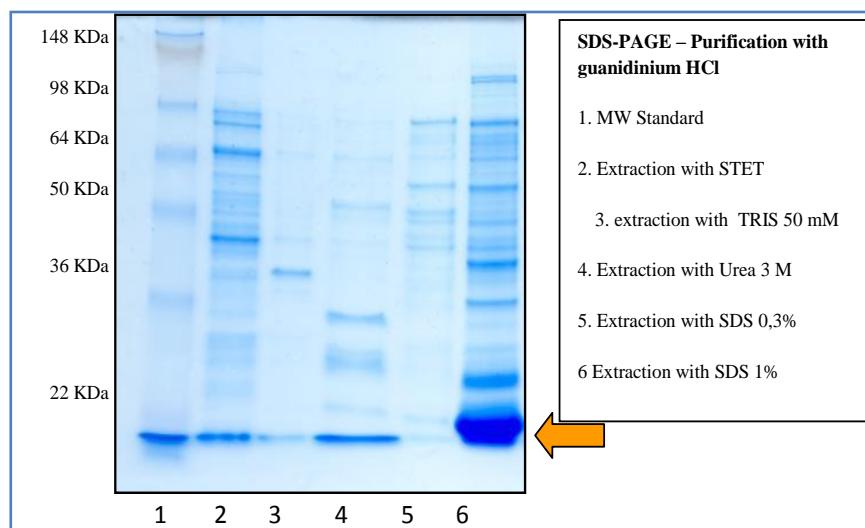


Figure 14: By adding GuHCl it is possible to enhance protein yield, but purity decreases.

Results

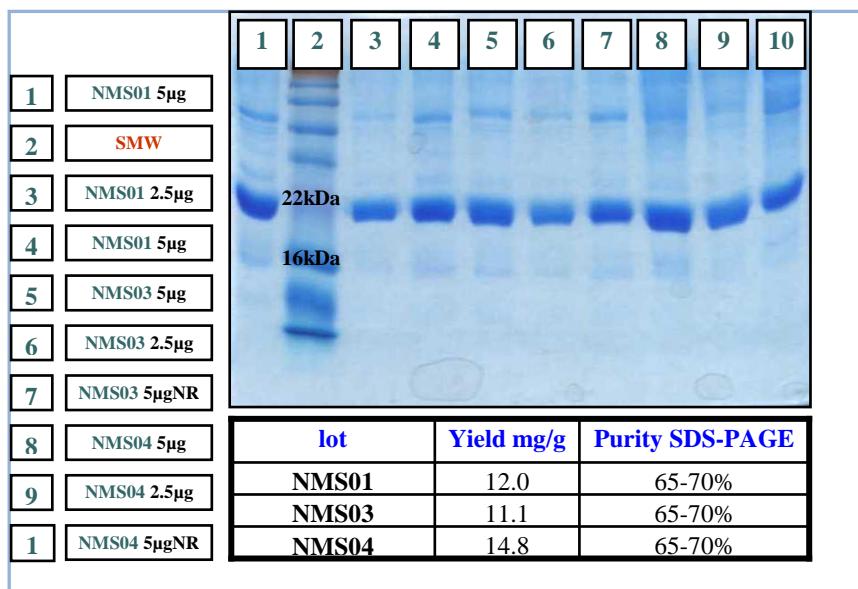


Figure 15: comparison of different lots of gp41-O purified with the dilution step. By this process yields and purity are enhanced.

Purification of inclusion bodies with GuHCl and denaturing Chelating

A purification procedure that avoids chromatographic steps is desirable from the point of view of an industrial production, because it is time- and cost-saving. Nonetheless, a 65-70% purity is still low for Liaison® application.

Since antigen purity is a key factor for the developement of a sensitive immunoassay, we added a chromatographic step, to further purify gp41-O. Chromatography on Me-chelating column allowed the elution of a concentrated protein (figure 16) with a significative increase of the purity (75%). Nevertheless final yield (mg/g) was not influenced by adding a further purification step (figure 16).

Results

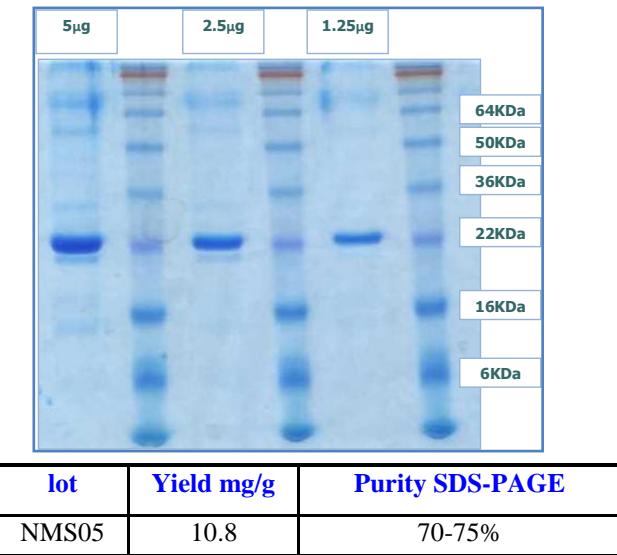


Figure 16: purification of gp41-O with chromatographic step. Yield was not reduced, and purity was enhanced.

Finally, gp41-O was compared with a commercial HIV glycoprotein: both proteins were in an aggregated form, as widely described in many publications (55-60) (figure 17).

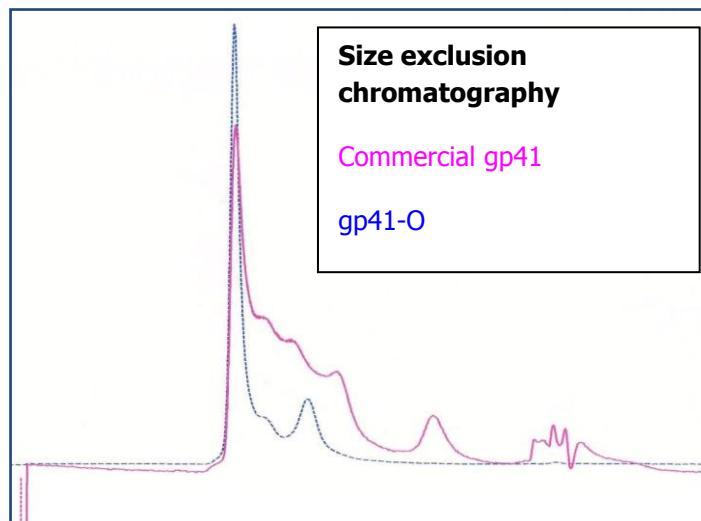


Figure 17: size-exclusion chromatography. Comparison between commercial gp41 and gp41-O: both proteins have a similar profile, and are eluted as insoluble aggregates.

IMMUNOCHEMICAL EVALUATION OF gp41-O

gp41-O was tested for its immunoreactivity.

A comparison with a commercial ELISA test (HIV TETRA ELISA, Bioteest) was performed: gp41-O, 0.5 μ g/ml, was adsorbed on the plate, and a positive serum was evaluated at different concentrations, to have preliminary data on sensitivity and selectivity of gp41-O. As negative control, a pool of negative sera was tested (figure 18).

Graphic below shows that gp41-O recognized also a 500-fold diluted HIV-1O positive serum, that was not detected by the commercial system. There was also a good separation between negative pool and positive sample.

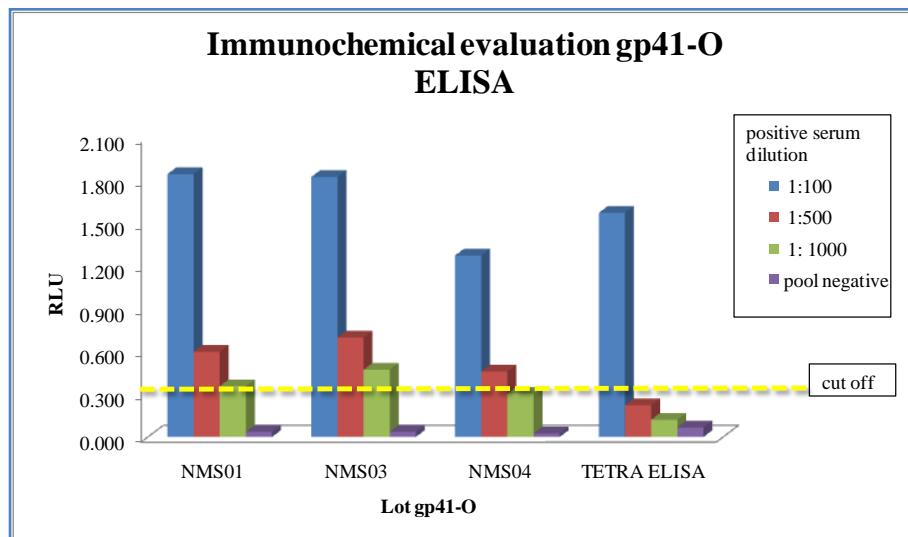


Figure 18: immunochemical evaluation of gp41-O. RLU=Relative Light Units

After demonstration of gp41-O reactivity on ELISA platform, we evaluated it on Liaison® system: the recombinant glycoprotein was coated on tosyl-activated paramagnetic microparticles (Dynal) at a concentration of 25 μ g/ml. The first evaluation of this antigen was via an indirect assay: specific anti-gp41-O antibodies in positive serum bound to antigen and were recognized by anti-human IgG conjugated to ABEI.

Positive serum was recognized at 1:50 and 1:100 dilutions, and a good separation between positive and negative sample is shown (figure 19).

Results

Furthermore, we tested various antigen lots, which showed comparable results, both on ELISA and in Liaison[®] platform: the process had, thus, a good reproducibility.

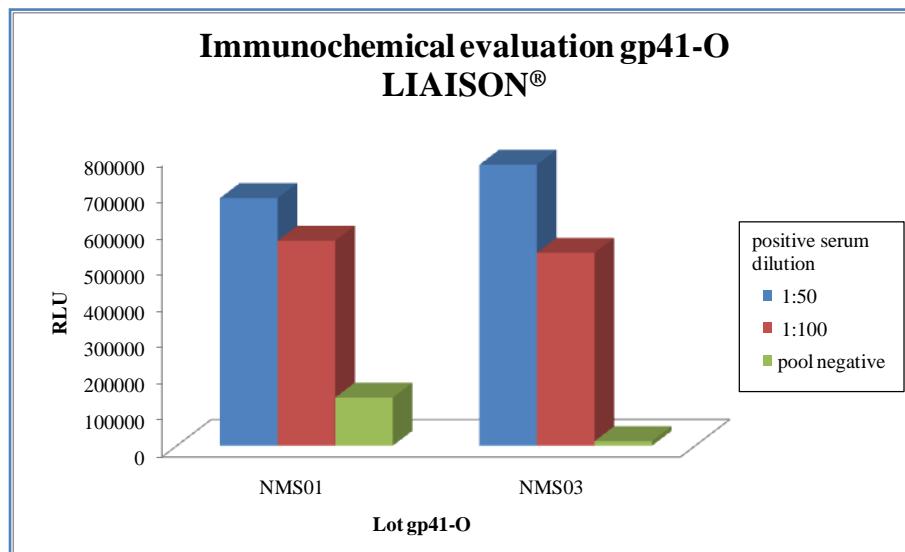


Figure 19: Immunochemical evaluation of gp41-O on indirect Liaison[®] assay. RLU=Relative Light Units. Typical results are shown.

Results

SOLID PHASE ANTIGENS: gp41-M

As done for gp41-O, a multiple alignment on gp41 from different HIV-1M strains was performed in order to identify the most conserved domains: we found a sequence of 143 aminoacids in the ectodomain as a good candidate for the solid phase (figure 20).

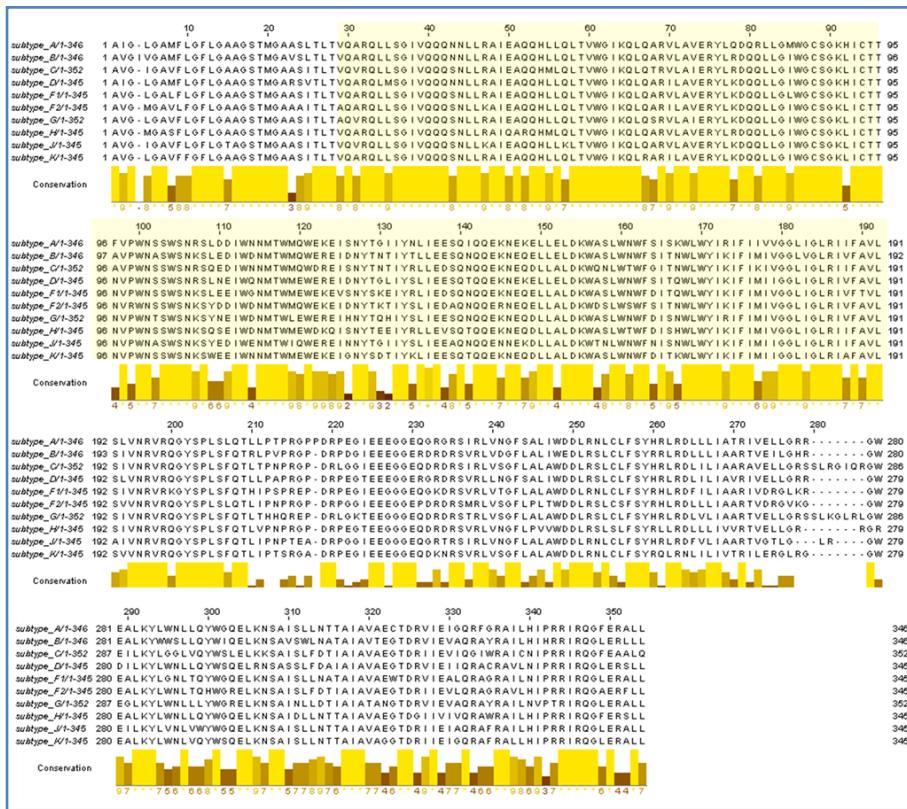


Figure 20: multiple alignment of different gp41-M. Evidenced sequence shows the most conserved part of ectodomain.

Synthetic gene encoding for gp41-M ectodomain portion was subcloned into pET30 vector between NdeI and XhoI restriction sites.

We made a plasmid stability test on *E.coli* BL21(DE3) cells carrying pET30gp41-M (table 3), plating cells on LB medium with or without antibiotic and IPTG. Since criteria suggested by Novagen (95) are respected, we considered the plasmid pET30-gp41-M as stable (table 3).

Results

Growth medium	#CFU (mean of three replicates)	Dilution factor
LB	157	10^{-6}
LB+KAN 30µg/ml	145	10^{-6}
LB+IPTG 1mM	3	10^{-4}
LB+KAN 30µg/ml +IPTG 1mM	1	10^{-3}

Table 3: plasmid stability assay of pET30-gp41-M.

Various lots of fermentation were performed in standard conditions (LB with antibiotic, 37°C, IPTG 1 mM when cellular density reaches $OD_{600nm}=0.6$), demonstrating that were reproducible (table 4):

lot	ON pre-inoculum (OD_{600})	0h (OD_{600})	1h (OD_{600})	2h (OD_{600})	3h (OD_{600})	Volume (ml)	Cell pellet (g)
NMS 236	3.2	0.6	1	1.1	1.2	1000	2.4
NMS 237	3.2	0.6	1	1	1.1	1000	2.4
NMS 238	3.0	0.6	0.9	0.9	1.1	1000	2.4
NMS 244	2.8	0.6	0.9	1	1.1	1000	2.7
NMS 245	2.8	0.6	0.9	1	1	1000	2.8
NMS 250	2.9	0.6	0.9	1	1	1000	2.4
NMS 251	2.9	0.6	1	1	1.1	1000	2.2

Table 4: comparison of various fermentation lots of BL21(DE3)pET30-gp41-M. Growth and yield were reproducible.

Results

gp41-M was well expressed in *Escherichia coli*: it was insoluble and accumulated in inclusion bodies. Purification procedure consisted of four phases:

- ✓ Phase 1. Inclusion bodies purification: cell pellet was resuspended in a phosphate buffer. There was a lysis step and a centrifugation: the proteic precipitate was washed and finally recovered with a phosphate buffer with GuHCl 7M pH 8.0.
- ✓ Phase 2. Size exclusion chromatography: solubilized protein was purified on gel filtration column Superdex 200. A multimeric protein was isolated and further purified.
- ✓ Phase 3. Reverse phase chromatography: proteic fraction from phase 2 was loaded on a column Source 15 RPC (GE Healthcare). gp41-M was eluted in a water/acetonitrile/trifluoroacetic acid 0.1% (v/v) buffer.
- ✓ Phase 4. Solubilization in stocking buffer. gp41-M was precipitated via dilution in a phosphate buffer. After a centrifugation step, proteic precipitate was solubilized in the final buffer, sterile filtered and stocked at -30°C.

Purification gave high yields and reproducible data (table 5).

lot	C (mg/ml)	V (ml)	Q (mg)	R _{280/260}	Cell pellet (g)	Total yield mg /g
NMS238	1.22	20	24.3	1.76	2.4	10.1
NMS244	1.39	20	27.8	1.72	2.7	10.2
NMS245	1.60	20	32.0	1.70	2.8	11.4
NMS250	1.63	19	30.9	1.73	2.4	12.9

Table 5: yields of various purification lots of gp41-M after phase 3.

