Novel Recombinant Multiepitope Proteins and Assay Concepts for Viral Infections

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Doctor of Philosophy

To

Jawaharlal Nehru University

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by

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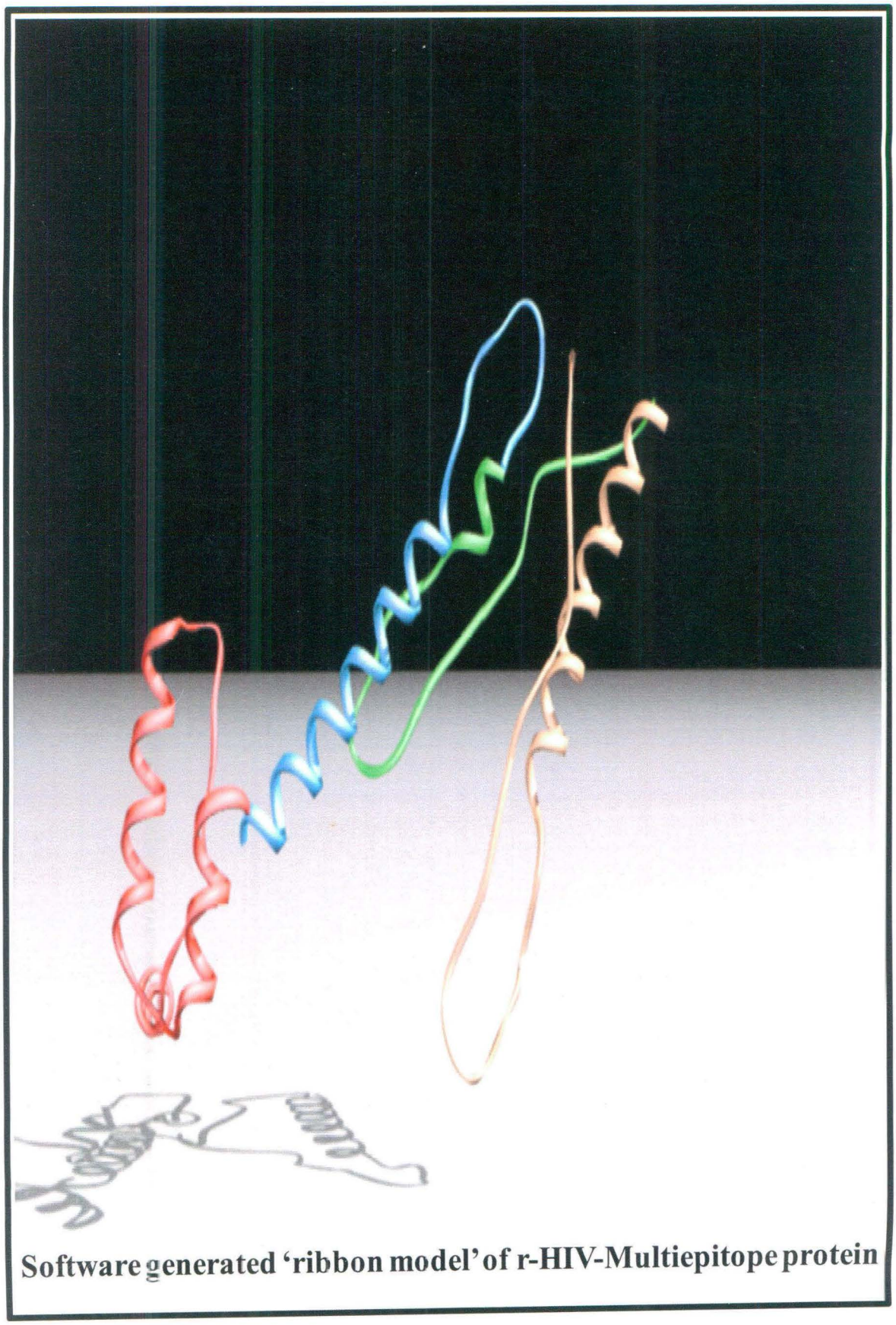

Software generated 'ribbon model' of r-HIV-Multiepitope protein

Cover picture- Software generated graphic visualization of ribbon model of r-HIV-Multiepitope protein. HIV specific four linear immunodominant epitopes were selected and linked with flexible linkers. Different epitopes are shown in different colours.

To my parents

Contents in brief

Declaration

I hereby declare that the research work embodied in this thesis entitled **'Novel Recombinant Multiepitope Proteins and Assay Concepts for Viral Infections'** has been carried out by me under the supervision of Dr. Navin Khanna at Recombinant Gene Products Group, International Centre for Genetic Engineering and Biotechnology, New Delhi.

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Certificate

This is to certify that the research work embodied in this thesis entitled '**Novel Recombinant Multiepitope Proteins and Assay Concepts for Viral Infections**' has been carried out at the Recombinant Gene Products Group, International Centre for Genetic Engineering and Biotechnology, New Delhi. This work is original and no part of this thesis has been submitted for the award of any other degree or diploma to any other university.

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Talha

Abbreviations

Amp	Ampicillin
Anti-HCV	antibodies to HCV
Anti-HIV	antibodies to HIV
Ab	Antibody
Ag	Antigen
ART	Anti-retroviral therapy
BBI	Boston Biomedica Incorporation
B-DNA	Branched DNA
BSA	Bovine serum albumin
bp	Base pair
C-terminal	Carboxy terminal
DNA	Deoxyribose nucleic acid
dNTPs	Deoxynucleoside triphosphates
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediamine tetracetic acid
ELISA	Enzyme linked immunosorbent assay
EIA	Enzyme immunoassay
g	Gram
Gu-HCL	Guanidine Hydrochloride
h	Hour/Hours
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellulr carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HTLV	Human T-cell leukemia virus
r-HCV-F-MEP	recombinant hepatitis C virus flexible multiepitope protein
r-HCV-MEP	recombinant Hepatitis C Virus multiepitope protein
r-HIV-MEP	recombinant Human immunodeficiency virus multiepitope protein
6x-His	A tag containing 6 histidine amino acid residues
HRPO	Horse radish peroxidase
IBs	Inclusion bodies
IU	Internatinal Unit
Kan	Kanamycin

kDa	Kilo dalton
kbp	Kilo base pairs
L	Litre
LB	Luria broth
LC	Liver cirrhosis
λ	Lambda (wavelength)
MAb	Monoclonal antbody
mRNA	Messenger RNA
min	Minutes
mg	Milligram
ml	Millilitre
mM	Millimolar
μ	Micro
NANB hepatitis	Non-A, non-B hepatitis
NAT	Nucleic acid testing
N-Terminal	Amino terminal
ng	Nanogram
Ni-NTA	Nickle-nitrilotriacetic acid
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAb	Polyclonal antibody
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
RIBA	Recombinant immunoblot assay
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
sec	Seconds
TB	Tuberculosis
TE	Tris E.D.T.A
TRF	Time-resolved fluorometric