Chromatographic profile, after gel filtration on Superdex 300 10/300 GL column, showed that gp41-M was in a stable, homogenous multimeric state (figure 21).

Results

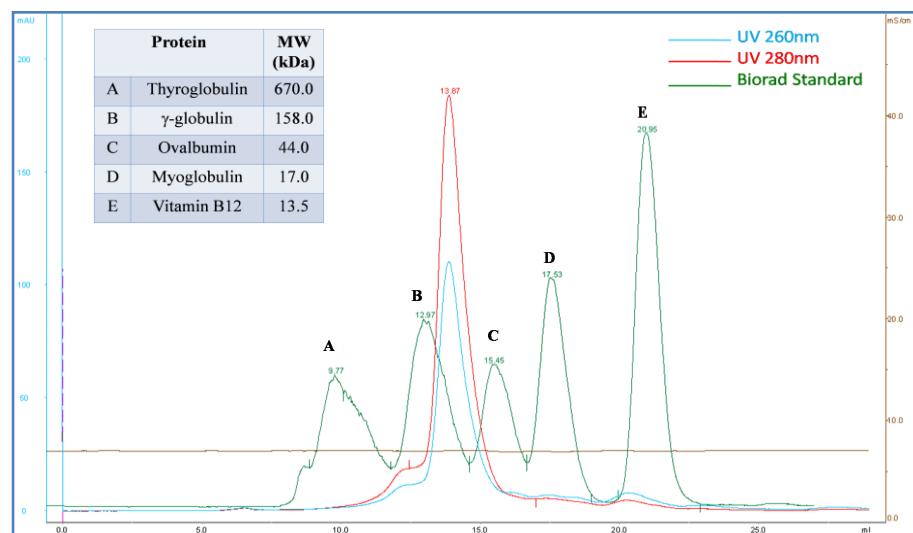


Figure 21: gel filtration of gp41-M. Retention time was compared with those of a standard mixture of proteins (Biorad), and molecular weight of multimeric form of gp41-M was calculated as 97 kDa

A freeze-thaw stability assay was also performed.

After freezing at -30°C, an aliquot of each lot of protein was thawed on ice: chromatographic analyses on Superdex 200 10/300 GL size exclusion column did not show any change in chromatographic profile in comparison with untreated protein (figure 22).

Results

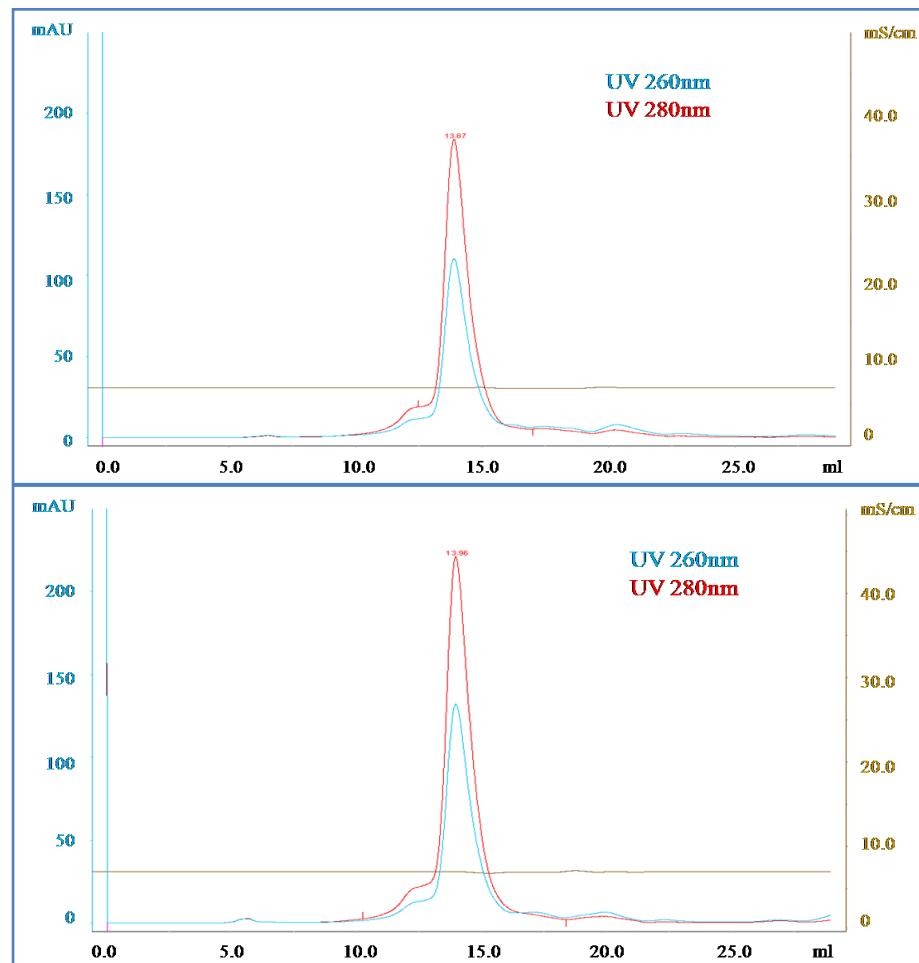


Figure 22: comparison of chromatographic profiles of gp41-M before (up) and after (down) a freeze-thaw process.

Results

IMMUNOCHEMICAL EVALUATION OF gp41-M

gp41-M was tested for its immunoreactivity: the recombinant glycoprotein was coated on tosyl-activated paramagnetic microparticles (Dynal) at a concentration of 25 μ g/ml. The first evaluation of this antigen was via an indirect assay: specific anti-gp41-M antibodies in positive serum bound to antigen and were recognized by anti-human IgG conjugated to ABEI.

Positive serum was recognized even at 1:3000 dilution, and a good separation between positive and negative sample is showed (figure 23). Furthermore, we tested various antigen lots, which showed comparable results, both on ELISA and in Liaison[®] platform: the process has, thus, a good reproducibility.

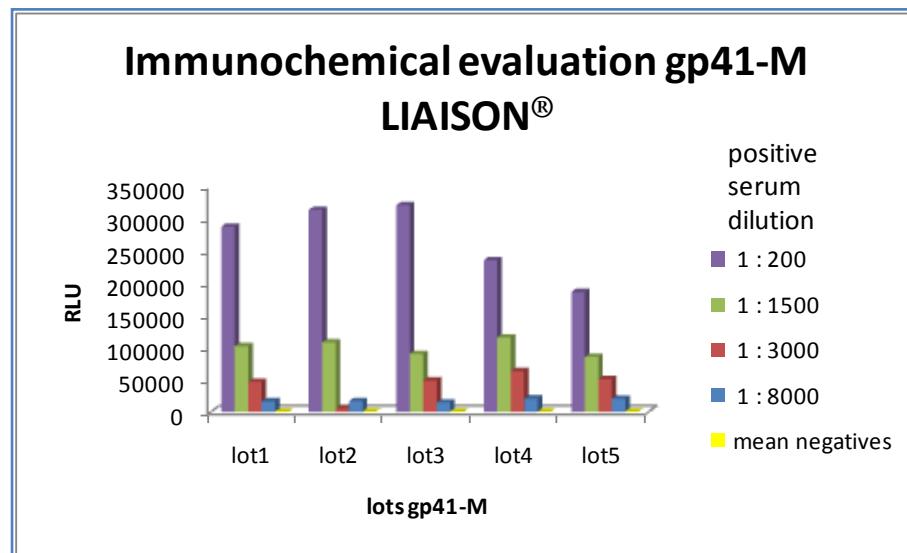


Figure 23: Immunochemical evaluation of gp41-M on indirect Liaison[®] assay. RLU=Relative Light Units

Results

SOLID PHASE ANTIGENS: gp35

As done for gp41-O and gp41-M, a multiple alignment on gp35 from different HIV-2 strains was performed in order to identify the most conserved domains: we found a sequence of 163 aminoacids in the ectodomain as a good candidate for the solid phase (figure 24).

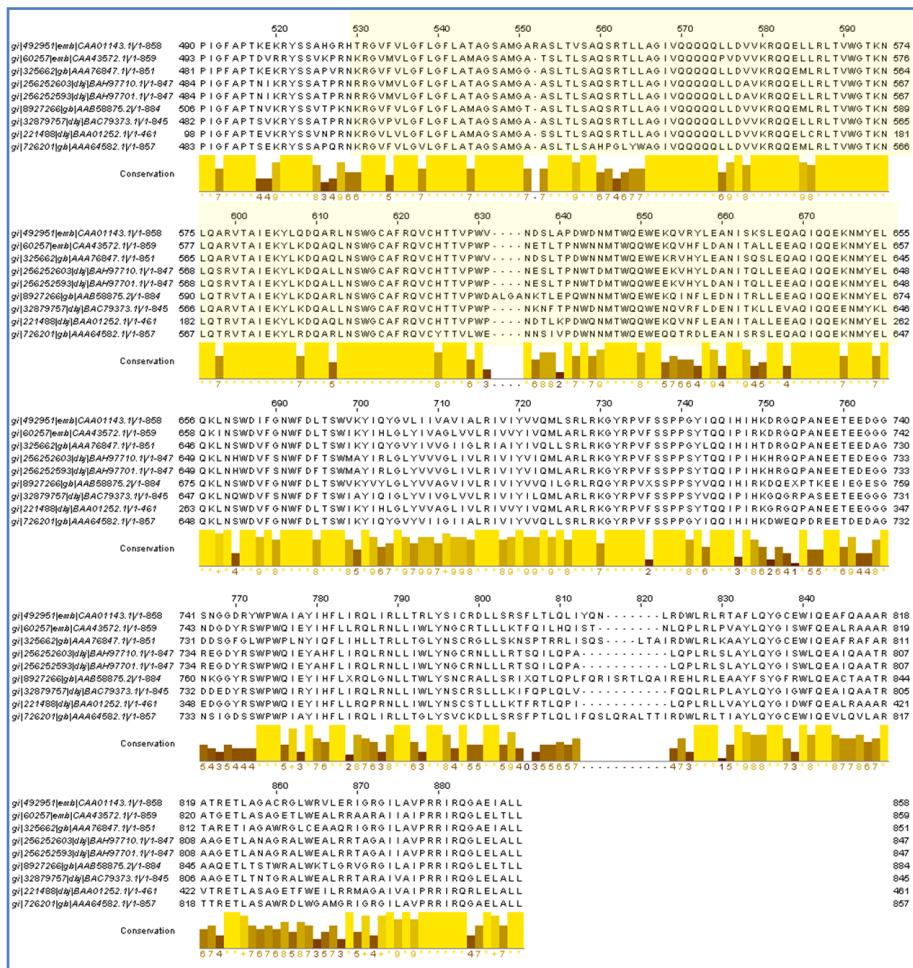


Figure 24: multiple alignment of different gp35. Evidenced sequence shows the most conserved part of ectodomain.

Results

Synthetic gene encoding for gp35 ectodomain portion was subcloned into pET24 vector between NdeI and XhoI restriction sites.

We made a plasmid stability test on *E.coli* BL21(DE3) cells carrying pET30gp35 (table 6), plating cells on LB medium with or without antibiotic and IPTG.

Since criteria suggested by Novagen (95) are respected, we considered the plasmid pET30-gp35 as stable (table 6).

Growth medium	#CFU (mean of three replicates)	Dilution factor
LB	438	10^{-6}
LB+KAN 30µg/ml	480	10^{-6}
LB+IPTG 1mM	2	10^{-4}
LB+KAN 30µg/ml +IPTG 1mM	1	10^{-3}

Table 6: plasmid stability test of BL21(DE3)pET30-gp35.

Since fermentation at standard conditions (LB with antibiotic, 37°C, IPTG 1 mM when cellular density reaches $OD_{600nm}=0.6$) showed no bacterial growth (table 7 and figure 25) and low protein production, two other conditions were tested:

- ✓ LB with antibiotic, 30°C, IPTG 1 mM when cellular density reaches $OD_{600nm}=0.6$
- ✓ LB with antibiotic, 37°C, IPTG 1 mM when cellular density reaches $OD_{600nm}=1.5$

Only with induction at 1.5OD cells grew at a satisfactory rate and protein production was enhanced (figure 25). Recombinant gp35 was produced as insoluble form, incorporated in inclusion bodies.

Results

Fermentations:						
temperature (°C)	OD ₆₀₀ at induction	0h (OD ₆₀₀)	1h (OD ₆₀₀)	2h (OD ₆₀₀)	3h (OD ₆₀₀)	volume (ml)
37	0.6	0.6	0.8	0.7	0.7	50
37	1.5	1.5	1.9	2.1	2.3	50
30	0.6	0.6	0.7	0.7	0.8	50

Table 7: comparison of various fermentation condition for BL21(DE3)pET30-gp-35.
Induction at 1.5OD allowed a greater growth.

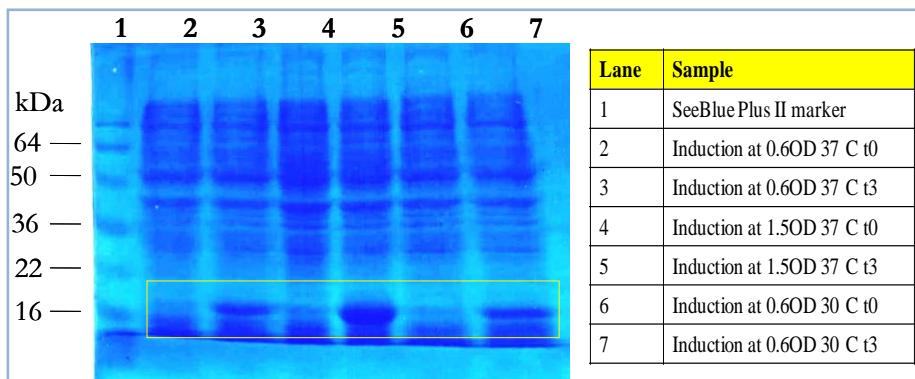


Figure 25: comparison of the three different induction conditions. Only with induction at 1.5 OD growth and protein production were satisfactory.

Purification procedure consisted of four phases, as for gp41-M:

- ✓ Phase 1. Inclusion bodies purification: cell pellet was resuspended in a phosphate buffer. There was a lysis step and a centrifugation: the proteic precipitate was washed and finally recovered with a phosphate buffer with GuHCl 7M pH 8.0.
- ✓ Phase 2. Size exclusion chromatography: solubilized protein was purified on gel filtration column Superdex 200. A multimeric protein was isolated and further purified.
- ✓ Phase 3. Reverse phase chromatography: proteic fraction from phase 2 was loaded on a column Source 15 RPC (GE Healthcare). gp35 was eluted in a water/acetonitrile/trifluoroacetic acid 0.1% (v/v) buffer.

Results

- ✓ Phase 4. Solubilization in stocking buffer. gp35 was precipitated via dilution in a phosphate buffer. After a centrifugation step, proteic precipitate was solubilized in the final buffer, sterile filtered and stocked at -30°C.

Retention time of third peak eluted with gel filtration was compared with retention times of a standard mixture of protein, and molecular weight was calculated. Recombinant gp35 seemed to be in hexameric form (figure 26).

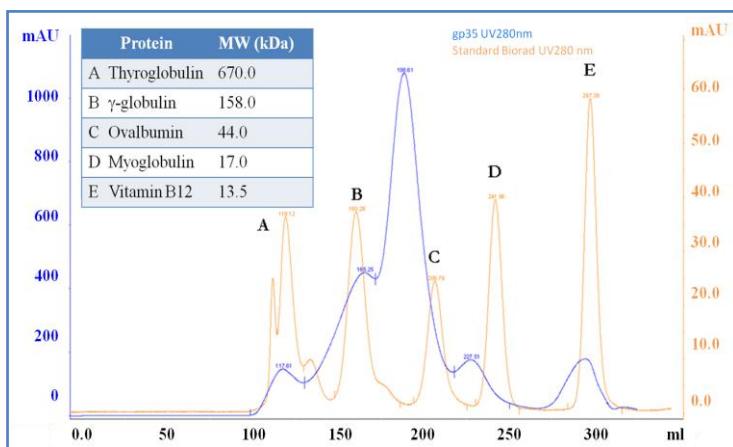


Figure 26: chromatographic profile of gp35. It was eluted from GFC as a hexameric aggregate.

A freeze-thaw stability assay was also performed.

After freezing at -30°C, an aliquot of each lot of protein was thawed on ice: chromatographic analyses on Superdex 200 10/300 GL size exclusion column did not show any change in chromatographic profile in comparison with untreated protein (figure 27).