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Introduction

Blood transfusion saves life and improves health. The Global Database on blood safety indicates that ~80% of the world's population does not have access to reliable and safe blood. Infection through blood supply is of particular concern in the under-developed countries. The National Blood Policy adopted in India is a step towards improving blood safety. Currently, blood banks carry out separate tests of variable test formats for HIV, HCV and HBV detection. This greatly increases the cost of transfusion. In this project, we have developed three individual Time-resolved Fluorometric (TRF) immunoassays for the detection of anti-HIV antibody, anti-HCV antibody and HBsAg in infected human sera. For the detection of anti-HIV antibodies, a novel recombinant multiepitope diagnostic intermediate, r-HIV-MEP was designed by fusing virus specific, immuno-dominant, linear and phylogenetically conserved epitopes of HIV. For the detection of anti-HCV antibodies, a novel recombinant multiepitope diagnostic intermediate HCV-MEP V2 (r-HCV-MEP), was designed. The r-HIV-MEP and r-HCV-MEP V2 were *in vivo* biotinylated in *E. coli,* purified to homogeneity and used as capture antigens to develop in-house TRF immunoassays for the detection of anti-HIV and anti-HCV antibodies, respectively. The assays were evaluated using well characterized serum panels from Boston Biomedica Inc. (BBI). The results suggested that both of these MEPs were extremely sensitive and specific for the detection of HIV and HCV infections. Monoclonal antibodies 21B and 5S specific for HBsAg were used to develop an in-house HBsAg TRF immunoassay. The HBsAg TRF immunoassay was evaluated by well characterized serum panel from BBI. The three individual in-house immunoassays were then combined to develop an HIV, HCV and HBV multiplexed TRF immunoassay. The multiplexed assay showed excellent sensitivity and specificity for the detection of all three infections. The use of fluorescent europium(III) chelate and europium(III) nanoparticles as reporters further simplified these immunoassays by easy measurement of time-resolved fluorescence directly from the dry wells of microtiter plate. The results show that a prototype multiplexed HIV, HCV and HBV TRF immunoassay was successfully developed. The 'know-how' of the design and production of novel r-HIV-MEP has been transferred to a leading diagnostics manufacturing company in India.

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1. Review of literature

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1.1. Blood bank safety and WHO recommendations

According to World Health Organization (WHO) estimates based on data collected from 172 countries (95% of the world's population), the annual global blood collection is 81.2 million units, out of which 55% is collected in countries with a high human development index (HDI) where only 18% of the world population lives. This is in contrast to the developing countries (medium and low HDI) where 45% of the global blood supply is available for almost 78% of the global population. Problems related to blood availability in many developing countries are multifactorial, and include lack of/ineffective implementation of nationally co-ordinated blood transfusion service, limited voluntary donor base, lack of quality assured screening for transfusion-transmissible infections, lack of technical expertise in immunohematology, and inappropriate clinical use, coupled with political and economic instability. In the Southeast Asia region (SEAR) countries, whole blood (WB) is transfused in the following clinical situations: obstetrical cases (30%), pediatric patients (14%), general surgery (10%), trauma cases (7%), and for a miscellaneous group of disorders, not clearly categorized. This one-third utilization in obstetric cases is due to pregnancy-related hemorrhage, an increasing number of cesarean sections and pregnancy aggravated anemia [Marwaha, 2010].

The provision of safe and efficacious blood and blood components for transfusion use involves a number of processes, from the selection of blood donors and the collection, processing and testing of blood donations, to the testing of patient samples, the issue of compatible blood and its administration to the patient. There is a risk of error in each process in this "transfusion chain" and a failure at any of these stages can have serious implications for the recipients of blood and blood products. Thus, while blood transfusion can be life-saving, there are associated risks, particularly the transmission of blood-borne infections. Screening for transfusion-transmissible infections (TTIs) to avoid transmitting infection from donors to recipients is a critical part of the process of ensuring that transfusion is as safe as possible. Effective screening for evidence of the presence of the most common and dangerous TTIs can reduce the risk of transmission to very low levels. Blood transfusion services should therefore establish efficient systems to ensure that all donated blood is correctly screened for specific TTIs and that only non-reactive blood

and blood components are released for clinical and manufacturing use. According to WHO recommendations mentioned in detail in 'Screening donated blood for transfusion-transmissible infections', each country should have a national policy on blood screening that defines national requirements for the screening of all whole blood and apheresis donations for transfusion-transmissible infections. Screening of all blood donations should be mandatory for the following infections and using the following markers [WHO, 2010]:

- HIV-1 and HIV-2: screening for either a combination of HIV antigen-antibody or HIV antibodies
- Hepatitis B: screening for hepatitis B surface antigen (HBsAg)
- Hepatitis C: screening for either a combination of HCV antigen-antibody or HCV antibodies
- Syphilis (*Treponema pallidum*): screening for specific treponemal antibodies.

Screening of donations for other infections, such as those causing malaria, chagas disease or HTLV, should be based on local epidemiological evidence.

The 'National Blood Policy' adopted in India, formulated by NACO (National Aids Control Organization) in 2002, followed by an 'Action Plan on Blood Safety' in 2003, was a step towards improving blood safety [Marwaha, 2010]. Currently under the Drugs and Cosmetics Act of India, it is mandatory to test blood for anti-HIV-1 and -2, anti-HCV, HBsAg and syphilis [Elhence, 2006]. This requires inexpensive screening tests. Currently blood is screened for above mentioned infections in individual immunoassays. This greatly increases the cost of transfusion. The detection of HIV and HCV is based on the detection of antibodies to these pathogens using a cocktail of several synthetic peptides and/or full-length recombinant protein antigens; the presence of HBV is identified through the detection of the virus-encoded HBsAg antigen.

In this study a prototype multiplexed immunoassay is developed, which proposes to make blood screening simple and cost-effective since only one diagnostic test has to be performed to screen for three viral infections (HIV, HCV and HBV). This technology will allow for rapid and economical screening of blood, making more safe blood available for transfusion.

1.1. Blood bank safety and WHO recommendations

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1.2. Human Immunodeficiency Virus

1.2.1. History and current epidemic patterns

The human immunodeficiency virus (HIV) was isolated in 1983 for the first time just 2 years after the description of a new disease affecting especially young homosexually active men; originally it had been named lymphadenopathy associated virus (LAV) and human T lymphotropic virus type III (HTLV-III) or AIDS related virus (ARV) [Stürmer et al., 2009]. HIV, a lentivirus of the family *Retroviridae,* is one of several viruses transmitted via blood transfusion [Allain et al., 2009]. In 2008, there were 2.7 million new HIV infections and 2 million HIV-related deaths. Globally, an estimated 33.4 million people lived with HIV in 2008 (Figure 1.1) [UNAIDS, 2009]. HIV infection induces a profound immune dysfunction, with abnormalities in every arm of the immune system resulting in the most severe clinical stage - Acquired Immune Deficiency Syndrome, AIDS [Chinen and Shearer, 2002]

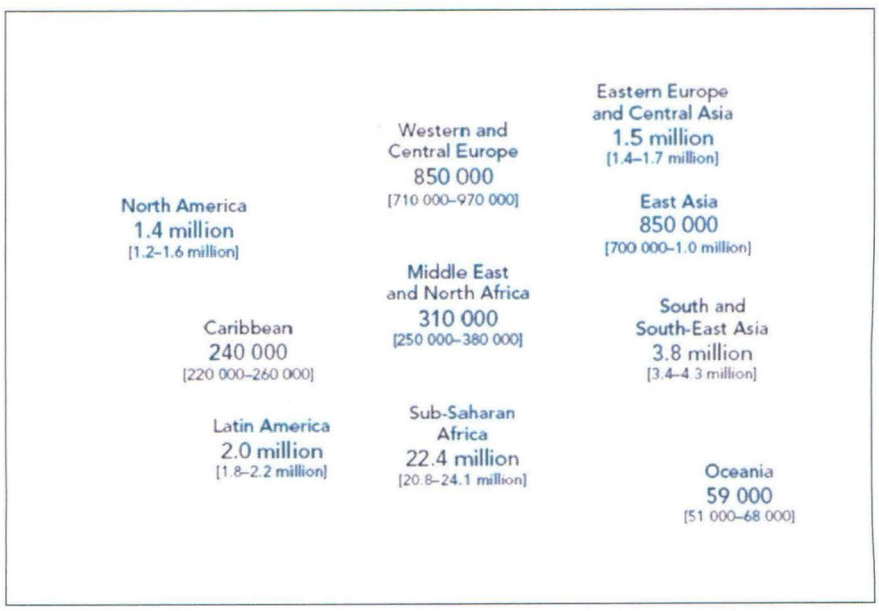

Figure 1.1. Adults and children estimated to be living with HIV in 2008 in different part of the world.