Results

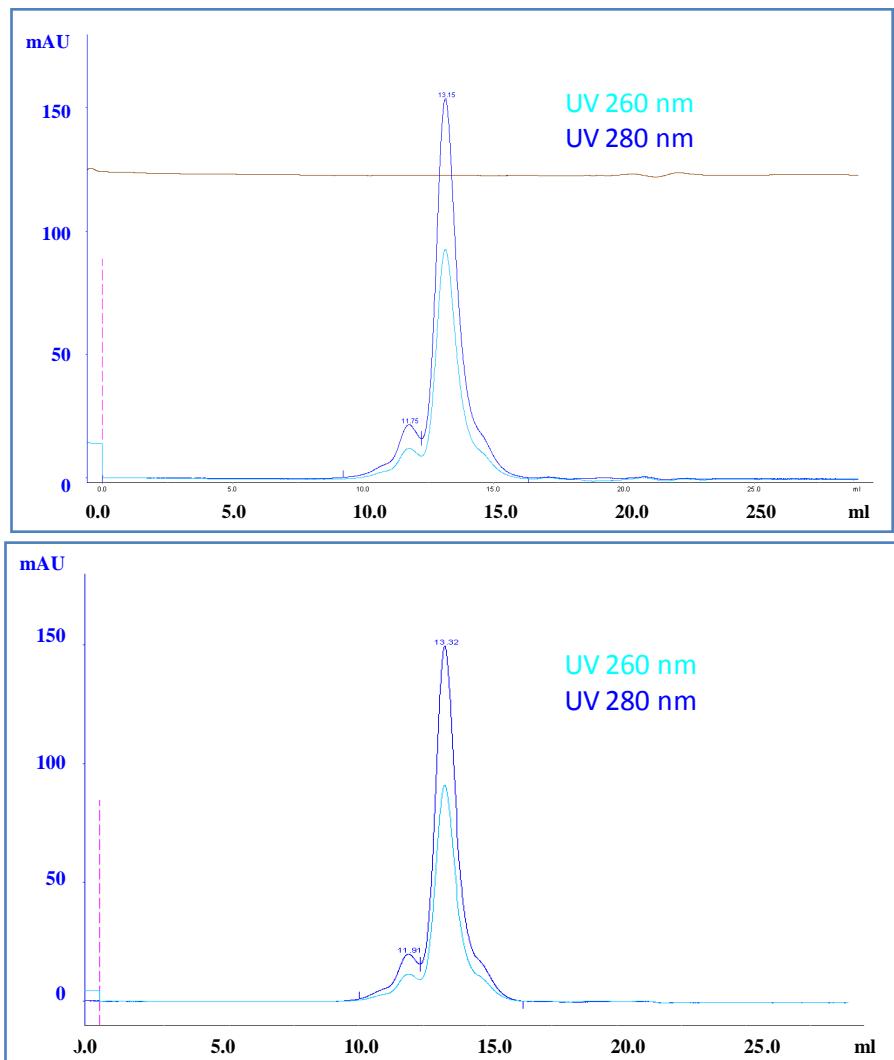


Figure 27: comparison of chromatographic profiles of gp35 before (up) and after (down) a freeze-thaw process.

IMMUNOCHEMICAL EVALUATION OF gp35

gp35 was tested for its immunoreactivity: the recombinant glycoprotein was coated on tosyl-activated paramagnetic microparticles (Dynal) at a concentration of 12.5 μ g/ml. The first evaluation of this antigen was via an indirect assay: specific anti-gp35 antibodies in positive serum bound to antigen and were recognized by anti-human IgG conjugated to ABEI. Positive serum was recognized even at 1:6400 dilution, and a good separation between positive and negative sample is shown (figure 28). Furthermore, we tested various antigen lots, which showed comparable results, both on ELISA and in Liaison® platform: the process has, thus, a good reproducibility.

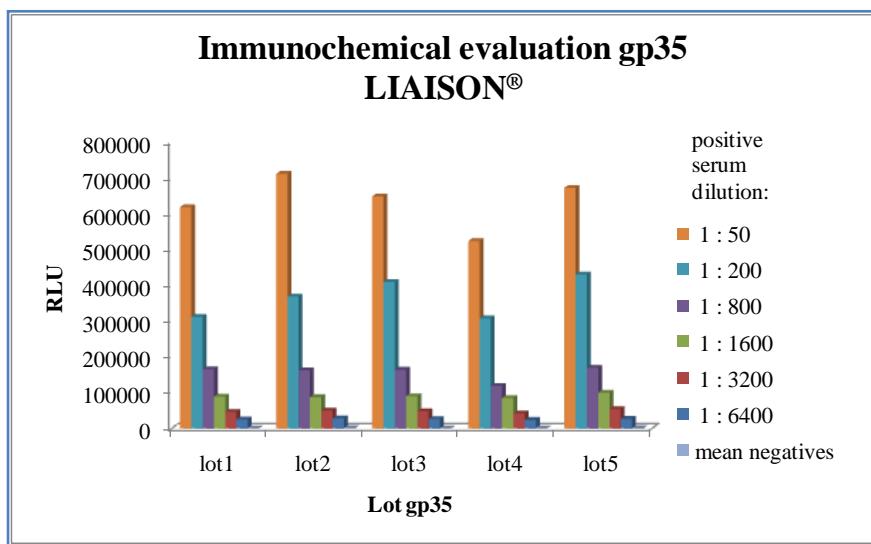


Figure 28: Immunochemical evaluation of gp41-M on indirect Liaison® assay. RLU=Relative Light Units

TRACER ANTIGENS: gp41-O

Sandwich immunoassays need the same protein both on solid phase and as tracer, because the specific antibody should make a bridge binding both proteins. Furthermore, Liaison® system needs soluble tracers for detection and signal generation.

For these reasons, the tracer must have the same immunogenicity of solid phase, but enhancing the solubility is necessary.

The simplest way to enhance the solubility of a protein is the fusion with soluble partners, such as thioredoxin (Trx), maltose binding protein (MBP) or NusA.

Thus, first of all, we fused gp41-O solid phase with these partners, in order to enhance solubility without affecting its immunoreactivity.

The only fusion partner that achieves a good result in enhancing solubility is MBP, but protein remained unstable, and after few hours precipitated.

We therefore searched for other fusion partners, and identified a folding helper, FH1: it is an endogenous prolyl-isomerase of *E. coli* (figure 29).



Figure 29: structure of FH1. Fused at N-terminus of gp41-O, it enhanced solubility and stability of the entire protein.

Results

It was fused to N-terminus of gp41-O with a flexible linker, which allowed the interaction between FH1 and gp41-O, without affecting structure and immunoreactivity. Synthetic gene FH1_gp41-O was subcloned into pET24 vector, between EagI and XhoI restriction sites.

FH1_gp41-O was also fused with two His-tag, C- and N-terminal, in order to facilitate the purification. We performed a plasmid stability test on *E.coli* BL21(DE3) cells carrying pET24-FH1_gp41-O (table 8), plating cells on LB medium with or without antibiotic and IPTG. Since criteria suggested by Novagen (95) are respected, we considered the plasmid pET24- FH1_gp41-O as stable (table 8)

Growth medium	#CFU (mean of three replicates)	Dilution factor
LB	710	10^{-6}
LB+KAN 30µg/ml	775	10^{-6}
LB+IPTG 1mM	4	10^{-4}
LB+KAN 30µg/ml +IPTG 1mM	0	10^{-3}

Table 8: plasmid stability test of pET24-FH1_gp41-O.

Various lots of fermentation were performed in standard conditions (LB with antibiotic, 37°C, IPTG 1 mM when cellular density reaches $OD_{600nm}=0.6$) demonstrating that were reproducible (table 9):

lot	ON pre-inoculum (OD_{600})	0h (OD_{600})	1h (OD_{600})	2h (OD_{600})	3h (OD_{600})	Volume (ml)	Cell pellet (g)
NMS82	3.6	0.6	1.1	1.6	1.8	1.5	4.0
NMS104	2.6	0.6	1.3	1.7	1.7	1.5	5.4
NMS115	2.2	0.7	1.1	1.3	1.7	3.0	7.0
NMS132	3.3	0.6	1.0	1.1	1.2	2.0	4.2
NMS145	3.4	0.7	1.2	1.7	2.0	1.5	4.7

Table 9: comparison of various fermentation lots of BL21(DE3)pET24-FH1_gp41-O. Growth and yield were reproducible.

Results

FH1_gp41-O was first purified on IMAC (Immobilized Metal Affinity Chromatography) Ni²⁺ functionalized. (figure 30).

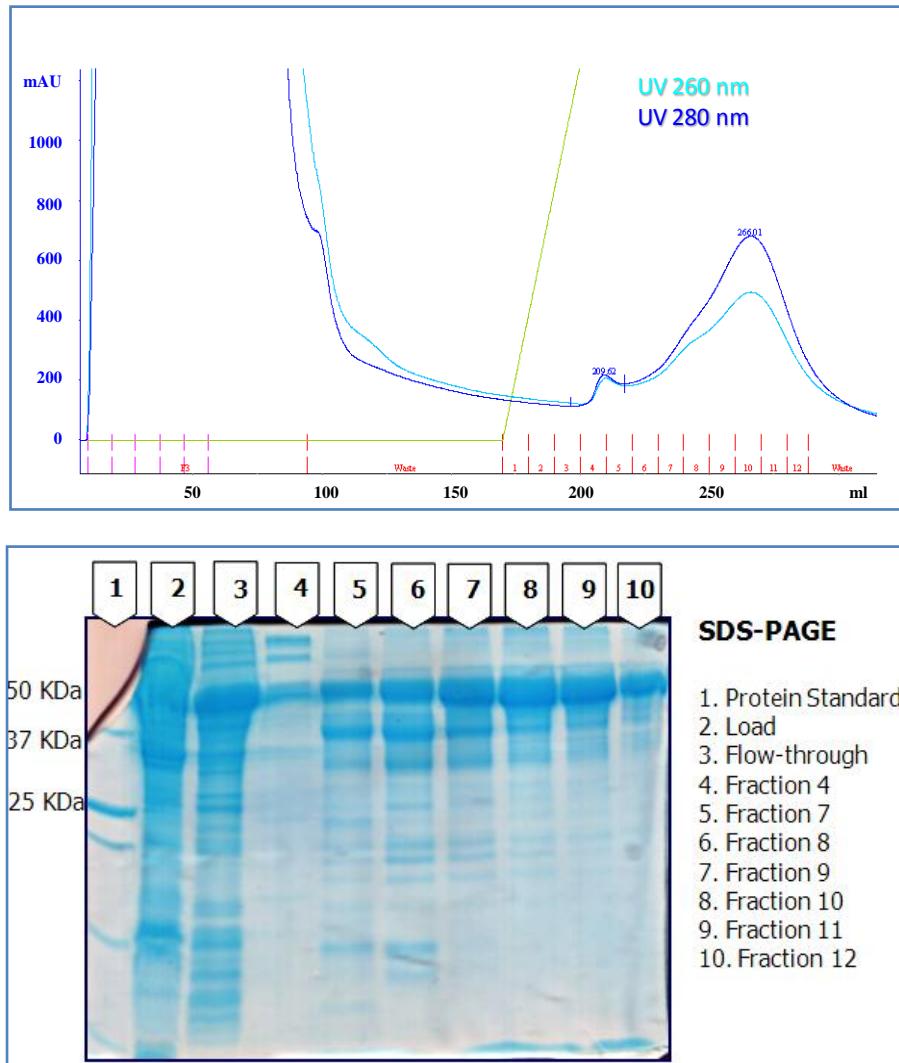


Figure 30: IMAC profile and SDS PAGE of selected fractions.

Selected fractions were loaded on gel filtration to further purify the different proteic forms. Chromatographic profile showed three different peaks (figure 31). Peak 1 (Retention volume = 48.83 ml) and peak 2 (retention volume = 58.01ml) were compared with a standard mixture of proteins (Biorad): molecular weights, thus, were calculated. Peak 2 is probably the hexameric

Results

form of gp41-O, while peak one was an aggregate, and the third peak contained contaminants and was discarded.

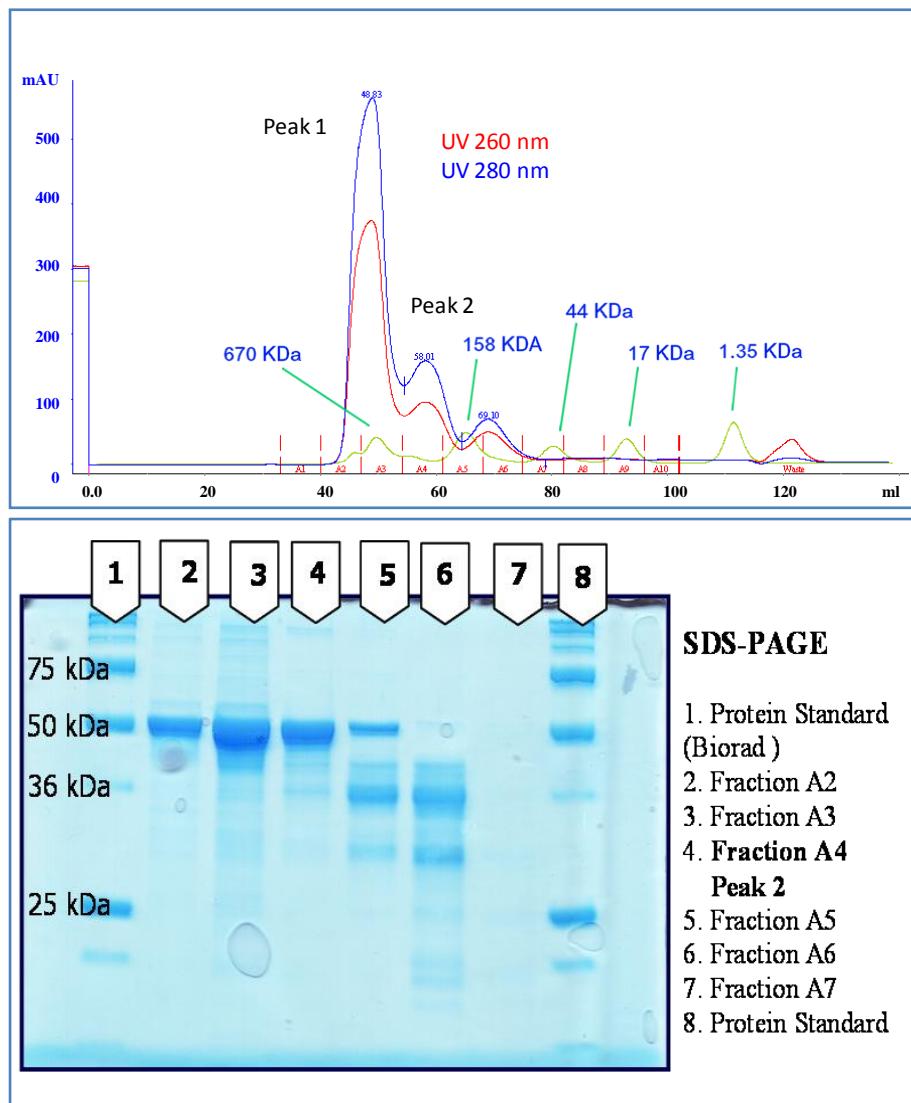


Figure 31: chromatographic profile and SDS page of gel filtration of FH1_gp41-O

Peak 1 and peak 2 were then conjugated to ABEI and the immunoreactivity was assessed (figure 32). Graphic in figure 32 shows reactivities of both peaks with different dilutions of a HIV-1O positive serum: both peak 1 and 2

Results

had a good reactivity and a great separation between positive and negative samples. We selected peak 2 both for its better reactivity and for its more reproducible molecular profile.

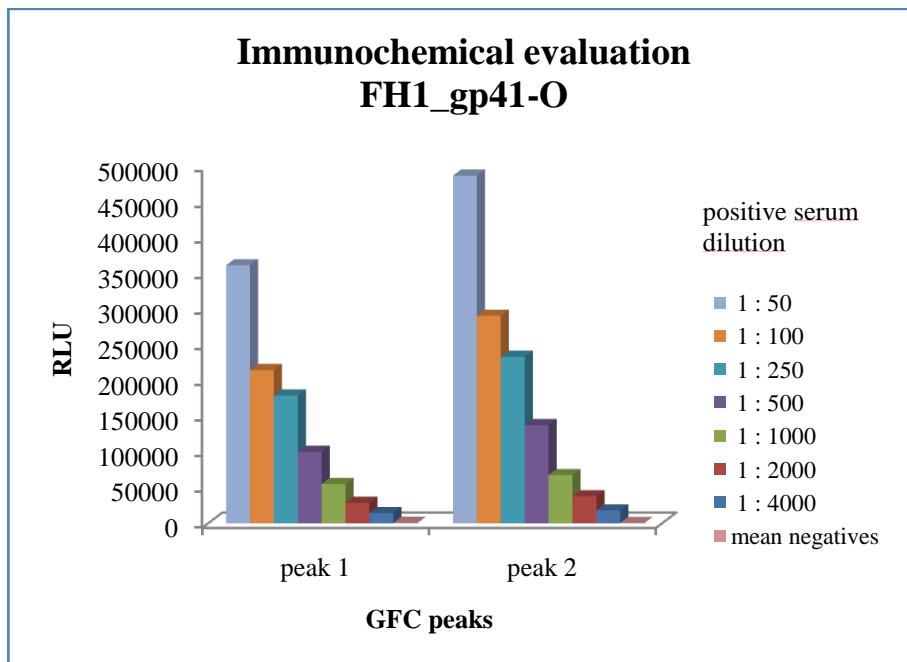


Figure 32: immunochemical evaluation of the two peaks from gel filtration of FH1_gp41-O. Both peaks recognized with high affinity different dilutions of a positive serum. This test was made on Liaison®, using gp41-O coated on paramagnetic beads as solid phase.

An immunochemical evaluation of peak 2 was also performed, coating the three solid phase antigens (gp35, gp41-M and gp41-O) on tosyl-activated paramagnetic microparticles, and detecting specific antibodies in sera HIV-1M, HIV-1O or HIV-2 positive (figure 33).

Results showed that gp41-O was specifically recognized by anti-HIV-1O antibodies in positive serum, even at a 1:12800 dilution. It was not recognized by anti-HIV-2 antibodies, and only a low cross-reactivity was seen with anti-HIV-1M antibodies, probably due to the similarity of sequences between gp41-M and gp41-O (figure 33).

Results

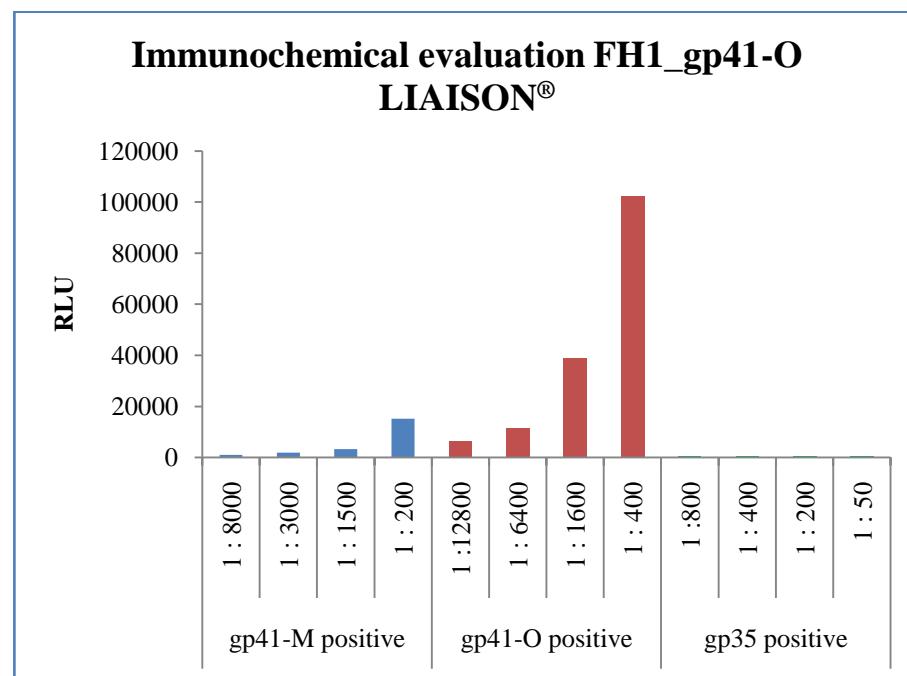


Figure 33: immunochemical evaluation of FH1_gp41-O. Test with cross-reactive sera demonstrates the specificity of gp41-O and FH1_gp41-O.

TRACER ANTIGENS: gp41-M

As for gp41-O, a soluble form of gp41-M is needed for the signal generation in Liaison® system. Again, conventional fusion partners did not succeed in enhancing solubility: we therefore followed the same procedure as for gp41-O. An endogenous prolyl-isomerase from *E.coli*, FH2, was fused to N-terminus of gp41-M with a flexible linker: we used two FH in tandem, in order to achieve the maximal flexibility and solubility. Synthetic gene FH2_gp41-M was subcloned into pET24 vector, between NdeI and XhoI restriction sites.

FH2_gp41-M was also fused with a C-terminal His-tag, in order to facilitate the purification. We made a plasmid stability test on *E.coli* BL21(DE3) cells carrying pET24-FH2_gp41-M (table 10), plating cells on LB medium with or without antibiotic and IPTG. Since criteria suggested by Novagen (95) are respected, we considered the plasmid pET24-FH2_gp41-M as stable (table 10).

Growth medium	#CFU (mean of three replicates)	Dilution factor
LB	27	10^{-6}
LB+KAN 30µg/ml	29	10^{-6}
LB+IPTG 1mM	13	10^{-4}
LB+KAN 30µg/ml +IPTG 1mM	1	10^{-3}

Table 10: plasmid stability test of pET24-FH2_gp41-M.

Various lots of fermentation were performed in standard conditions (LB with antibiotic, 37°C, IPTG 1 mM when cellular density reaches OD_{600nm}=0.6) demonstrating that were reproducible (table 11).

Results

lot	O.N. Pre- inoculum (OD ₆₀₀)	0h (OD ₆₀₀)	1h (OD ₆₀₀)	2h (OD ₆₀₀)	3h (OD ₆₀₀)	volume (l)	Cell pellet (g)
NMS06	3,5	0,56	1,1	1,43	1,65	500	1,55
NMS51	4,0	0,68	1,3	1,7	1,8	2000	6,2
NMS116	3,0	0,8	1,3	1,5	1,8	1500	4,5
NMS141	3,7	0,65	1,7	2	2,1	1500	6
NMS169	3,7	0,6	1,2	1,4	1,6	1500	3,9
NMS174	2,6	0,57	1,2	1,4	1,6	1000	2,5
NMS176	3,3	0,6	1,3	1,6	1,8	1000	4,1
NMS177	3,3	0,63	1,3	1,5	1,7	1000	4

Table 11: comparison of various fermentation lots of BL21(DE3)pET24-FH2_gp41-M. Growth and yield are reproducible.

Solubility tests showed that FH2_gp41-M is soluble in 50 mM Tris, 5 mM β-mercaptoethanol, 100 mM NaCl, 1 M Urea, pH 8.0.

The purification protocol with a strong reducing IMAC and refolding on gel filtration column in a phosphate buffer allowed the elution of an aggregate of about 600kDa, and preliminary immunochemical evaluation showed a low reactivity.

In our experience, the correct quaternary multimeric form of HIV glycoprotein is needed for the reactivity, and depends on the correct folding during purification. Therefore, in the case of FH2_gp41-M, we changed the purification protocol, gradually replacing 7M guanidinium with a non denaturating/non reducing buffer during IMAC, so allowing an on column refolding (figure 34).

Results

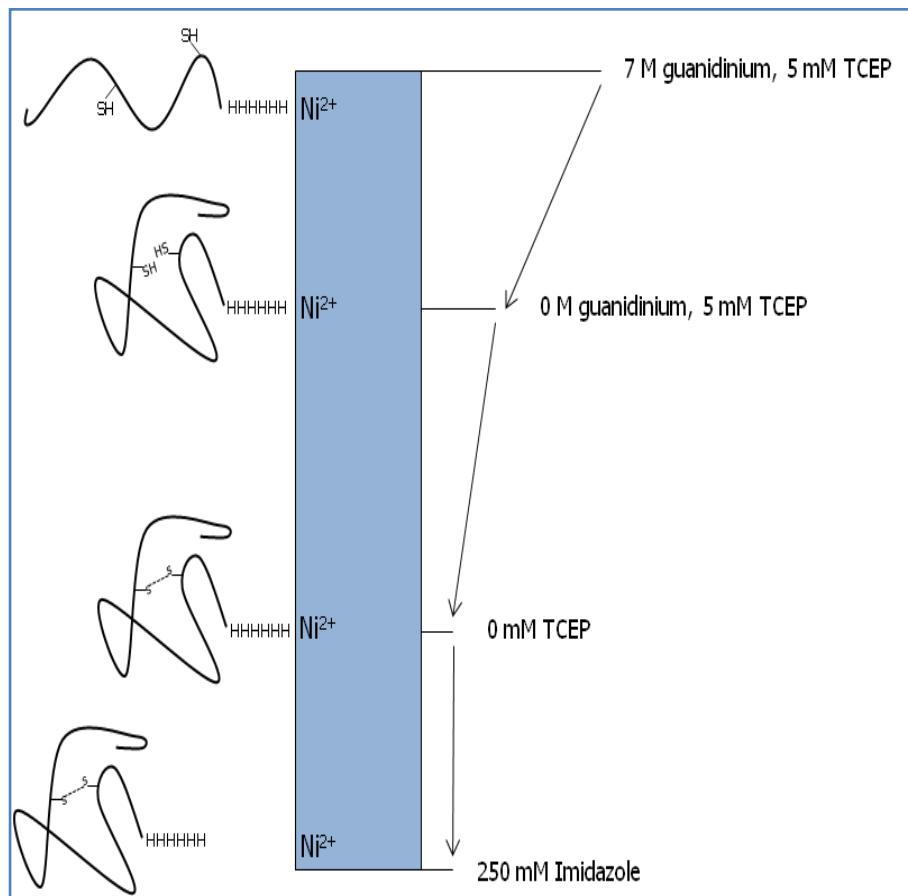


Figure 34: on column refolding. During an overnight flow, FH2_gp41-M folded correctly.

IMAC purification is made of four steps at a controlled temperature (4°C):

- ✓ Step 1: capture of denatured protein on IMAC resin and washing steps (figure 35).

Results

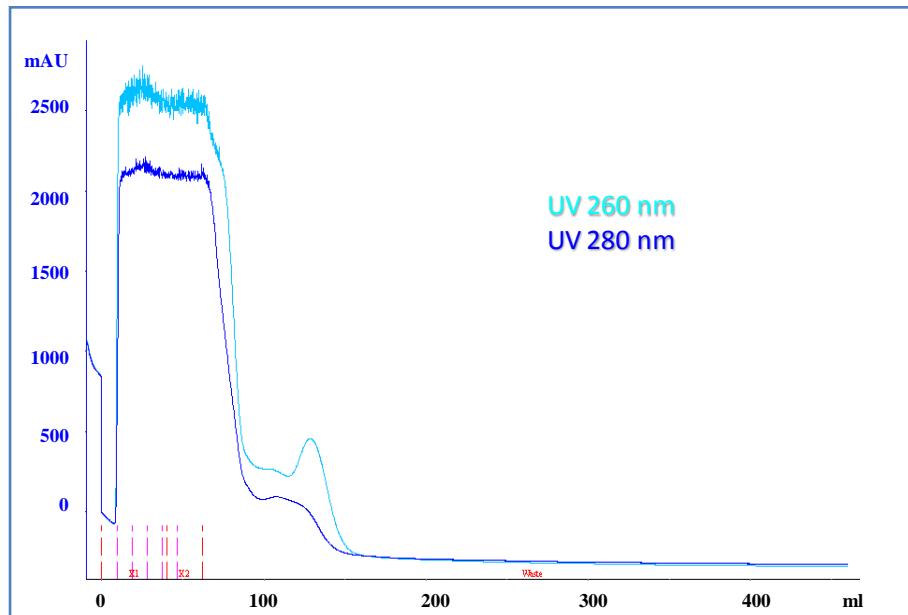


Figure 35: IMAC chromatographic profile of FH2_gp41-M during loading and washing steps.

- ✓ Step 2. Protein refolding of immobilized protein on IMAC resin. During this step there is a decrease in denaturing buffer. This decreasing gradient promoted the correct folding of gp41-M, in an immunologically active form. TCEP maintained the reduced state of disulfide bridges
- ✓ Step 3. Disulfide bridges formation. During this step, the environment of the protein is modified from reducing to non reducing, and disulfide bridges are thus promoted.
- ✓ Step 4: elution of refolded protein from IMAC. Renatured protein was eluted in one step, with imidazole (figure 36).

Fractions eluted from IMAC were loaded on GFC column for further purification and characterization (figure 37). Chromatographic profile showed three isolated forms of FH2_gp41-M (figure 37). SDS PAGE analysis confirmed that all the three peaks contained only FH2_gp41-M.

Results

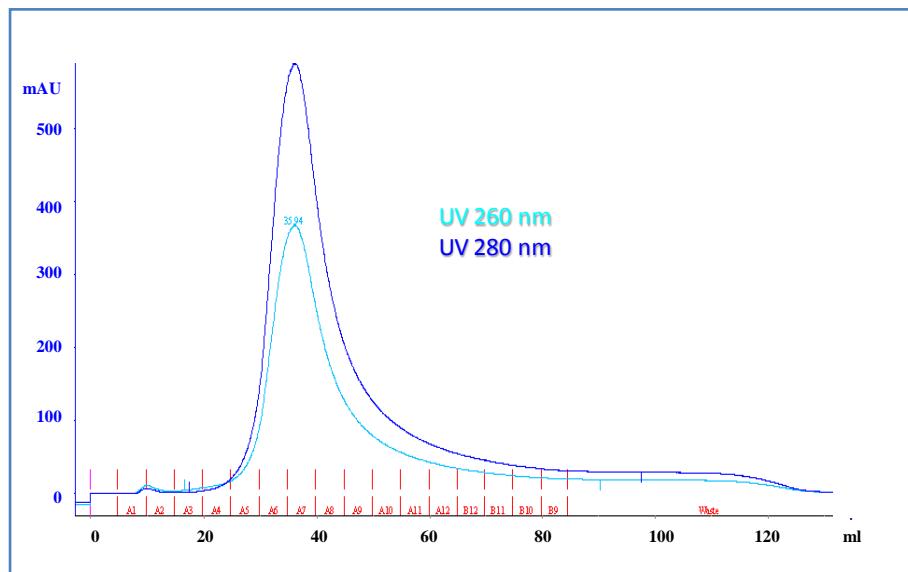


Figure 36: chromatographic profile of FH2_gp41-M after IMAC purification

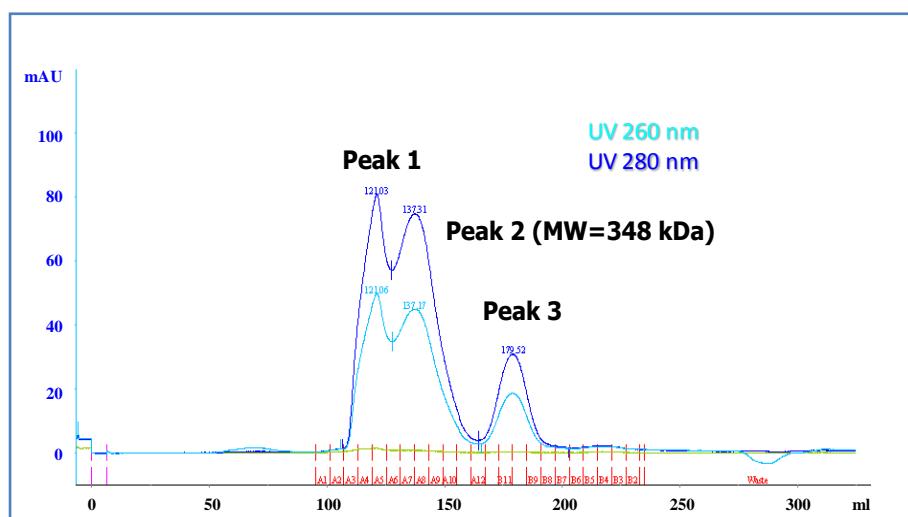


Figure 37: chromatographic profile of FH2_gp41-M after GFC purification

Conjugation with ABEI was performed on all the three peaks, but peak 2 was the most reactive and stable.

Results

Retention time of proteic form corresponding to peak 2 was compared with retention times of a standard mixture of proteins (Biorad), and molecular weight was calculated. Peak 2 has a molecular mass of 348kDa, consistent with a hexameric form of gp41-M.

Peak 2, conjugated to ABEI, was evaluated for its reactivity on Liaison® platform (figure 38). Graphic below shows that FH2_gp41-M successfully recognized positive sera even at 1:8000 dilution. A good reproducibility between various lots is also shown.

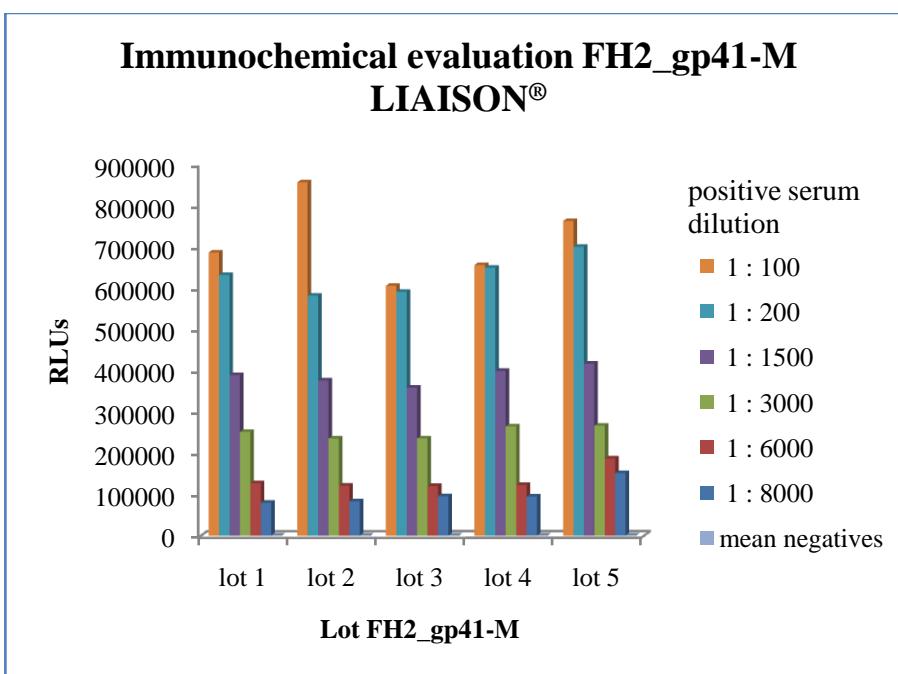


Figure 38: immunochemical evaluation of FH2_gp41-M.