1.2.2. The virus

HIV RNA, which integrates into the host genome as a provirus, is approximately 9.2 kilobases in length [Muesing et al., 1985]. Like all viruses, HIV can replicate only inside cells, commandeering the cell's machinery to reproduce. Retroviruses have

genes composed of ribonucleic acid (RNA) molecules whereas the genes of humans and most other organisms are made of deoxyribonucleic acid (DNA). However, once inside the cell, HIV and other retroviruses use the enzyme reverse transcriptase to convert their RNA into DNA, which can be integrated into the human cell's genes. [Klimas et al., 2008]. The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins. These proteins are divided into three classes, the major structural proteins (Gag, Pol and Env), the regulatory proteins (Tat and Rev) and the accessory proteins (Vpu, Vpr, Vif and Nef) (Figure 1.2) [Gallo et al., 1988].

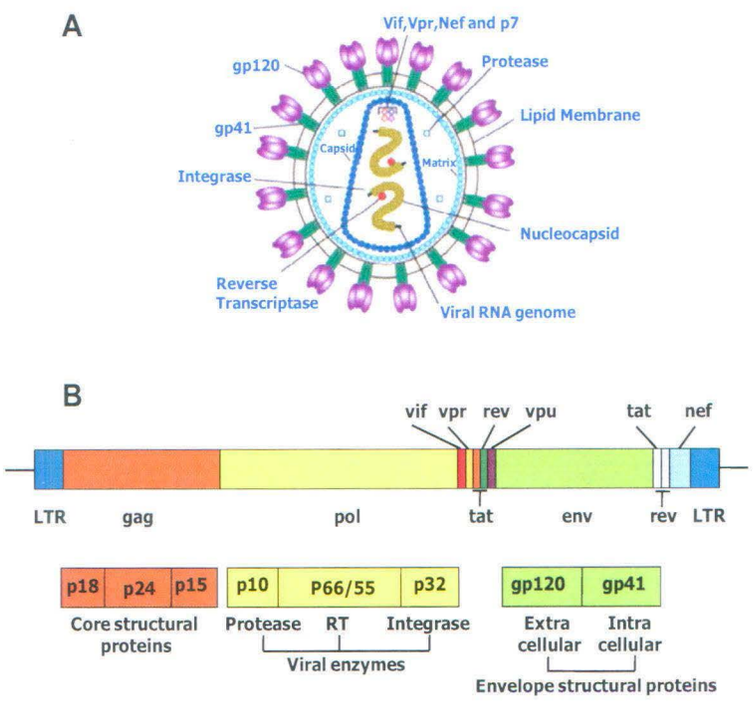

Figure 1.2. Human Immunodeficiency virus (HIV): model structure and genome organization. *(A) Model structure of HIV. (B) The integrated form of HIV-1, also known as the provirus, is approximately 9.8 kilobases in length. Both ends of the provirus are flanked by a repeated sequence known as the long terminal repeats (LTRs). The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins. These proteins are divided into three classes, the major structural proteins (Gag, Pol, and Env), the regulatory proteins (Tat and Rev) and the accessory proteins (Vpu, Vpr, Vif, and Nef).*

1.2.3. Genotypes of HIV and its geographical distribution

Two genetically distinct viral types of HIV have been identified, HIV-1 and HIV-2 [Butler et al., 2007]. HIV-1 is the type associated with disease in the United States, Europe, central Africa, Asia and most other parts of the world. HIV-2 has been found mainly in infected individuals in western Africa and 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. All HIV types and subtypes are thought to be derived from zoonotic introductions from nonhuman primates [Tebit et al., 2007]. HIV-1 variants are classified into three major groups: group M (main/major), 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 subtypes, or clades (A to K). Sub-subtypes and circulating recombinant forms (CRFs) have emerged over the past few decades [Buonaguro et al., 2007]. Genetic variation for HIV-1 is especially high, with rapid turnover of HIV-1 virions. Over 20 different CRFs have been defined within group M alone. HIV-1 diversity in the worldwide epidemic is shown in Figure 1.3 [Ariën et al., 2007]. The HIV-1 subtypes C and A account for the majority of HIV cases in the pandemic, but the other viral forms circulate globally. HIV-1 subtype B is predominant in North America, Western Europe, and Australia [Klimas et al., 2008]. The first report of HIV/AIDS infection in India was in 1986, and since then the virus has spread rapidly throughout the country. Both HIV serotypes 1 and 2 exist in India, and HIV-1 subtype C is the most common subtype reported [Agoramoorthy and Hsu, 2005].

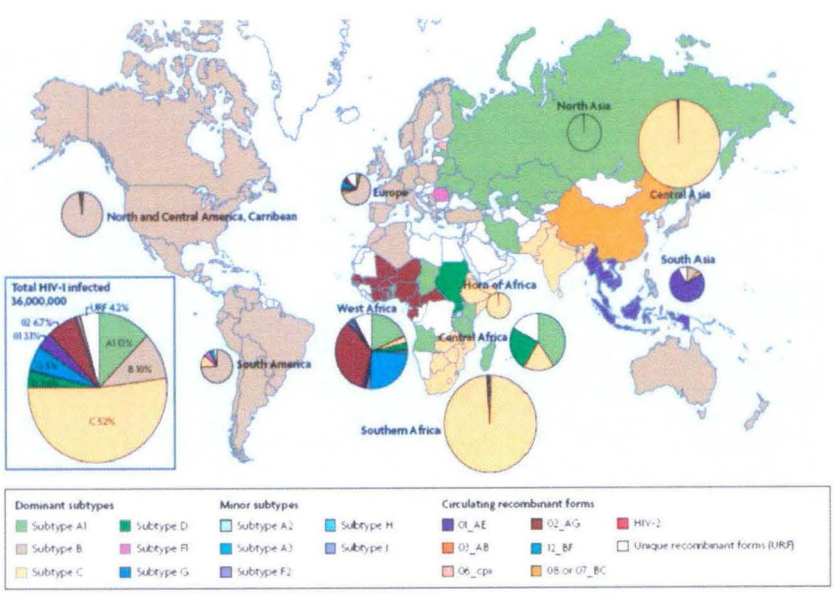

Figure 1.3. HIV-1 diversity in the worldwide epidemic. *The frequency of each HIV-1 subtype and recombinant form was estimated in each country based on published findings. The countries are colour-coded based on the dominant HIV-1 group main (M) subtype. The countries coloured grey have a low level of HIV-1 prevalence or were not represented in the scientific literature related to HIV-1 subtype prevalence. The pie charts depict the proportion of each subtype or recombinant form in each geographical region. The size of the pies is proportional to the number of HIV-1 infected individuals in that particular region.*

1.2.4. Disease

1.2.4.1. Transmission

The HIV transmission is driven by two forces leading to a worldwide spread: sexual intercourse and blood contamination. The latter occurs mostly by intravenous drug consumption in the western world, and by blood transfusion in combination with a highly increasing HIV incidence in developing countries [Stürmer et al., 2009]. HIV is one of several viruses transmitted via blood transfusion, through blood or blood products [Allain et al., 2009]. HIV is also spread through the practice of reusing or sharing syringes and needles with drugs. [Klimas et al., 2008]. The commonest route of transmission of HIV-1 is through heterosexual intercourse [McMichael, 2006]. HIV is spread most commonly by having unprotected sex with an infected partner. The virus can enter the body through the lining of vagina, vulva, penis, rectum or mouth during sex. The risk of infection during intercourse is greatly increased by concurrent sexually transmitted diseases, rough sex, or a partner with a very high viral load such as that seen in primary infection and again in late-stage disease [Klimas et al., 2008]. Postnatal transmission of HIV-1 by breast milk is more important than previously believed and approximately doubles the risk of mother to child transmission [Grant and De Cock, 2001].

1.2.4.2. Clinical symptoms

Seroconversion refers to the development of antibodies to HIV and usually takes place between 1 and 6 weeks after HIV infection has occured. Whether or not HIV infection causes initial symptoms, an HIV-infected person is highly infectious during this initial period and can transmit the virus to another person. The main HIV symptoms are summarized below [UNAIDS, 2008]-

- Primary HIV infection - may be asymptomatic or experienced as Acute retroviral syndrome
- Clinical stage 1 - asymptomatic or generalized swelling of the lymph nodes
- Clinical stage 2 - includes minor weight loss, minor mucocutaneous manifestations, and recurrent upper respiratory tract infections
- Clinical stage 3 - includes unexplained chronic diarrhoea, unexplained persistent fever, oral candidiasis or leukoplakia, severe bacterial infections, pulmonary

tuberculosis, and acute necrotizing inflammation in the mouth. Some patients with clinical stage 3 have AIDS.

- Clinical stage 4 - includes 22 opportunistic infections or cancers related to HIV. All patients with clinical stage 4 have AIDS.

1.2.4.3. Therapy

Most of the currently available drugs for the treatment of HIV inhibit functions of viral enzymes. HIV has a high mutation rate due to the infidelity of the reverse transcriptase. Because of an Anti-Retroviral Therapy (ART), which is not fully suppressive, HIV mutants are selected which replicate in the presence of the drug; hence, the drug is bound insufficiently to the enzyme [Stürmer et al., 2009]. There are presently six classes of ART available that interrupt viral replication: (a) nucleoside/nucleotide reverse transcriptase inhibitors, (b) nonnucleoside reverse transcriptase inhibitors, (c) protease inhibitors, (d) fusion inhibitors, (e) CCR5 antagonists, and (f) integrase inhibitors. Each of these classes of drugs affects the HIV virus at a different stage in its life cycle. Current treatment consists of highly active antiretroviral therapy (HAART), that is, at least three drugs belonging to two classes of antiretroviral agents [Klimas et al., 2008]. Time scale of the availability of antiviral drugs for the treatment of HIV is shown in Table 1.1 [Stürmer et al., 2009].

1.2.4.4. Vaccine

Vaccine development has progressed slowly, due to the tremendous capacity of this virus to escape immune pressure as well as the number of strain variations. Nonetheless, with an improved understanding of effective host response to the infection and the ability to deliver viral antigens to the immune system in novel ways, vaccine development is being pursued vigorously [Gallo, 2005]. Since 1987, thousands of healthy human volunteers have participated in the testing of 30 candidate vaccines in >80 Phase I/II trials. A June 2005 study estimated that $682 million has been spent on AIDS vaccine research annually [Klimas et al., 2008]. A number of vaccine concepts are being pursued including live attenuated vaccines, subunit vaccines, and live recombinant vaccines. This essential research effort recently suffered a baffling setback. The first trial of a vaccine designed to elicit strong cellular immunity has shown no protection against infection. More alarmingly, the vaccine

seemed to increase the rate of HIV infection in individuals with prior immunity against the adenovirus vector used in the vaccine [Sekaly, 2008]. The field is still a long way from a testable vaccine that will efficiently induce neutralizing antibody responses.

Table 1.1 Time scale of the availability of antiviral drugs for the treatment of HIV

Year	Anti-HIV drugs and their targets *
1983	HIV-1 isolated
1987	Zidovudine (RT)
1991	Didanosine (RT)
1992	Zalcitabine (RT)
1995	Stavudine (RT), Saquinavir (PR)
1996	Nevirapine (RT), Indinavir (PR), Ritonavir (RT)
1997	Nelfinavir (PR)
1998	Lamivudine (RT)
1999	Abacavir (RT), Amprenavir (PR), Delavirdine (RT)
2000	Lopinavir + ritonavir (PR)
2001	Tenofovir (RT)
2003	Emtricitabine (RT), Atazanavir (PR), Fosamprenavir (PR), Enfurtide (gp41)
2005	Tripanavir (PR)
2006	Darunavir (PR)
2007	Maraviroc (CCR5)
2008	Raltegravir (IN), Etravirine (RT)
2009	Vicriviroc (CCR5), Elvitegravir (IN), Rilpivirine (RT)

** RT, Reverse Transcriptase; PR, Protease; IN, Integrase; CCR5, Chemokine Coreceptor for HIV.*

1.2.5. Diagnosis

After HIV infection, early immunological and virological blood markers appear in a chronological order and, in particular: HIV RNA, HIV p24 antigen (a protein coded by the *gag* gene), and antibodies to HIV antigens. Viral RNA (viremia) is measurable in plasma as early as within 2 weeks after infection (in general in 10-12 days). Its titres increase exponentially, up to about 1 million copies of RNA/ml within a couple

of months [Buttò et al., 2010]. Many diagnostic tools are available that are based on both detection of HIV-specific antibodies and virus antigen, or nucleic acid. As technology evolves, HIV testing assays are being improved, providing better sensitivity and specificity. Qualitative and quantitative tests that are being used in laboratories, from the HIV antibody-based assays to the new tests for the detection of HIV nucleic acids are summarized below-

1.2.5.1. Qualitative tests

First, second, third and fourth generation Enzyme Immunoassays (EIAs)