TRACER ANTIGENS: gp35

As for gp41-O and gp41-M, a soluble form of gp35 is needed for the signal generation in Liaison® system. Again, conventional fusion partners did not succeed in enhancing solubility: we therefore followed the same procedure as for gp41-O and gp41-M. As for gp41-M, FH2 was fused to N-terminus of gp35 with a flexible linker: also in this case, we used two FH in tandem, in order to achieve the maximal flexibility and solubility. Synthetic gene FH2_gp35 was subcloned into pET24 vector, between NdeI and XhoI restriction sites.

FH2_gp35 was also fused with a C-terminal His-tag, in order to facilitate the purification. We made a plasmid stability test on *E.coli* BL21(DE3) cells carrying pET24-FH2_gp35 (table 12), plating cells on LB medium with or without antibiotic and IPTG. Since criteria suggested by Novagen (95) are respected, we considered the plasmid pET24-FH2_gp35 (table 12).

Growth medium	#CFU (mean of three replicates)	Dilution factor
LB	87	10^{-6}
LB+KAN 30µg/ml	79	10^{-6}
LB+IPTG 1mM	0	10^{-6}
LB+KAN 30µg/ml +IPTG 1mM	0	10^{-3}

Table 12: plasmid stability test of pET24-FH2_gp35.

Various lots of fermentation were performed in standard conditions (LB with antibiotic, 37°C, IPTG 1 mM when cellular density reaches OD_{600nm}=0.6), demonstrating that were reproducible (table 13).

Results

Lot	O.N. Pre- inoculum (OD ₆₀₀)	0h (OD ₆₀₀)	1h (OD ₆₀₀)	2h (OD ₆₀₀)	3h (OD ₆₀₀)	volume (l)	Cell pellet (g)
NMS04	3.5	0.6	1.2	1.5	1.5	0.5	1.9
NMS13	4.3	0.6	0.9	1.3	1.5	1.5	4.5
NMS29	4.1	0.7	-	-	1.7	2.0	7.2
NMS119	2.9	0.6	1.2	1.3	1.6	1.0	3.0
NMS130	2.8	0.6	0.9	1.1	1.4	2.0	6.0

Table 13: comparison of various fermentation lots of BL21(DE3)pET24-FH2_gp35. Growth and yield were reproducible.

Purification protocol, similarly to FH1_gp41-O, started with an IMAC: Histidine-tag at N-terminus of FH2_gp35 was immobilized on a Nickel activated affinity column, and a relatively pure protein was eluted with imidazole after few washing steps. Chromatographic profile from IMAC (figure 39) showed a single peak, which was characterized by the presence of gp35 with a relatively high purity, as demonstrated by a SDS PAGE analysis.

Results

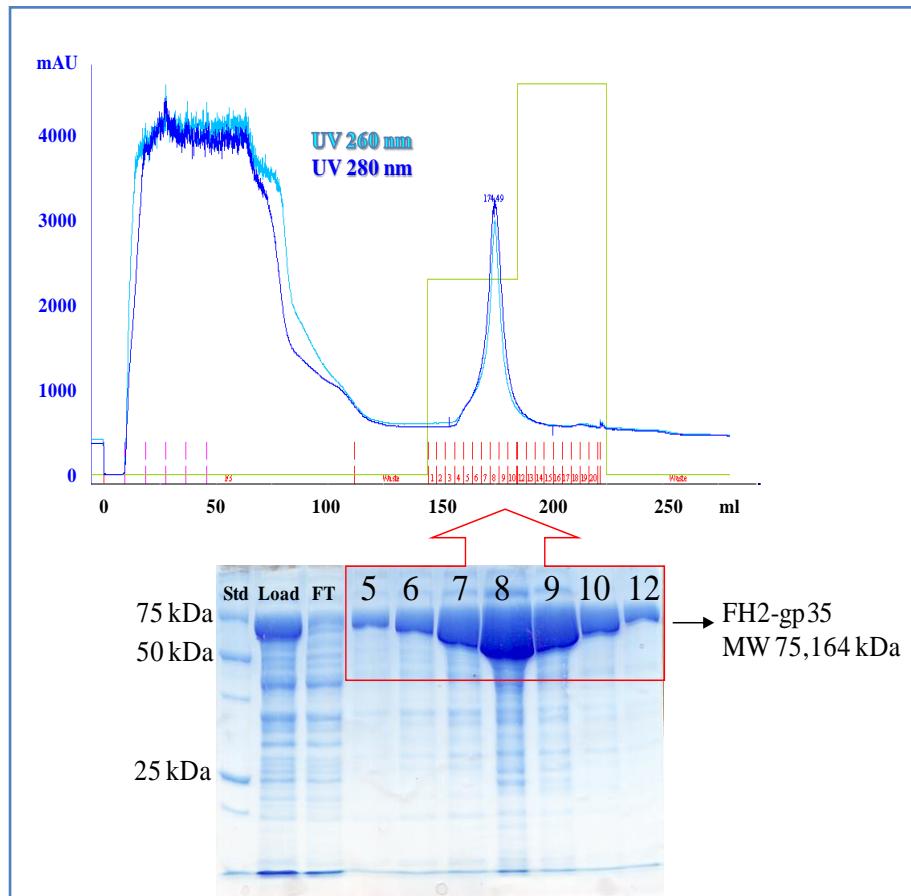


Figure 39: IMAC profile and SDS PAGE analysis on selected fractions. Purification on affinity column resulted in a pure and concentrated protein. SDS analysis confirmed that the single peak observed on IMAC was constituted by FH2_gp35. Numbers on top of gel lanes refer to the number of the fraction eluted by IMAC. Std: standard molecular weights; Load: total sample loaded on IMAC; FT: Flow through, impurities which did not bind to the IMAC column.

Selected fractions from IMAC were pooled together and loaded on a gel filtration column, to further purify the protein (figure 40). Chromatographic profile identified three isolated different peaks. The three forms were analyzed on SDS PAGE (figure 40), demonstrating that there were three different aggregation states of the same protein.

Results

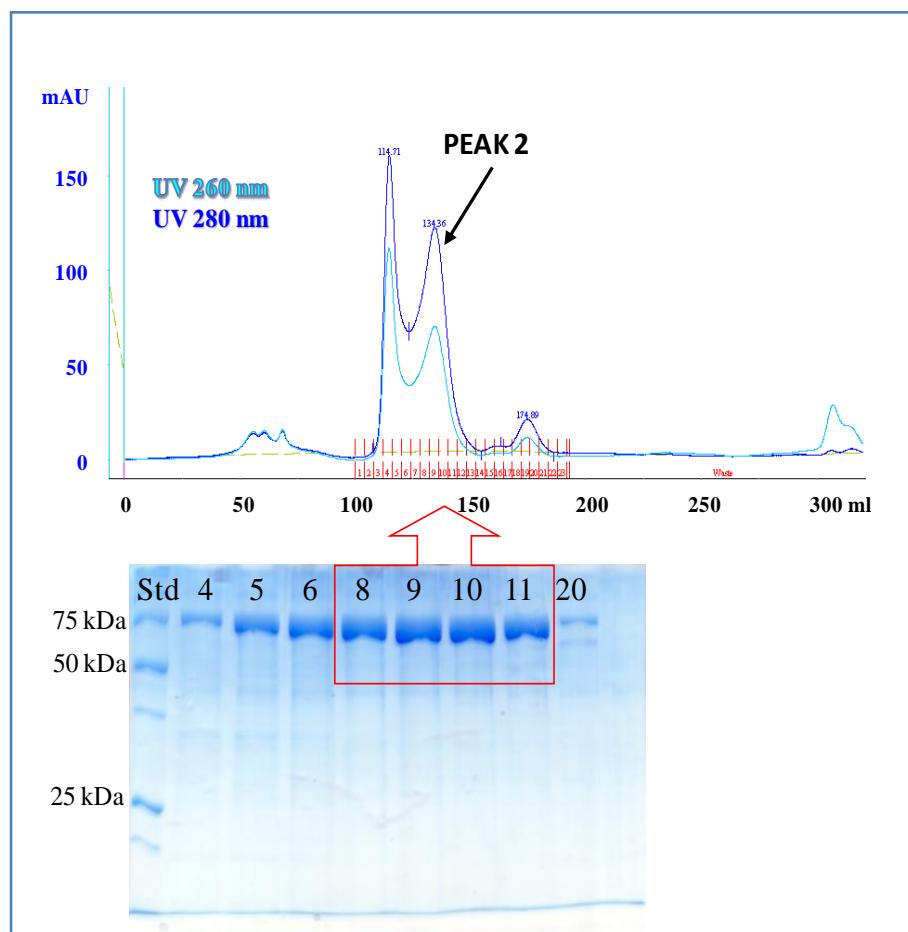


Figure 40: GFC profile and SDS PAGE analysis on selected fractions. Purification on gel filtration column resulted in a pure and concentrated protein, in three different aggregation states. SDS analysis confirmed that the three peaks observed on GFC are constituted by FH2_gp35. Numbers on top of gel lanes refer to the number of the fraction eluted by GFC: 4, 5 and 6 are fractions from peak 1; 8, 9, 10 and 11 from peak 2; and 20 from peak 3. Std: standard molecular weights.

Conjugation with ABEI was performed on all the three peaks, but peak 2 was the most reactive and stable.

Retention time of proteic form corresponding to peak 2 was compared with retention times of a standard mixture of proteins (Biorad), and molecular weight was calculated. Peak 2 has a molecular mass of 450kDa, consistent with a hexameric form of gp35.

Results

Peak 2, conjugated to ABEI was evaluated for its reactivity on Liaison® platform (figure 41). Graphic below shows that FH2_35 successfully recognized positive sera even at 1:6400 dilution. A good reproducibility between various lots is also shown.

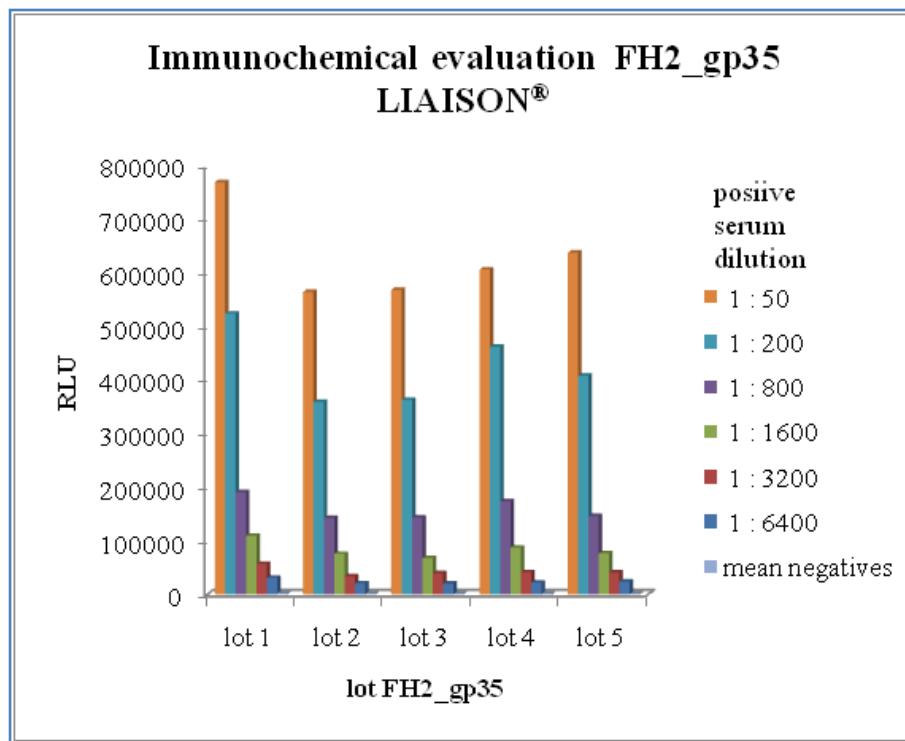


Figure 41: immunochemical evaluation of FH2_gp35.

Results

**Phase 2:
Detection of p24 capsid antigen**

ANTIGEN p24

The capsid protein p24 is one of the main structural proteins of human immunodeficiency virus. There are about 1500 p24 molecules composing virus capsid in a mature virion, therefore p24 is the most abundant protein produced during virus replication and can be detected in the very early stage of asymptomatic phase after HIV infection. In the late stage of AIDS, p24 can be detected again because of rapid replication of viruses and decline of HIV specific antibody production. Hence p24 detection provides a means to aid the early diagnosis of HIV infection, track the progression of disease and assess the efficacy of antiretroviral therapy. Additionally, p24 is one of the most conserved viral proteins and shows very little antigenic variations among the strains of HIV-1 isolated.

A recombinant p24-M was produced, as a positive standard.

Synthetic gene encoding for p24-M was subcloned into pET24 vector, via NdeI-XhoI restriction sites, generating a C-term 6xHis-tagged protein, in order to facilitate the purification procedure.

Growth medium	#CFU (mean of three replicates)	Dilution factor
LB	120	10^{-6}
LB+KAN 30µg/ml	132	10^{-6}
LB+IPTG 1mM	15	10^{-4}
LB+KAN 30µg/ml +IPTG 1mM	1	10^{-3}

Table 14: plasmid stability assay of pET24-p24M

Various lots of fermentation were performed in standard conditions (LB with antibiotic, 37°C, IPTG 1 mM when cellular density reaches OD_{600nm}=0.6) demonstrating that were reproducible (table 15).

Results

lot	ON pre-inoculum (OD ₆₀₀)	0h (OD ₆₀₀)	1h (OD ₆₀₀)	2h (OD ₆₀₀)	3h (OD ₆₀₀)	Volume (ml)	Cell pellet (g)
NMS37	3.9	0.55	1	1.2	1.35	500	3.82
NMS38	3.9	0.55	1	1.3	1.38	500	4.02
NMS42	3.1	0.55	0.89	1.1	1.2	2000	14.73
NMS72	3.3	0.6	1.4	1.2	1.6	1500	7.9
NMS98	3.45	0.7	1.29	1.42	1.619	1500	5.55
NMS233	3.7	0.60	1.2	1.3	1.6	1000	3.8

Table 15: comparison of various fermentation lots of BL21(DE3)pET24-p24M. Growth and yield were reproducible.

p24 was expressed in BL21(DE3) cells and purification process has been developed and optimized. The antigen was abundantly expressed in a soluble form. An IMAC purification was performed on soluble fraction of cellular lysate: p24 was purified with high yield and purity (figure 42). To further clean and characterize the protein, a GFC was also performed (figure 43). Chromatographic profile showed a pure and concentrated protein, probably in a dimeric form.

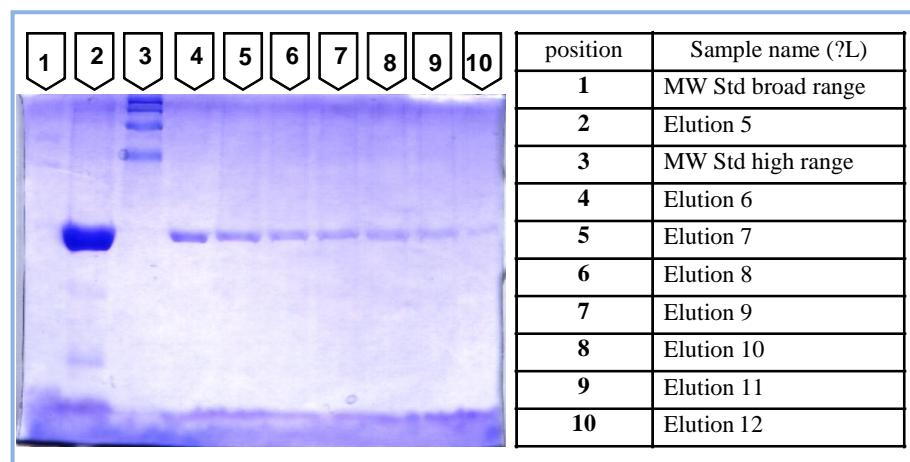


Figure 42: SDS PAGE analysis of the selected fractions eluted from IMAC. p24 was pure and concentrated.