All antibody assays are based on the principle of a specific antigen-antibody reaction. In 1985, first generation EIA appeared. These assays employed "whole virus" antigens, obtained from cell cultures. Detection of antibodies bound to HIV antigens used an "indirect" approach. Their capacity to detect early HIV antibodies averaged a little more than 40 days after infection. In 1987, the second generation EIA was introduced. They used the same indirect format as the first generation assays, but the difference was the use of HIV recombinant antigens and peptides, instead of the full viral lysate. The introduction of recombinant antigens increased the specificity and sensitivity of the test. These tests reduced the window period, being able to detect antibodies as early as 33-35 days after infection. In the '90s, the problem of the huge variability of HIV became progressively evident. EIA kits started to include antigens from the HIV-2 virus, in order to ensure recognition of antibodies directed against both HIV-1 and HIV-2. In addition, new antigens from viruses of the HIV-1 groups M, N, and O were included. In 1994, third generation EIA was designed on a new format. Recombinant HIV-1 and HIV-2 proteins and/or peptides, bound on the solid phase, react with the patient serum. Antigen-bound antibody is revealed by the addition of the same viral antigen conjugated with an enzyme molecule. This "sandwich" format ensured higher sensitivity and specificity, since all potential classes of anti-HIV antibodies (IgG, IgM and IgA) could be revealed. This generation of tests drastically reduced the "window period", bringing it to about 22 days after infection. Recently, fourth generation assays have been introduced. These assays are able to reveal the presence of both the antibodies and p24, the major antigen of HIV and have further reduced the window period, at almost the levels of the detection of virus RNA. However, high sensitivity tests can still have a lower specificity. Third

and fourth-generation EIA have an average specificity of 99.5% to 99.9%. Due to the possibility of non-specific reactivity inherent in any assay, all reactive screening test results must be confirmed, in order to exclude the risk of reporting non-specific reactivity as "positive" for HIV infection [Buttò et al., 2010; Stürmer et al., 2009].

Confirmatory assays

The most commonly used confirmatory assays are western blot (WB) and line immune assays (LIA). The WB is a confirmatory assay that is only carried out if the sample is reactive in the screening assay. Many of the commercially available WBs include antigens from both HIV-1 and HIV-2. The WB is a methodology for which denatured HIV proteins are blotted on strip of a nitrocellulose membrane, which is then incubated with patient serum. If the serum contains antibodies against the various viral proteins, they will bind to the corresponding protein. This antigen-antibody reaction is revealed using an enzyme-labeled secondary antibody and a substrate. A colorimetric reaction reveals the presence of HIV proteins recognized by antibodies as bands on the strip. For HIV-1, proteins detectable by WB can be divided into three groups: the Env (envelope) glycoproteins (gp41, gp120, gp160), the Gag or nuclear proteins (p17, p24/25, p55) and the Pol or endonuclease-polymerase proteins (p34, p40, p52, p68). Most of the commercially available WBs also include a protein from HIV-2 in order to detect both HIV-1 and HIV-2 infections. The result of a WB may be either positive or negative or (in case of an incomplete pattern of visible bands) indeterminate, which may reflect borderline or non-specific reactivity. A great disadvantage of the WB assay is its high price. Assays similar to WB, generically called LIA, based on recombinant proteins and/or synthetic peptides capable of detecting antibodies to specific HIV-1 and/or HIV-2 proteins, have been developed. Examples of this technology include the INNOLIA, Pepti-Lav, and RIBA assays. In general, these assays produce fewer indeterminate results as compared to WB, but are equally expensive [Buttò et al., 2010].

Rapid tests

Today, a number of rapid HIV tests are available, also referred to as rapid/simple (R/S) test devices. Many rapid tests contain a 'built-in' internal control, *e.g.* a control band indicating whether the samples and reagents have been added correctly. At

present, many rapid tests are based on the principles of the second or third generation EIA with antigens from both HIV-1 and HIV-2, and very few are structured as fourth generation tests. Rapid tests can present some problems of sensitivity.

1.2.5.2. Quantitative tests

Nucleic acid testing (NAT) allows the quantitative determination of the HIV-RNA viral load in plasma. Different methods have been applied for HIV-1 nucleic acid detection such as PCR, nucleic acid sequence-based amplification (NASBA), branched DNA (b-DNA), and finally Taqman-PCR as a further refinement. The commercially available tests have high sensitivity and high throughput performance and have the ability to detect HIV-RNA during early seroconversion. Nucleic acid testing has nearly completely replaced virus isolation for all viruses and older methods of virus detection like immunofluorescence microscopy, since NAT is faster, and more sensitive [Stürmer et al., 2009]. The quantitative detection of HIV RNA in plasma is used as a prognostic marker, to monitor antiretroviral therapy and to estimate infectiousness. Today, 'ultra'-sensitive tests that detect as few as 50 RNA copies per cubic millimetre of plasma are commercially available. Viral load testing is, therefore, an indispensable clinical tool. Unfortunately, due to the need of skilled personnel and of expensive dedicated instrumentation, as well as the lack of the necessary organization to monitor HIV-positive ART – treated individuals, many developing countries cannot routinely use the currently available NAT assays. Finally, the problem of the increasing HIV variability can have a serious impact on NAT sensitivity. NAT screening in geographic regions where multiple subtypes and CRFs (circulating recombinant forms) are present can result in a failure to detect infection, since the primers and probes used may not bind and amplify to the nucleic acid of some HIV variants. Similar problems with the use of NAT can arise in countries where HIV-2 infection is endemic. All these reasons, together with the still complicated procedure for testing, drastically limit the use of HIV RNA testing in the diagnosis of HIV infection [Buttò et al., 2010].

1.2.5.3. Currently available kits

Currently, there are several diagnostic kits for HIV, available in the market. Some of them are described in table 1.2.

Table 1.2. Diagnostic kits for HIV

Kits	Kit antigens / antibody	No. of antigens	Assay Type
Abbott HIV-1/HIV-2 EIA	r-gp160, r-p24 of HIV-1 r-gp160 of HIV-2	3	Sandwich EIA
Abbott HIV 1/2 gO	r-gp160, r-p24 of HIV-1, r-gp160 of HIV-2, group O and HIV-2 envelope peptides	5	AxSYM Sandwich MEIA
GS rLAV EIA (Bio-Rad)	r-gp41, Viral Lysate	1+viral lysate	Indirect EIA
GS HIV-1/HIV-2 PLUS O EIA (Bio-Rad)	r-gp160, r-p24 of HIV-1, group O and HIV-2 envelope peptides	4	Sandwich EIA
Vironostika HIV Uni-Form II plus O	r-gp160, r-p24 of HIV-1, group O and HIV-2 envelope peptides	4	Sandwich EIA
Genscreen HIV Ultra	r-gp160, group O and HIV-2 envelope peptides, anti-p24 MAb and PAb	3 + anti-p24 Ab	Sandwich EIA
Vironostika HIV Uniform II Ag/Ab	r-gp160, group O and HIV-2 envelope peptides, anti-p24 MAb	3 + anti-p24 Ab	Sandwich EIA

1.3. Hepatitis C Virus

1.3.1. History and current epidemic patterns

Hepatitis C virus was first detected in 1989 using molecular biology techniques after extensive testing of serum from experimentally infected animals. Ever since its discovery, it became clear that this virus was the major cause of acute hepatitis after a blood transfusion that was neither related to hepatitis A nor to hepatitis B (hence the early name for this disease, non-A, non-B hepatitis) [Mukhopadhya, 2008]. Geographic pattern of HCV prevalence is shown in figure 1.4. It has been estimated that the global prevalence of Hepatitis C virus (HCV) infection is around 3%, with 170 million persons chronically infected with the virus and 3 to 4 million persons newly infected each year [Kamal, 2008].

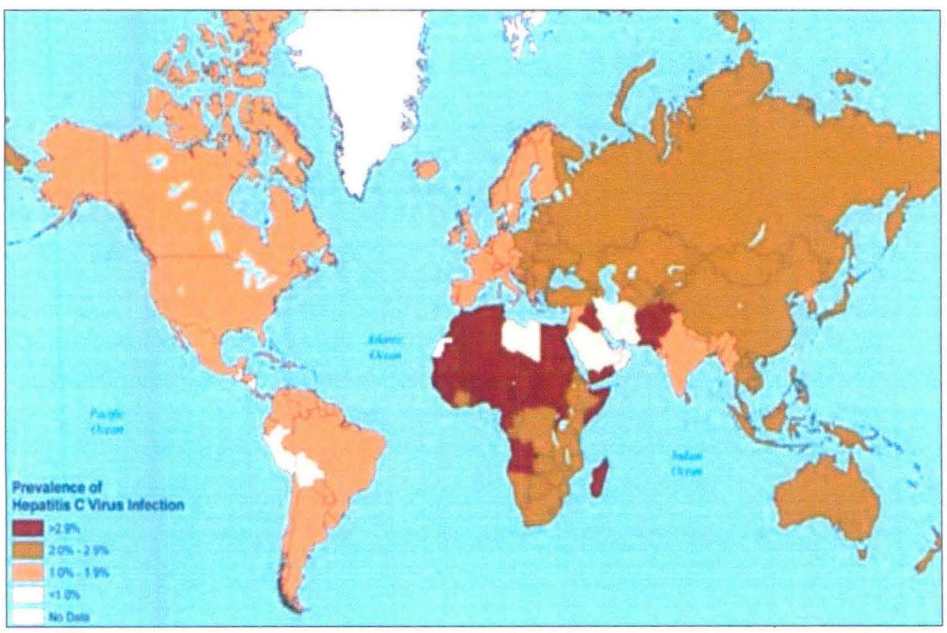

Figure 1.4. Geographic pattern of HCV prevalence

1.3.2. The virus

Hepatitis C virus (HCV), an important human pathogen, is a small (40 to 60 nm in diameter), enveloped RNA virus, classified in the *Hepacivirus* genus of the *Flaviviridae* family. The HCV genome is a single-stranded, positive-sense RNA molecule approximately 9.5 kilo bases (kb) in length, with a single open reading frame encoding a polyprotein of 3,010 to 3,033 amino acid residues, depending upon

the strain. The polyprotein is processed by virus and host-cell encoded proteases, into several structural and non-structural polypeptides. There is also a non-coding region (NCR) of 324-341 nucleotides at the 5' end and a 3' NCR of variable length including a poly (U) tract [Bartosch and Cosset, 2006; Czepiel et al., 2008]. The three structural proteins are the core and two-envelope polypeptides. The several non-structural proteins have various enzymatic functions [Tellinghuisen et al., 2007]. Model structure of HCV and genome organization is shown in Figure 1.5 [Irshad et al., 2008]. Like other RNA viruses, the HCV genome exhibits substantial heterogeneity, which is the result of mutations that occur during viral replication. Within an infected person, HCV exists as a population of closely related yet heterogeneous variants, called quasispecies that result from the rapid development of mutations in the viral genome [Mukhopadhya, 2008].

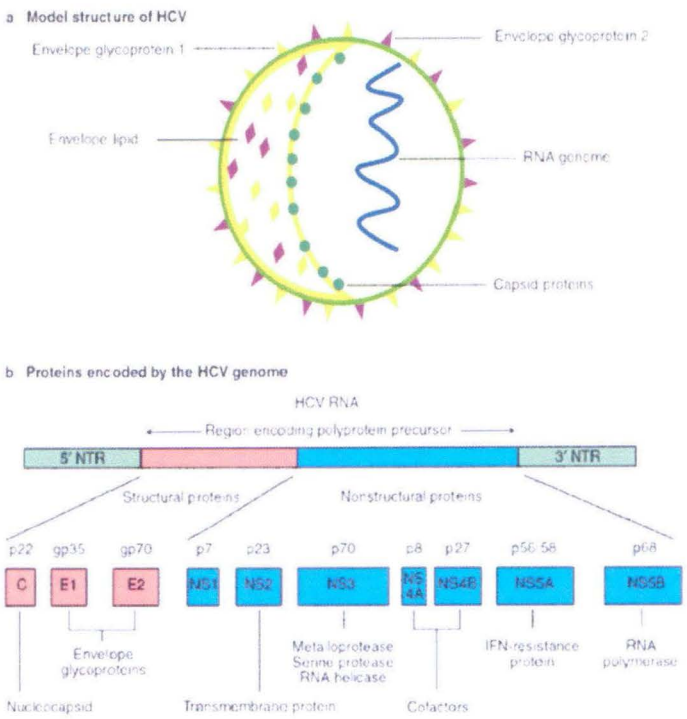

Figure 1.5. Hepatitis C virus (HCV): model structure and genome organization. *(a) Model structure of HCV. The left-hand side of the illustration shows the viral surface of envelope lipids and glycoproteins; the right-hand side shows the RNA genome encapsulated by capsid proteins. (b) Proteins encoded by the HCV genome. HCV is formed by an enveloped particle harboring a plus-strand RNA of ~9.6 kb. The genome carries a long open-reading frame (ORF) encoding a polyprotein precursor of 3010 amino acids. Translation of the HCV ORF is directed via a ~340 nucleotide long 5' non-translated region (NTR) functioning as an internal ribosome entry site; it permits the direct binding of ribosomes in close proximity to the start codon of the ORF. The HCV polyprotein is cleaved co- and post-translationally by cellular and viral proteases into ten different products, with the structural proteins [core (C), E1 and E2] located in the N-terminal third and the nonstructural (NS2–5) replicative proteins in the remainder. Putative functions of the cleavage products are shown.*

1.3.3. Genotypes of HCV and its geographical distribution

There are 6 major (11 total) known genotypes of HCV and many subtypes [Czepiel et al., 2008]. They are dispersed regionally to a certain degree and respond to treatment differently as well. There is high mutation rate in HCV, which is on account of imperfect proof reading ability of the viral RNA-dependent RNA polymerase. As a result, different mutants of the parent strain co-exist as quasispecies in a single infected individual. Genotype of the virus does not appear to influence disease presentation or severity of disease but has been identified as a major predictor of response to antiviral therapy. Most of the reported studies from India seem to suggest a north south divide, wherein genotype 3 predominates in the north, east and west India, whereas genotype 1 is more common in south India [Mukhopadhya, 2008]. The worldwide geographical distribution of HCV genotypes is shown in Figure 1.6.