Results

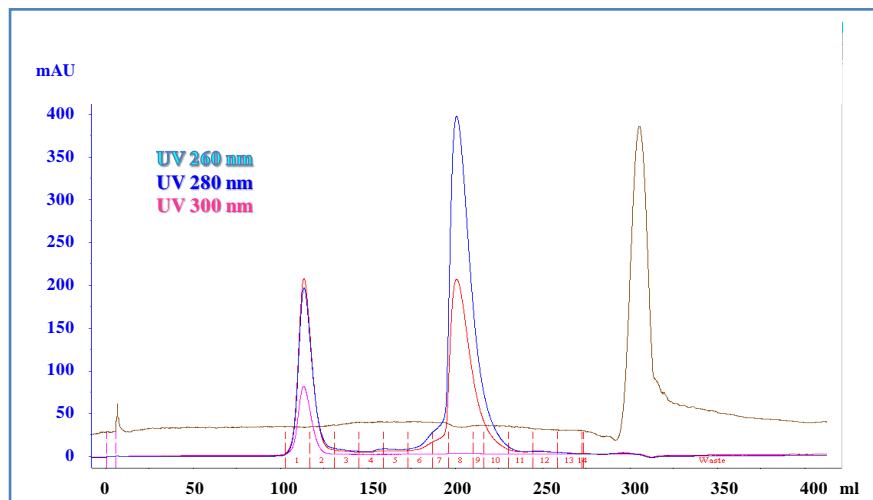


Figure 43: GFC chromatographic profile. p24 was a dimer.

p24 was immobilized on a SensiQ® chip, to evaluate the affinity of anti-p24 monoclonal antibodies (purchased from Innogenetic and Biomaric). SensiQ® analysis (figure 44) showed a high affinity of antibodies for recombinant p24: binding between antigen and commercial antibodies was stable.

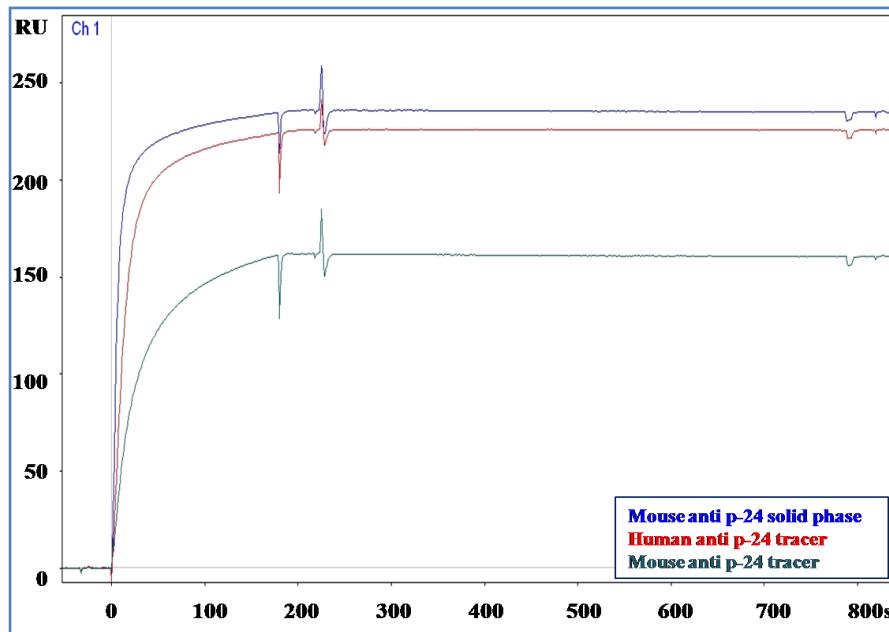


Figure 44: binding analysis of monoclonal antibodies and recombinant p24.

Results

Liaison® system is designed for a sandwich immunoassay: thus, it is necessary that antibodies on solid phase and tracer antibodies recognize different epitopes on p24.

For this purpose, a binding analysis was performed in a sandwich format (figure 45). Solid phase commercial antibodies were immobilized on SensiQ® chip. Recombinant p24 was then added, and a fast binding was observed. No dissociation is detectable, even for a long time. The tracers anti-p24 are then added, and, again, a fast binding is observed: this analysis demonstrated, thus, that a sandwich occurred between solid phase antibodies, p24, and tracers. Dissociation of p24 or tracer antibodies was not detectable, demonstrating the stability of the binding.

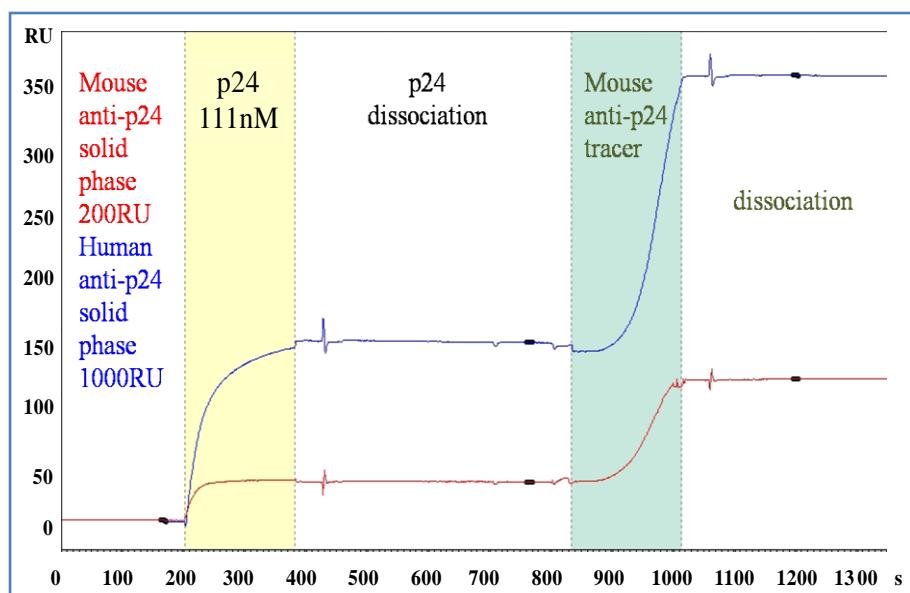


Figure 45: SensiQ® analysis on solid phase and tracers antibodies.

Reactivity of anti-p24 monoclonal antibodies was also tested on different standards, compared with recombinant p24 on Liaison® platform (figure 46). A comparison was performed between:

- ✓ World Health Organization standard (WHO STD), at different dilutions in negative serum
- ✓ Biorad standard p24, at different concentrations
- ✓ Recombinant p24, at different concentrations
- ✓ Viral lysate, at different dilution in negative serum.

Results

Streptavidinated microparticles were used as solid phase for the detection of HIV antigen p24. Monoclonal antibodies conjugated with biotin (Biomaric and Innogenetics), and tracer antibodies conjugated to ABEI built the immunocomplex with p24 in patients' sera and were captured by the beads. Figure 46 shows that the assay had a good reactivity even at low antigen concentration.

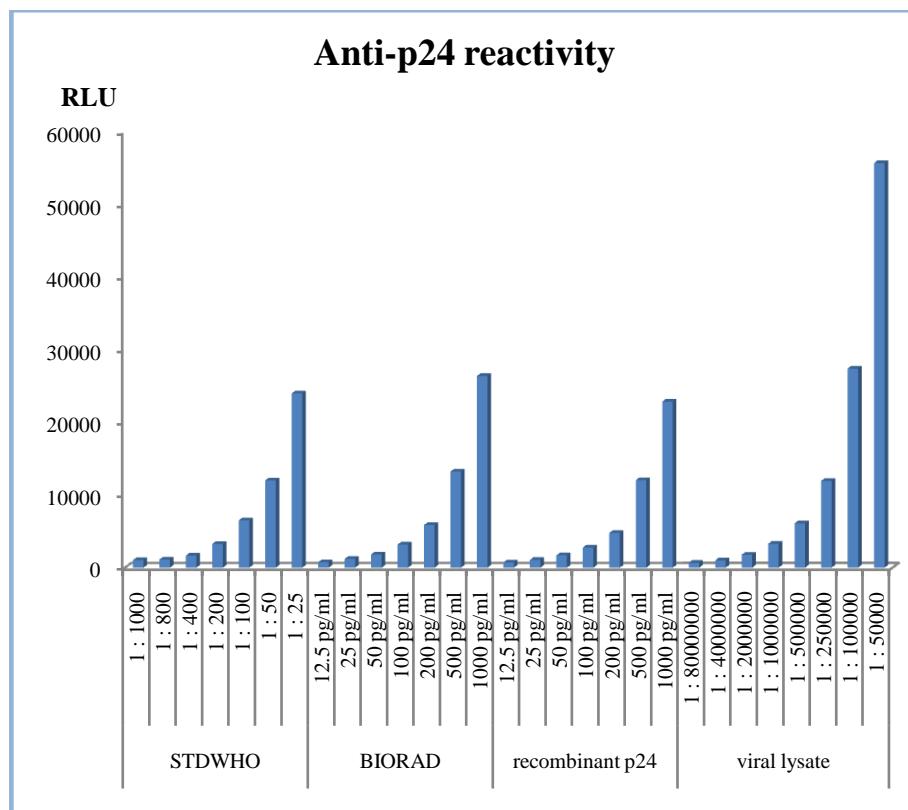


Figure 46: Liaison® assay on monoclonal antibodies anti-p24.

EVALUATION OF SENSITIVITY AND SPECIFICITY OF THE FINAL ASSEMBLATE

Lot of immunochemical evaluation was performed on the final assemblate, in order to assess specificity and sensitivity. On 1393 negative samples (1011 Sera from Novara hospital and 382 TRINA donors) Liaison® had a specificity of 99.43% in the first test and 99.93% in the re-test (table 17).

COMBO	First Test	Retest
Negative samples	1393	1393
Samples Abs > 20000 RLU Samples Ag > 800 RLU	8	1
SPECIFICITY	99.43%	99.93%
Requested specificities	99.4%	99.6%

Table 17: evaluation of sensitivity. 1011 Sera from Novara hospital and 382 TRINA donors were screened on Liaison® platform. Sensitivity is satisfactory.

We also tested the final kit on patients from a hospital in Lyon: among these, 117 were negative, 31 were HIV-1 positive, 4 were HIV-2 positive and 3 were indetermined on western blot. Table 18 shows the results of Liaison® HIV COMBO assay in comparison with Architect and Modular commercial kits. Liaison® detected all the primary infections that result in indeterminate western blot, and all the positive sera both for HIV-1 and HIV-2.

False positive were less than the other commercial kit. No false negative sera were detected.

A key point in the diagnosis of HIV infection is the early detection of seroconversion: thus, we tested the reactivity of our system on a commercial panel of different seroconversions, and compared the performances of Liaison® and Architect Combo (Abbott) (table 19). As shown in table 19, Liaison® system detected all the requested seroconversions like Architect, and, in case of PRB924-4 sample, it had a better sensitivity than the other commercial assays.

Results

Number of sera from Lyon hospital	Description	Recognized by Liaison® Combo HIV	Recognized by Architect	Recognized by Modular
15	indeterminate WB, primary infection	15	15	15
31	HIV-1 positive	31	31	31
4	HIV-2 positive	4	4	4
117	negative	114	100	97

Table 18: comparison of different diagnostic kits. Liaison® Combo HIV has a better sensitivity than other commercial kits on negative samples.

seroconversion type	days from initial bleed to first positive sample infection	Liaison® HIV Combo	Abbott Architect Combo
PRB922-1	0	+	+
PRB922-2	4	+	+
PRB922-3	7	+	+
PRB922-4	11	+	+
PRB924-1	0	-	-
PRB924-2	2	-	-
PRB924-3	8	-	-
PRB924-4	10	+	-
PRB924-5	26	+	+
PRB924-6	33	+	+
PRB924-7	35	+	+
PRB924-8	40	+	+

Table 19: evaluation of seroconversions. Liaison® Combo detected all the seroconversion required by european common technical specifications. The results is comparable with Abbott Architect; as evidenced in yellow, Liaison® detected seroconversion PRB924 at an earlier stage than Architect.

DISCUSSION

Discussion

The fourth-generation HIV combination assay described in this work meets sensitivity, specificity, and precision requirements that permit safe and effective detection of sera from HIV-infected individuals. Individual antibody and antigen detection formats have been combined into a single immunoassay exhibiting an antibody sensitivity equivalent to that of third-generation single-format HIV antibody assays and an antigen sensitivity equal to that of single-format antigen tests. The combined-format assay accelerates detection of HIV infection by 16 days within the seroconversion window period defined by seroconversion PRB924 in comparison with Architect (Abbott). The assay is designed to detect p24 antigen prior to seroconversion, with a seroconversion window that is reduced compared to those of assays that detect only antibodies elicited against HIV. The quantitative antigen sensitivity of the combined immunoassay was estimated at 1.25 IU of p24/ml of serum, a value closely approaching that of single-format antigen detection assays (80). In contrast to the antigen sensitivity of the Liaison® Combo, other combined-format assays are manyfold less sensitive (65 to 250 pg/ml) than the single-antigen detection formats (8 to 15.7 pg/ml). Of course, comparisons of quantitative sensitivity are primarily dependent on the quantitative standard, and for that reason we selected three different standards for our studies (WHO standard, Biorad p24 standard and viral lysate).

Although antigen sensitivity is of particular value because it is the key driver of seroconversion sensitivity (80-85), antibody sensitivity must be maintained in order to detect specimens that may contain barely detectable levels of antibody or very low levels of both antibody and antigen. Sensitivity is the most important performance characteristic of an HIV screening or diagnostic assay, and it should not be compromised for any reason. The reduction in the seroconversion window achieved with the Liaison® Combo was consistent with sensitive (1.25IU/ml) antigen detection. Close inspection of the commercial seroconversion panels (and their associated vendor data) selected for this study revealed that in all selected seroconversion panels, the combined immunoassay was reactive against the earliest positive bleed detected by a single-format antigen (or antibody) assay. The data clearly demonstrate that the combined format detects specimens from HIV-antigenemic seronegative individuals and therefore reduces the risk of transfusion-transmitted HIV infection due to seronegative donations during the window period. Ly (85) and others (80,

Discussion

86-88) have attempted to provide a frame of reference across multiple seroconversion panels and multiple HIV combination assays. Comparisons between combination assays are most meaningful when identical seroconversion panels are evaluated across assays run concurrently. Liaison® Combo is sensitive for p24 antigen from multiple HIV-1 group M subtypes. Remarkably, the assay displayed no difference in antigen sensitivity across group M and group O isolates.

Antibody sensitivity in the combined format was equivalent to or better than that of a related, sensitive, third-generation single-format antibody assay. The combined immunoassay readily detected all specimens from individuals infected with known or unknown HIV-1 group M subtypes , HIV-2 or HIV-1 group O. The antibody sensitivity of the Liaison® Combo is consistent with the goal of maintaining sensitivity as the single most important performance characteristic of an HIV screening or diagnostic assay.

The specificities of the combined format for blood donor and diagnostic populations were nearly equivalent and met or exceeded the specificity characteristics usually associated with third-generation single-format antibody assays. The Liaison® Combo is an appropriate screening method for low-incidence populations (blood donors) because of the low rate of potential deferrals, and it is an appropriate diagnostic assay for higher-risk populations because of the low rate of false-reactive results, coupled with excellent sensitivity and a high positive predictive value. Numerous reports indicate that the specificities of HIV combination assays appear to vary significantly (82, 83, 86, 88). HIV combination immunoassays may suffer from a lack of specificity because each of the two individual formats (antigen detection or antibody detection) may elicit different negative population profiles (means and SD), may require different cutoff values for optimal sensitivity and specificity, and must necessarily utilize more reagents (e.g., antigens and monoclonal antibodies) in the combined format, providing additive contributions to nonspecific binding (89). These factors must be reconciled in the combined format in order to achieve a specificity equivalent to or better than that of third-generation single-format antibody assays. We solved these problems by using two different integrals for the detection of the antigen and the antibodies: in this way, we avoid any problem of cross-reactivity or difference of cut-offs. The assay can easily discriminate between antigen and antibody positive sample, giving an important suggestion also for the therapy.

Discussion

The Liaison[®] Combo is a highly sensitive, specific, and reproducible fourth-generation test that provides a clear sensitivity advantage over third-generation single-format antibody assays. Technical factors that may cause combination assays to be less sensitive or less specific than single-format assays have been overcome, resulting in an assay with a specificity suitable for screening or diagnostic purposes, an antigen sensitivity approaching that of single-format antigen tests, and an antibody sensitivity equivalent to that of a related third-generation HIV antibody assay. It is also user-friendly and fast: time to first result is less than one hour, and it can manage up to 80 tests/hour. The thermal controlled compartments allow an on-board stability for all reagents of more than 4 weeks.

Chapter 2

*A new real-time pcr method for
plasmid copy number detection in
*E.coli**

INTRODUCTION

PLASMID COPY NUMBER (PCN)

Plasmids are frequently used as a vehicle to carry foreign genes into a bacterial host cell. Recombinant gene expression systems using bacterial plasmids are widely used to produce many types of valuable proteins, such as therapeutics and vaccines (72-74).

The production of recombinant proteins in *Escherichia coli* bacteria is affected by the number of plasmids, their structural and segregational stability, which have an essential impact on productivity (75). Maintenance of the segregational stability of plasmids is often a major problem during the fermentation process. Uneven distribution of plasmid-based expression vectors to daughter cells during bacterial cell division results in an increasing proportion of plasmid free cells during growth. This is a major industrial problem leading to reduction of product yields and increased production costs during large-scale cultivation of vector-carrying bacteria. For this reason, a selection must be provided that kills the plasmid free cells. The most conventional method to obtain this desired selection is to insert some gene for antibiotic resistance in the plasmid and then grow the bacteria in the presence of the corresponding antibiotic (76).