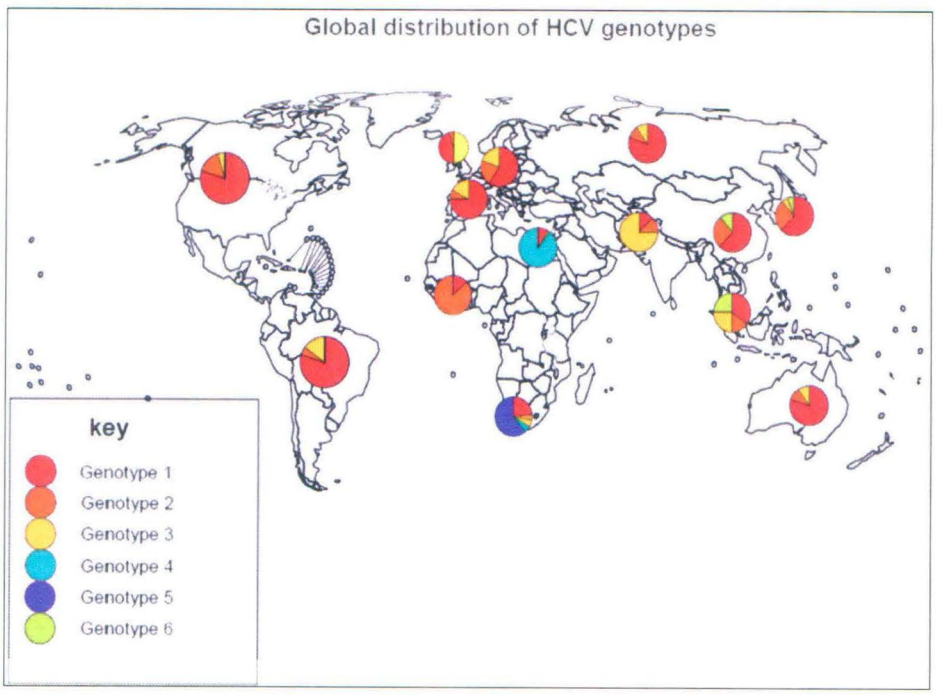

Figure 1.6. HCV diversity in the worldwide epidemic. *The frequency of each HCV genotype was estimated in each country. The pie charts depict the proportion of each genotype in each geographical region.*

1.3.4. Disease

1.3.4.1. Transmission

HCV is spread primarily by contact with blood and blood products. Blood transfusions and the use of shared, unsterilized, or poorly sterilized needles and syringes have been the main causes of the spread of HCV. The major high-risk groups for hepatitis C are people who have frequent exposure to blood products. These include patients with hemophilia, solid-organ transplants, chronic renal failure, or cancer requiring chemotherapy, health care workers who suffer needle-stick accidents, injection drug users, including those who used drugs briefly many years ago and infants born to HCV-infected mothers. Other groups who appear to be at increased risk for hepatitis C are people with high-risk sexual behaviour, multiple partners, sexually transmitted diseases, and tattooing/ body piercing [Czepiel et al., 2008; Kamal, 2008; Mukhopadhya, 2008].

1.3.4.2. Clinical symptoms

The incubation period of HCV is usually 15–150 days from the moment of exposure, with an average of 40–50 days. No characteristic signs or symptoms are reported in10–30% of the cases. The symptoms may include mild stomach discomfort, sometimes flu-like symptoms, myalgia, arthralgia and low degree fever. Jaundice and liver enlargement occur in approximately one third of patients. Serum alanine aminotransferase (ALT) activity is usually elevated and may be 10 times higher than normal. In 70–80% of patients there is a progression into a chronic process. In the course of long-standing chronic hepatitis, gradual damage to the liver develops. Basically, most individuals with chronic hepatitis do not exhibit any symptoms for a long time. The only sign of the disease may be fatigue. Dyspepsia or itching appears rarely. Serum ALT levels are normal or slightly elevated. Infrequently, serum γ-glutamyl transpeptidase, alkaline phosphatase and bilirubin levels are slightly increased. After ~20 years, liver cirrhosis develops and its typical symptoms and complications occur in about 20% of patients. Mortality among patients with liver cirrhosis induced by HCV infection – resulting from the development of portal hypertension, liver failure or primary liver cancer (hepatocellular carcinoma - HCC) –

reaches 2–5% yearly, whereas the prevalence of HCC development is about 1–4% per year [Czepiel et al., 2008].

1.3.4.3. Therapy

Various regimens that have been approved as therapy for hepatitis C are: mono-therapy with alpha interferon; combination-therapy with alpha interferon and ribavirin; mono-therapy with pegylated interferon; and combination-therapy with pegylated interferon and ribavirin (Table 1.3) [Stürmer et al., 2009]. Currently, a combination of pegylated interferon α (PEG-IFN) and ribavirin over a period of 24–48 weeks is used in the treatment of chronic hepatitis C. Introduction of pegylated interferon α has improved the effectiveness of the therapy for HCV infection. PEG-IFN α is the interferon conjugated with polyethylene glycol with a long half-life and improved bio-availability. Due to the introduction of the polyethylene glycol molecule, the drug remains longer in the bloodstream and its blood level is more stable. The effectiveness of treatment with interferon α and ribavirin reaches 37–42%, whereas that of PEG-IFN and ribavirin in patients with a non-1 genotype amounts to 78%, and to 55% in patients with a 1 genotype [Czepiel et al., 2008].

Table 1.3. Time scale of the availability of antiviral drugs for the treatment of HCV

Year	Drugs for the treatment of HCV
1989	HCV, identified by its genome sequence interferon α for treatment[a]
1998	Interferon α + ribavirin
2001	Pegylated interferon α + ribavirin
2009	Albinterferon α -2b

[a] *Interferon delays virus particle production in the cell and alters the immune response, but is not involved in the inhibition of the action of the HCV polymerase or protease*

1.3.4.4. Vaccine

As with any RNA virus, rapidly mutating genomes such as HCV pose a huge obstacle to antiviral/ vaccine development and represent a major challenge to the field [Stoll-Keller et al., 2009]. In addition, HCV acquires a lipid envelope, which originates from host membranes. Thus, the lipid composition of the HCV envelope resembles that of the host cell membrane and this clever mimicry probably allows HCV to avoid

detection by the immune system [Sharma, 2010]. Formidable obstacles remain to be overcome before a generally useful HCV vaccine is available. However, the lack of any other animal model than the chimpanzee, has confounded the development of antiviral compounds active against this agent [Irshad et al., 2008].

1.3.5. Diagnosis

There have been remarkable advances in diagnostic testing for hepatitis C virus (HCV) over the past decade. This has included progressive improvement in both the sensitivity and specificity of tests for antibodies to HCV (anti-HCV). These tests now provide rapid means of identifying individuals who have been infected with hepatitis C. Qualitative and quantitative tests for HCV RNA provide a molecular basis for determining the presence of viremia. Qualitative tests for HCV RNA have become the gold standard of successful antiviral therapy. Finally, determining the HCV genotype and viral load has become increasingly important in guiding the duration of combination therapy with interferon and ribavirin [Scott and Gretch, 2007]. As a result, HCV testing has become the mainstay of both diagnosis and management of patients with hepatitis C [Stürmer et al., 2009]. We review the currently available qualitative and quantitative diagnostic tests for hepatitis C-

1.3.5.1. Qualitative tests

The most commonly used assay for anti-HCV is the enzyme immunoassay (EIA), in which viral antigens are embedded in the wells of a microtiter plate. Antibodies, directed against any of these antigens, in patients' sera, will adhere to the well. Rapid detection of antibodies is facilitated by adding anti-immunoglobulins containing a colorimetric marker. The advantages of this technique include ease of automation, highly reproducible results, and low cost.

First, second and third generation of anti-HCV EIA

In 1989, the scientists at Chiron reported first-generation EIA for HCV using recombinant HCV protein C-100-3, from NS4 region of HCV polyprotein [Kuo et al., 1989]. This immunoassay detected anti-HCV antibodies in 80% of patients developing NANB (Non A Non B hepatitis) after transfusion and in 60% of patients with all the clinical signs of NANB but without an identifiable source of infection.