The addition of antibiotics into the growth medium is the simplest and most broadly used method to preserve a high number of plasmids in bacterial cells (77). Although this is acceptable on laboratory scale, the broader use of antibiotics on an industrial scale is not desirable due to environmental pollution. Nevertheless, to ensure a high production of recombinant proteins, it is necessary to maintain an optimal plasmid copy number in bacterial cells. This level must be sufficient for the desired gene dosage effect, yet not so high that it induces a metabolic burden and loss of cell resources (78). To better understand and to optimize the recombinant protein production process, the accurate and rapid quantification of plasmid copy numbers is essential.

Plasmids can be described as either low (1–10 copies), medium (11–20 copies), or high-copy number plasmids (as high or greater than 700 copies per cell). Various techniques have been described for determining plasmid copy-number, such as methods based on UV spectrophotometry, radiolabelling of nucleic acids, hybridization of DNA extracts to probes, or measurement of plasmid-encoded activities (76). Most methods, though, are labour-intensive and time consuming. A capillary electrophoresis-based

Introduction

method has been described for rapid determination of plasmid copy number, but this technique requires relatively specialized equipment that is not available in most laboratories. In contrast, thermal cyclers for quantitative real-time PCR (qPCR) analyses of DNA are more common and a qPCR-based approach for determining the number of multi-copy genes has been described. In brief, the threshold cycle value, CT, of the gene of interest is compared to the CT value of a single copy reference gene. The difference in CT values is then used to derive copy number (79). Although q-PCR is one of the most common methods for the quantification of plasmids in a cell, a protocol with a universal calibrator has still not yet described.

In this study we provide a fast and simple method for the detection of plasmid copy number using a universal plasmid as standard reference.

MATERIALS AND METHODS

BACTERIAL STRAINS, PLASMIDS AND CULTIVATION:

E. coli strain, XL1-Blue (Stratagene) and BL21(DE3) (Novagen), were used as bacterial hosts for the plasmids.

The plasmids used in this study were pGA4PCNSTD (2961bp), obtained from Geneart (Regensburg, Germany), and pET24 (5236bp) which was purchased from Novagen. Synthetic plasmid PGA4PCNSTD encodes for both kanamycin resistance and DKS (a single copy gene in *E.coli* genome, encoding for 1-deoxyxylulose-5-phosphate synthase) and carries ampicillin resistance. In this way, a unique plasmid can be used to build three different standard curves, suitable for almost all commercial vectors. A gene encoding for HIV glycoprotein was subcloned into pET24.

These plasmids were transformed into *E.coli* competent cells following Novagen procedure, slightly modified as follows: about 200ng of purified DNA were used to transform XL1-Blue and BL21(DE3) *E.coli* competent cells. After incubation on ice for 30 minutes, bacteria were heat-shocked at 42.0°C for 30 seconds and returned on ice for 5 minutes. SOC medium (Novagen) was added for cellular recovery, and competent cells were incubated at 37°C, 80rpm, for 60 minutes. *E.coli* cells were plated on LB agar with kanamycin (30ug/ml).

E. coli host cells were inoculated from freshly streaked overnight LB agar plates into 10 ml LB media and allowed to grow in an incubator shaker (180rpm) at 37°C overnight (approximately 15 h) to the stationary phase.

BACTERIAL DNA ISOLATION AND PURIFICATION

Genomic or total DNA were isolated from 1 ml cell pellets spun down at 13,200 rpm for 1 min.

In this study we compared two different methods for total DNA purification: the first method has been reported in previous publication (Pushnova et al., 2000) and was adopted with the following modifications. First, to ensure complete cell lysis, mechanical destruction of cell walls was performed by mixing with a syringe with 0.7 mm needle, after addition of 10% SDS and Proteinase K, followed by incubation at 50 °C. Second, in order to further purify the DNA obtained, one volume of 100% isopropanol was added to precipitate the DNA after chloroform extraction and then washed with one

Materials and methods

volume of 70% ethanol before rehydration in one volume double deionized water (ddH₂O) at 65°C for 1 h.

With the second method, total DNA was isolated with Qiagen QIAamp DNA Mini Kit, following manufacturer's instructions.

Plasmid DNA for standard curves was extracted from the host cells according to the Promega Wizard® Plus Minipreps DNA Purification System.

PRIMERS

Primer sets for DXS, Kanamycin resistance and Ampicillin resistance were generated using Visual OMPTM:

PRIMER NAME	SEQUENCE	amplification length	Tm
DXS FW	CGAGAAACTGGCGATCCTTA	113bp	63.9°C
DXS REV	GCTTCATCAAGCGGTTTCACAAA		64.0°C
KAN FW	TAATGGCTGGCCTGTTGAAC	86bp	65.1°C
KAN REV	AATCACCATGAGTGACGACTGA		64.0°C
AMP FW	CTACGATAACGGGAGGGCTTA	96bp	64.3°C
AMP REV	CTGGTTATTGCTGATAATCTGGAG		64.3°C

QUANTITATIVE REAL-TIME PCR ASSAY

The ABI PRISM® 7000 instrument (Applied Biosystems) was used for QPCR amplification and detection. QPCR was prepared in triplicates or quintuplicates of 50µl reaction mixture in MicroAmp optical 96-well reaction plates and sealed with optical caps (Applied Biosystems). Each reaction well contains 20µl of template DNA (normalized at 2.5pg/µl), 25µl of 2XSYBR® Green PCR Master Mix (Biorad), and 400nM each of forward and reverse primers. Concentrations of plasmid and genomic DNA standards were measured by absorbance at 260 nm.

The ratio of absorbance at 260 and 280nm, (OD260/OD280), was routinely found to be between 1.8 and 2.0, indicating minimum protein contamination. Serial ten-fold dilutions of standard plasmid were conducted in triplicates to establish the standard curves. The standard curve is a plot of the threshold

Materials and methods

cycle (Ct) versus log concentration (Co). For any unknown total DNA sample, by interpolating its Ct value against the standard curve, the absolute quantity of both plasmid and genomic DNA would be obtained. PCN was obtained from the ratio plasmidic DNA: genomic DNA. Negative control was included in the experimental runs: the negative control was set up by substituting the template with ddH₂O and that routinely had a high Ct value which represented the lower detection limit.

Real-time QPCR was conducted with the following cycling conditions: 95°C for 2 min, followed by 35 cycles of 95°C for 10s and 62°C for 45s each.

RESULTS AND DISCUSSION

CONSTRUCTION OF STANDARD CURVES

Plasmid DNA for standard curves was extracted as described in materials and methods section, and serial diluted for the generation of standard calibration concentrations: a mathematical relation correlates weight of DNA and its length. In this way it is possible to easily calculate the number of copies of standard plasmid for each extraction.

$$\#bp \times 1.096 \times 10^{-21} = g\ DNA$$

In the case of pGA4-PCNSTD:

$$3422\text{bp} \times 1.096 \times 10^{-21} = 3.7 \times 10^{-6}\text{pg DNA/copy}$$

With these results, it is possible to construct a standard curve with increasing concentrations of DNA (figures 47 and 48).

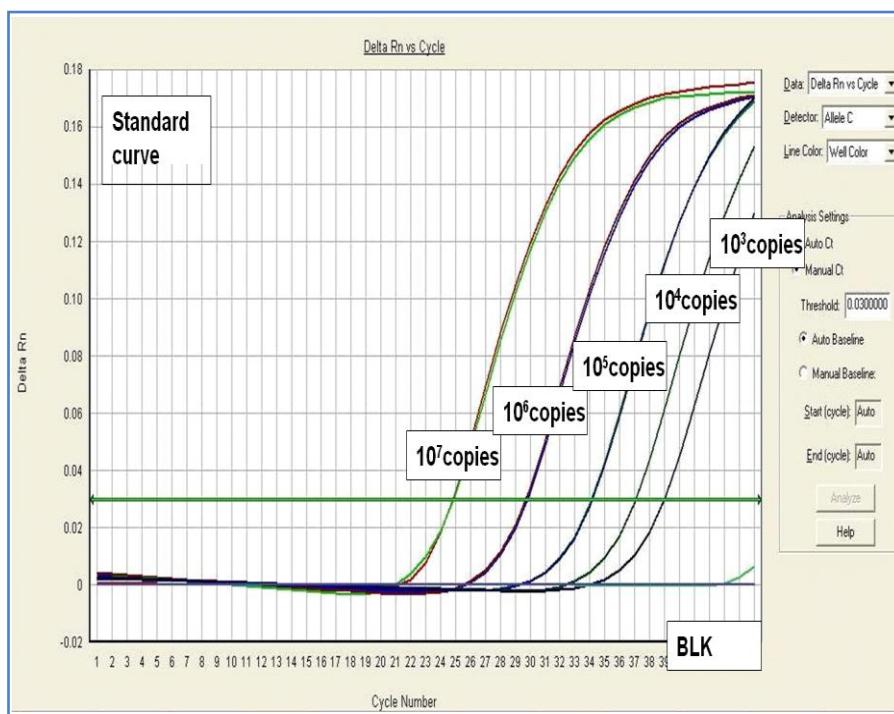


Figure 47: real time PCR on standard plasmid. BLK=blank

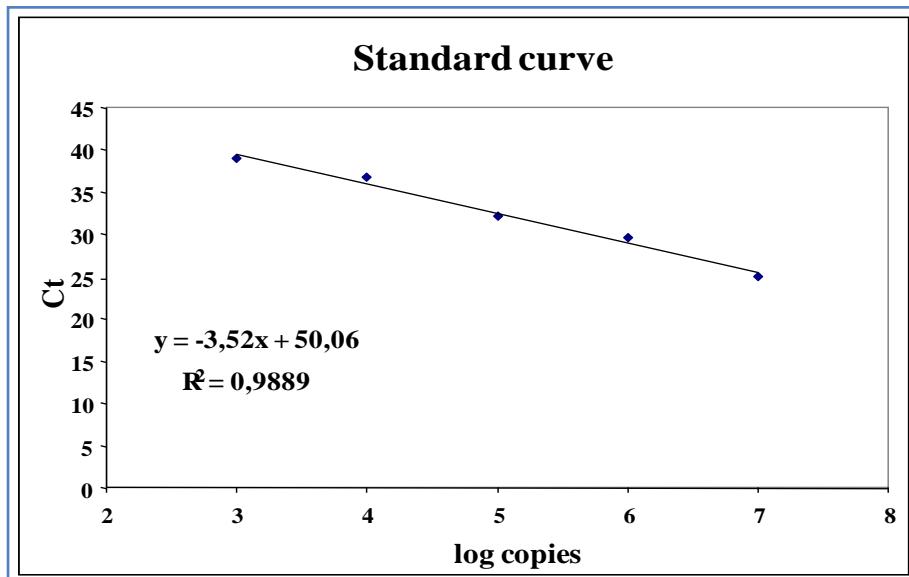


Figure 48: standard curve with increasing concentration of plasmidic DNA.

The DNA dosage was measured with a spectrophotometer; to assess the reliability of the instrument we performed a real time pcr on PCNSTD and on a commercial standard, dosed by the manufacturer. As shown in figure 49, standard curves were perfectly overlaid.

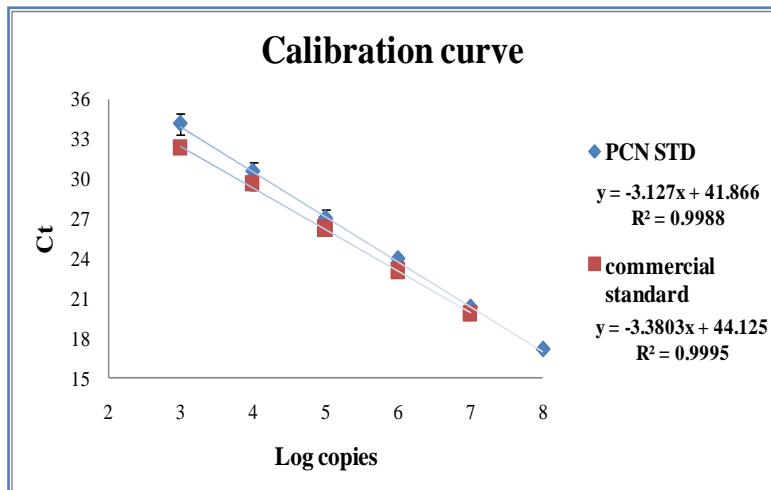


Figure 49: calibration curves of PCNSTD and of a commercial standard: the doses were perfectly overlaid

COMPARISON BETWEEN PLASMIDIC AND GENOMIC DNA

Another interesting point to be assessed is the difference between plasmids and genomic DNA: standard curve is indeed built on plasmidic DNA, while plasmid copy number will be measured on a mixture of genomic and plasmidic DNA. To assess if the use of a plasmid as a standard reference could be rightful, we performed a parallel real time pcr both on PCNSTD and on genomic DNA of untransformed XL1-Blue cells (which contain a single copy of DXS gene) (figure 50).

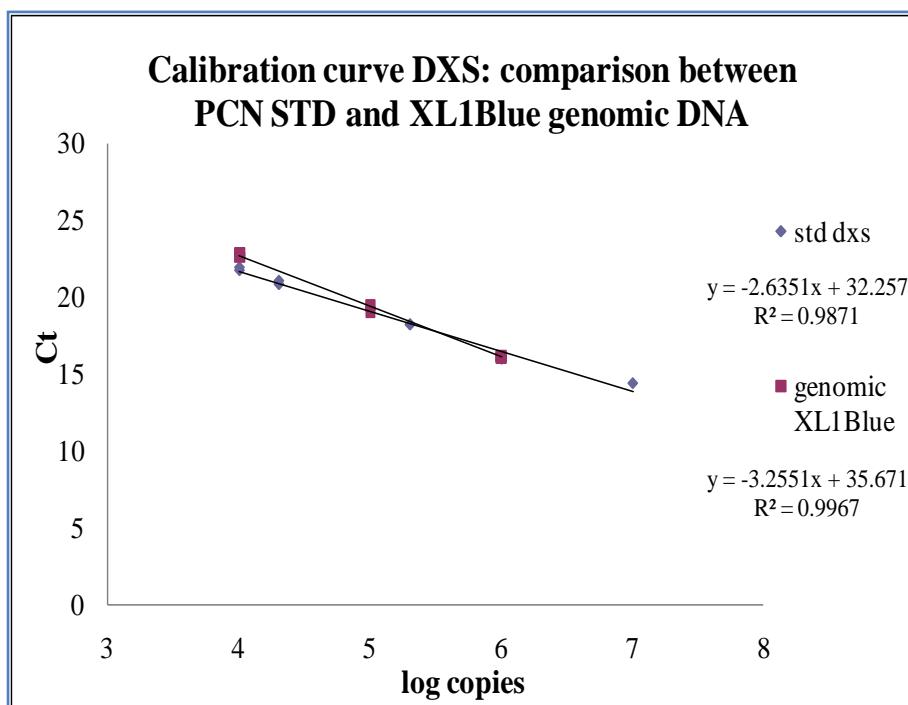


Figure 50: comparison between genomic DNA and PCN STD. In both real time pcr DXS gene was amplified, starting from known and increasing concentration of DNA. Plasmidic and genomic DNA were amplified with the same efficiency, and regression lines were similar.

As shown in figure 50, genomic and plasmidic DNA were amplified with the same efficiency, and standard curves are overlaid. We therefore have a good standard, suitable both for plasmidic and genomic DNA analyses.

COMPARISON BETWEEN BL21(DE3) AND XL1-BLUE *E.coli* STRAINS

All the plasmid copy number evaluations are performed on BL21(DE3) strain: since this strain is still not completely sequenced, we compared genome from XL1-Blue with that from BL21, to assess if there is a single copy DXS also in this productive strain. Known amount of DNA from both strains was amplified, at increasing dilutions. Graphic below shows a perfect overlay between standard curve from XL1-Blue and BL21 (figure 51).

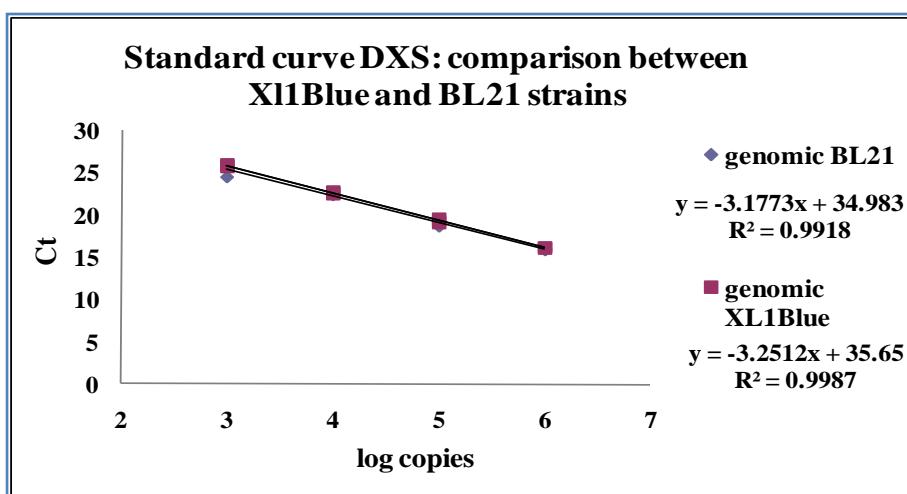


Figure 51: comparison between XL1-Blue and BL21 genomes: both strains have a single copy DXS/cell

To further demonstrate that a single copy of DXS is present in a BL21 cell, we assessed if the system is able to discriminate between a single copy/cell and a two copies/cell sample: we therefore amplified known amounts of genomic DNA of BL21(DE3) and a two-fold dilution of each sample (figure 52).

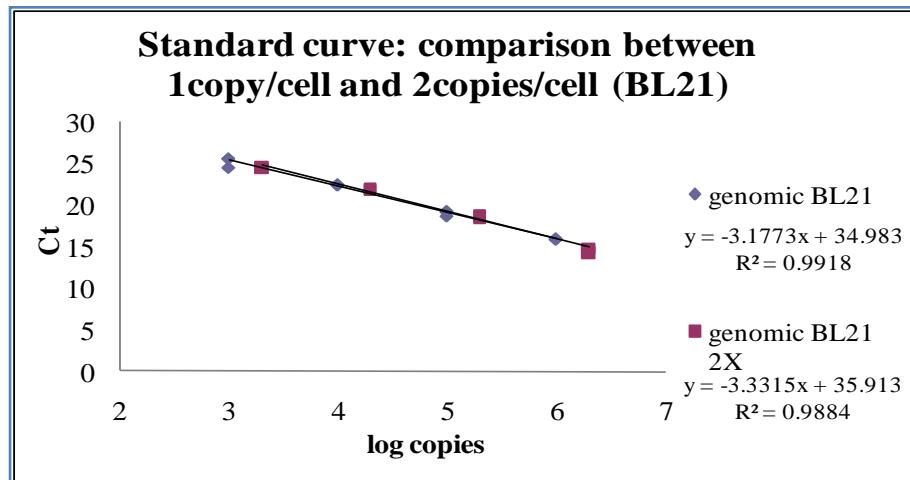


Figure 52: comparison between 1 copy/cell and 2 copies/cell.

As shown in figure 52, the system can easily discriminate between a single copy/cell and a 2 copies/cell samples. It is rightful, according to these results, to measure plasmid copy number in *E. coli* BL21 using, as standard reference, a vector carrying DXS.

The amplification reaction for PCN measurement is performed on a mixture of unknown amount of genomic and plasmidic DNA: to assess if the efficiency of amplification is affected by the nature and the concentration of DNA, we performed a real time pcr on mixture with different ratios of genomic and plasmidic DNA, amplifying Kan sequence: from the worst event for plasmidic DNA (genomic:plasmidic 2:1) to the maximal plasmidic concentration in a genomic environment (genomic:plasmidic 1:1000) (figure 53).

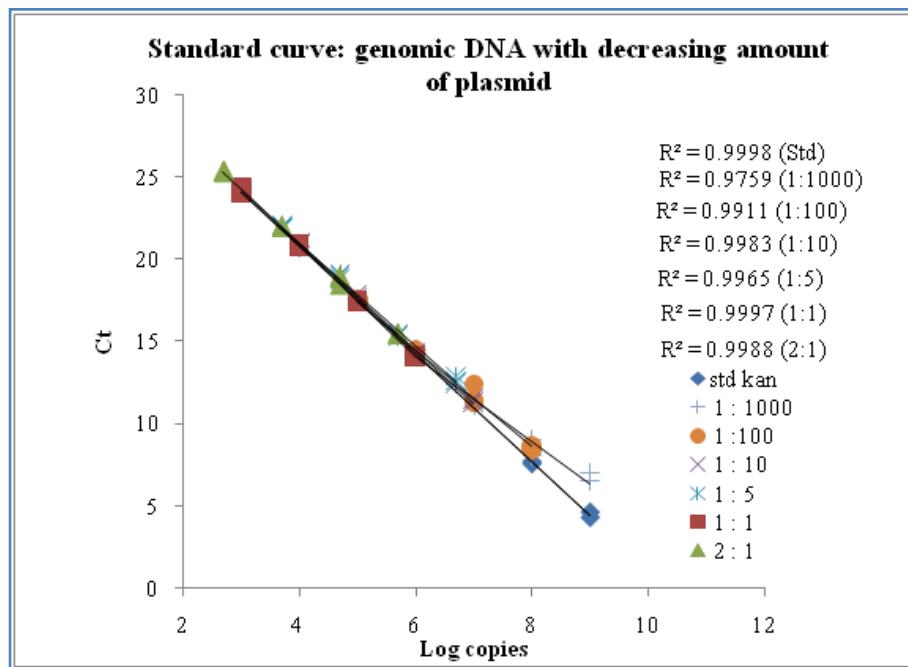


Figure 53: assessment of efficiency of amplification reaction. Amplification occurred with different mix of genomic and plasmidic DNA with the same efficiency.

As shown in figure 53, efficiency was not affected by the dilution factor.

MEASUREMENT OF PLASMID COPY NUMBER

Plasmid copy number was evaluated first on BL21(DE3) competent cells transformed with an empty vector (pET24), and compared with PCN of BL21(DE3) transformed with pET24 in which an exogenous gene had been subcloned: we wanted to evaluate if the efficiency of the extraction or of the amplification was affected by the presence of an exogenous gene (figure 54)..

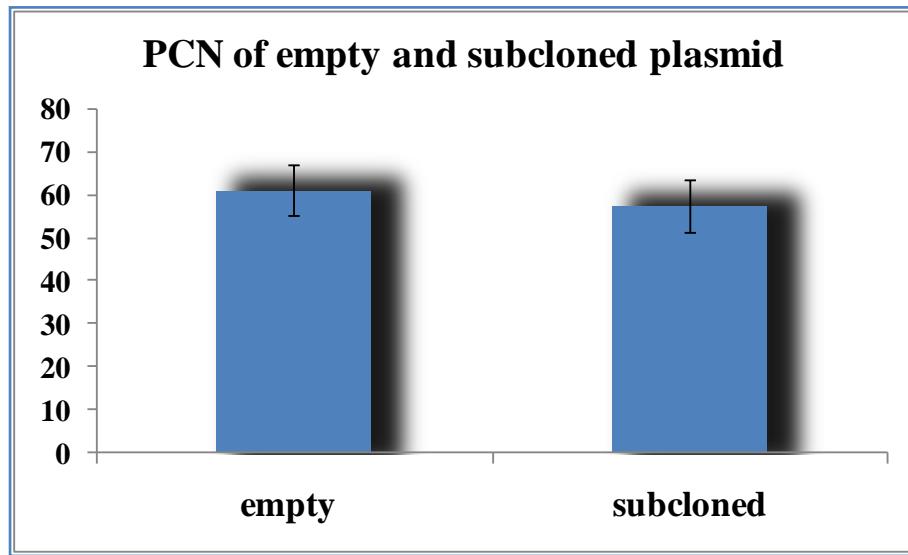


Figure 54: comparison between empty plasmid and vector carrying an exogenous gene: the efficiency of extraction and amplification is not affected by the cloning step, unless the protein is toxic for the host cell.

As shown in figure 54, PCN was calculated in both *E. coli* transformants: measurements were done on three different clones and in triplicate for each condition. PCN was calculated as 61 copies/cell for empty plasmid and 58 copies/cell for subcloned vector: efficiency of extraction and amplification is not affected by the cloning, nor by the single clone growing.

The production of recombinant proteins in *Escherichia coli* bacteria is affected by the number of plasmids, their structural and segregational stability, which have an essential impact on productivity: assessment of PCN is one of the methods to control the production process.

We therefore calculated PCN for all the antigens produced in HIV kit. DNA extraction was done on three different clones for each antigen, and real time

PCR was performed in quintuplicate for each sample: figure 55 below shows the mean values for each antigen. PCN was calculated even after one year from the first measure, to confirm the stability of the cell line.

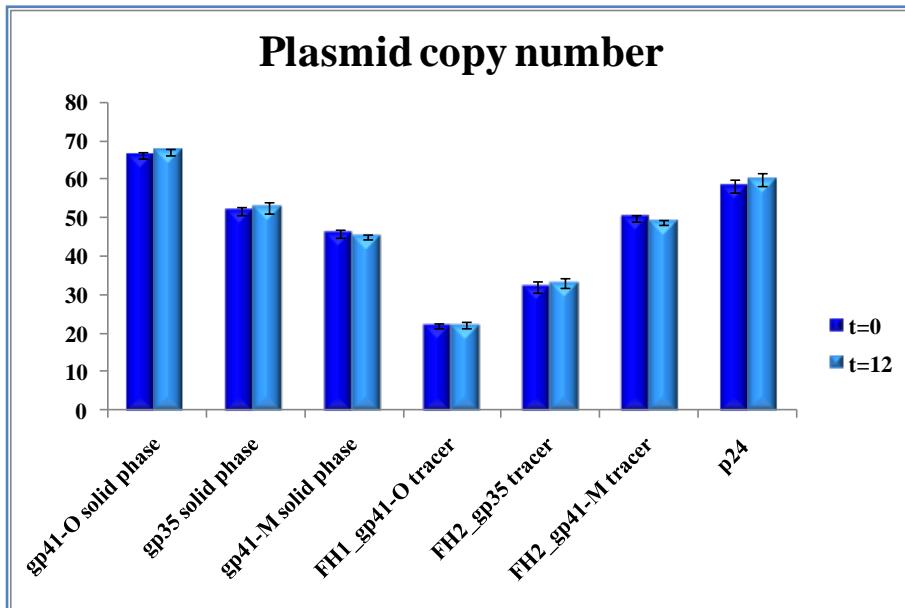


Figure 55: PCN of the cell lines used for the production of the component of HIV kit. Even after 12 months, plasmid was stable and plasmid copy number did not decrease.

In conclusion, with this new, easy and rapid method it is possible to monitor the PCN during the production on an industrial scale. We have demonstrated that a unique plasmidic internal standard is sufficient for the detection of plasmid copy number for the most common bacterial strains. With this standard, indeed, it is possible to amplify a single-copy genomic sequence of *E.coli*, and the two most common antibiotic resistances (ampicillin and kanamycin). From an industrial point of view, it is of fundamental importance to be sure that plasmid copy number remains constant, so that the starting material for industrial production do not vary even after a long time. Thus, it is important to evaluate if cryoconservation and clonal expansion do not affect PCN. We applied this method to check plasmid stability on productive strains of *E.coli*. The differences in PCN depended only by the nature of the plasmid and of the gene: marked evidences showed that, in some cases, cell lost their plasmid and did not produce the target protein anymore, probably because of the toxicity of the protein. With this method

Conclusions and discussion

we demonstrated that bacterial cells that produce HIV glycoproteins had a stable PCN, which was not affected by cryoconservation nor by clonal expansion: we demonstrated that, even after 12 months, plasmid copy number did not vary significantly.

This new method could also be applied for the monitoring of the fermentations, to see if the growing and induction conditions have any effect on plasmid copy number.

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Riassunto

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Il rilevamento combinato e simultaneo di anticorpi anti-HIV e della proteina virale del core distingue i saggi immunologici di quarta generazione (saggi combinati) da quelli di terza generazione (saggi “sandwich” a doppio antigene)

Prima dell'introduzione dei saggi di quarta generazione, i kit commerciali per la diagnosi dell'infezione da HIV erano basati unicamente sul rilevamento della proteina del core virale di HIV (p24) oppure sul rilevamento degli anticorpi, specialmente quelli rivolti contro le proteine transmembrana del virus.

Questi anticorpi compaiono durante la sieroconversione dei pazienti infetti da HIV, e rimangono ad alti livelli durante tutto il corso dell'infezione.

I saggi immunodiagnostici di quarta generazione hanno lo scopo di ridurre il “periodo finestra” (il periodo di tempo che intercorre tra l'infezione e la sieroconversione), per poter ottenere una diminuzione del rischio residuo di infezione da HIV trasmessa con le trasfusioni.

Il formato di saggio che combina il rilevamento contemporaneo sia dell'antigene che degli anticorpi anti-HIV permette di ridurre il periodo finestra, in quanto la proteina del core (p24) appare in maniera transiente nel sangue nei primi momenti dell'infezione, precedendo di alcuni giorni la comparsa degli anticorpi. Si ritiene che il test dell'antigene in un formato combinato sia in grado di ridurre il periodo finestra di circa due settimane rispetto ai saggi per i soli anticorpi.

Nonostante l'aumento della sensibilità durante la sieroconversione, i kit di quarta generazione forniscono un valore aggiunto alla diagnosi solo nel caso in cui la sensibilità e la specificità delle singole parti non venga compromessa nel formato combinato.

Con questo lavoro, è stato disegnato e costruito un saggio di quarta generazione che, basandosi sulla chemiluminescenza, rileva sia l'antigene p24, sia gli anticorpi anti-HIV.

Il sistema Liaison® prevede che si formi un sandwich tra una fase solida (microparticelle paramagnetiche a cui vengono legate le glicoproteine di HIV) e un tracciante (gli stessi antigeni della fase solida, in forma solubile e coniugate a molecole di ABEI, che, se eccitate, emettono energia sotto forma di luce).

Se il campione testato è positivo, gli anticorpi specifici per gli antigeni di HIV permetteranno la formazione del sandwich e la generazione del segnale

che verrà rilevato da un fotomoltiplicatore ed analizzato da un software specifico.

In particolare, le linee guida della comunità europea suggeriscono che i saggi immunochimici per la diagnosi da HIV siano in grado di riconoscere sia HIV-1 (e in particolare i gruppi M e O), sia HIV-2.

Per questo sono state prodotte, in forma ricombinante in *Escherichia coli*, le glicoproteine gp41-M, gp41-O e gp35: gli antigeni, insolubili e purificati come corpi d'inclusione, sono stati coniugati alle microparticelle paramagnetiche in modo da costituire la fase solida del saggio.

Per quanto riguarda i traccianti, è stato necessario aumentare la solubilità delle proteine.

Per quanto riguarda la gp41-O, è stata fusa con un *folding helper* (FH1), una prolil-isomerasi di *Escherichia coli*, grazie alla quale è stata raggiunta una solubilità sufficiente, mantenendo un'elevata specificità.

Per quanto riguarda gp41-M, invece, è stato necessario fondere due *folding helper* in tandem (FH2), per poter ottenere una solubilità adeguata. Anche il processo di purificazione, per questo antigene, è stato modificato: è stato aggiunto uno step di refolding in colonna, che ha permesso di ottenere una proteina pura, solubile e reattiva.

Anche con la gp35 è stato necessario fondere due *folding helper* in tandem, ma una purificazione standard con IMAC e gel filtrazione è stata sufficiente per aumentare la solubilità e la purezza, mantenendo la reattività.

Infine, per la parte di rilevamento dell'antigene, è stata prodotta una p24-M in forma ricombinante, per avere uno standard durante l'ottimizzazione del saggio. Il sandwich viene formato da due anticorpi monoclonali commerciali reattivi per due epitopi diversi della proteina.

La reattività è stata testata sia con la metodica della *surface plasmon resonance*, sia in ELISA, sia nel formato Liaison®.

L'intero kit ha dimostrato di avere una sensibilità e una selettività pari, e in certi casi superiore, ai kit commerciali di quarta generazione già presenti sul mercato (specificità 99.43% al primo test e 99.93% al re-test).

La seconda parte di questo lavoro illustra una nuova metodica per la determinazione del plasmid copy number in *Escherichia coli*, messo a punto nel nostro laboratorio. Si tratta di una reazione di pcr *real time* che amplifica in parallelo un gene a singola copia nel genoma di *E.coli* (il gene DXS) e il gene per la resistenza all'antibiotico. Per ottenere un dato quantitativo, sono

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sono state costruite delle curve standard con concentrazioni note di un plasmide che porta entrambi i geni (pGA4PCNSTD). Interpolando i valori di amplificazione del campione ignoto, è possibile ottenere il valore assoluto delle copie di DNA genomico e plasmidico. Facendo poi il rapporto tra le due specie di DNA, si ottiene facilmente il plasmid copy number.

Con questo lavoro è stato dimostrato che il metodo messo a punto permette di monitorare facilmente la quantità di plasmidi presenti in una cellula batterica. È stato dimostrato che l'efficienza di amplificazione non è influenzata dalla quantità e dalla specie di DNA presente nella reazione. È stato inoltre dimostrato che è possibile applicare questo metodo ai ceppi batterici produttivi, come ad esempio il ceppo BL21(DE3). Grazie allo standard interno unico, è possibile applicare questo metodo a tutti i plasmidi con resistenza a kanamicina o ampicillina.

E' stato infine valutato se la crioconservazione o l'espansione clonale avessero qualche effetto sul plasmid copy number: è stato applicato questo metodo per valutare la stabilità plasmidica nei ceppi trasformati utilizzati per la produzione degli antigeni di HIV. Anche dopo 12 mesi di conservazione, il plasmid copy number non è variato in modo significativo.

Questo nuovo metodo, infine, può essere anche applicato per monitorare la stabilità dei plasmidi durante le fermentazioni, per controllare se le condizioni di crescita e induzione possono avere degli effetti sul plasmid copy number.