Many limitations of the first-generation EIA were overcome by the introduction of the second-generation EIA for anti-HCV (EIA-2). This assay contains antigens from the core and non-structural proteins - NS3 and NS4 regions of the HCV genome [Bhattacherjee et al., 1995]. Approximately 92-95% of patients in whom chronic hepatitis C is suspected, can be detected using this second-generation EIA. The use of this test shortened the window from blood transfusion to the first detection of anti-HCV to approximately 10 weeks compared with an average of 16 weeks with the first-generation EIA. An improved version of 2nd generation has been approved by US FDA for screening blood donors. This third generation EIA (EIA-3) contains re-configured core and NS3 antigens and an additional antigen from the NS5 region of the HCV genome [Bresters, 1992]. This test offers a slight improvement in sensitivity over the EIA-2 test, particularly in low-risk settings such as a blood bank. The time from infection to anti-HCV seroconversion is shortened to 7-8 weeks in approximately 30% of patients [Barrera et al., 1995]. However, because of the distinct geographical distribution of the HCV, worldwide, all these EIAs do not perform to the required sensitivity in all parts of the world.

Recombinant immunoblot assays (RIBA)

Because of the high false-positive rate of EIA assays, particularly in low prevalence settings such as blood banks, supplemental tests for anti-HCV have been developed. These tests contain the same antigens as the corresponding EIA assay. However, in the commonly used recombinant immunoblot assays (RIBA, Chiron Corporation, Emeryville, CA) individual HCV antigens are displayed on a nitrocellulose strip. As a result, antibodies against specific HCV antigens can be identified. A positive RIBA assay requires at least two reactive bands. The RIBA 2.0 assay, which contains the same antigens as the EIA-2 assay, has been the most commonly used supplemental assay for anti-HCV. A RIBA 3.0 assay, which has an additional antigen from NS5 region of HCV genome, has been approved for use by blood banks as a supplemental test for EIA-3 positive test results. This test has the advantage over the RIBA-2 assay of fewer indeterminate results and a better correlation with the presence of viremia [Tobler et al., 2000]. However RIBA formats remain extremely expensive, and because of difference in the genotypes of circulating HCV worldwide, RIBA may not perform to similar levels of sensitivity in all parts of the world.

Rapid dot assays

These represent one of the earlier rapid test formats and are usually supplied in kits as individual cassettes with extraction and wash buffers included. The test principle involves a flow of fluid containing the analyte through a porous membrane and into an absorbent pad. A second layer, or sub-membrane, inhibits the immediate back-flow of fluids, which can obscure results. These tests can be used to detect both antibodies and antigens. To detect antibodies and antigens, the corresponding capture is bound or immobilized as a dot or line on the membrane. This reagent "captures" the analyte as it flows through the membrane. Earlier flow-through tests used enzyme immunoassay (EIA) principles to generate signal, but later these tests have successfully used colloidal gold [Damen et al., 1995].

Lateral flow assay system

Lateral flow tests are also called immunochromatographic strip (ICS) tests. ICS tests are used for the specific qualitative or semi-quantitative detection of many analytes including antigens, antibodies, and even the products of nucleic acid amplification tests. One or several analytes can be tested for simultaneously on the same strip. Urine, saliva, serum, plasma, or whole blood can be used as specimens. Extracts of patient exudates or fluids have also been successfully used. To perform the test, a sample is placed on the sample pad on one end of the strip. The tracer reagent is solubilized and binds to the antigen or antibody in the sample and moves through the membrane by capillary action. If specific analyte is present, the tracer reagent binds to it, and a second antibody or antigen, immobilized as a line on the nitrocellulose, then captures the complex. If the test is positive, a pink/purple line develops. All tests include an internal procedural control line that is used to validate the test result. Appearance of two lines, therefore, indicates a positive result, while a negative test produces only one line.

1.3.5.2. Quantitative tests

There are 3 types of tests available for assessing the quantity of circulating HCV RNA in patients with chronic hepatitis C: quantitative reverse transcription– PCR, real-time PCR, and branched-chain DNA (b-DNA). Quantitative PCR tests include MONITOR 2.0 (Roche Diagnostics) and SuperQuant (National Genetics Institute); they provide

comparable results. The b-DNA method differs from reverse transcription–PCR tests in that the detection signal is amplified rather than target RNA. The third generation assay (VERSANT b-DNA 3.0, Bayer Corp, Tarrytown, NY) has a lower limit of detection of 615 IU/mL and an upper range of 7.7 million IU/mL. It is highly reproducible and the specificity ranges from 96% to 98.8%. A critically important advance in molecular diagnostics has been the adaptation of real-time PCR methods to quantify HCV RNA. Using TaqMan technology, real-time PCR yields quantitative results with comparable sensitivity to qualitative tests. In addition, real-time PCR can accurately quantify HCV RNA levels over a linear range exceeding 6 logs (ie, 10 IU/mL to 100 million IU/mL) for purposes of therapeutic monitoring. Therefore, a single test result serves the purpose of both quantitative and qualitative HCV NATs. The assay is faster and more cost-effective than the other techniques and has already replaced other NAT testing platforms at many institutions. However, real-time PCR assays are presently available only as in-house tests. Because the initial HCV RNA quantification techniques reported results in different units, direct comparisons were often difficult. With the adoption of a World Health Organization international standard, units from different assays are now interconvertible. However, because there is still variability between the various assays, it is recommended that clinicians use the same assay throughout the treatment course of any given patient [Scott and Gretch, 2007].

1.3.5.3. Currently available kits

Currently, there are several diagnostic kits for HCV, available in the market. Some of them are described in table 1.4.

Table 1.4. Diagnostic kits for HCV

Manufacturer Format	Kit Antigens	Number of antigens	Assay Type
Ortho HCV 3.0 (Ortho diagnostics)	C22-3, c200 and NS5	3	Indirect EIA
Abbott HCV EIA 3.0 (Abbott Laboratories)	HC-43, c100-3, NS5	3	Indirect EIA
Abbott HCV EIA 2.0	C100-3, HC-31, HC-34 coated beads	3	Solid phase polystyrene beads, Indirect EIA
Abbott IMx HCV MEIA	C200, c22-3, HC34, HC 31	4	For use on the IMx system
Chiron RIBA HCV 3.0	C33c, NS5, 5-1-1, c100, c22	5	SIA (Strip immunoblot assay)
Abbott Matrix HCV	Abbott NS4 (yeast), NS4 (E.coli), NS3, Core	3	Immunoblot, For use on matrix system
Monolisa Anti-HCV (Sanofi diagnostics)	Core, r-NS 3, NS 4	3	Indirect EIA

1.4. Hepatitis B Virus

1.4.1. History and current epidemic patterns

Hepatitis B virus (HBV) was identified in 1970 and is one of the most widespread and pathogenic viruses in the world, causing liver cirrhosis and hepatocellular carcinoma after years or decades of chronic infection [Allain et al., 2009]. Hepatitis B virus is one of the major global public health problems. HBV infection is the 10th leading cause of death and HBV related hepatocellular carcinoma (HCC) is the 5th most frequent cancer worldwide. It is estimated that approximately 2 billion people – one third of the world's population has serological evidence of current or past infection with HBV. Of these, an estimated 350 million are chronically infected with HBV and approximately 1 million persons die annually from HBV-related chronic liver diseases, including severe complications such as liver cirrhosis (LC) and HCC [Datta, 2008]. Geographic pattern of HBsAg prevalence is shown in Figure 1.7. Prevalence of HBsAg ranges between 0.1% and 25% in populations of blood donors, the highest being found in West Africa, the lowest in Western Europe and North America [Allain et al., 2009].

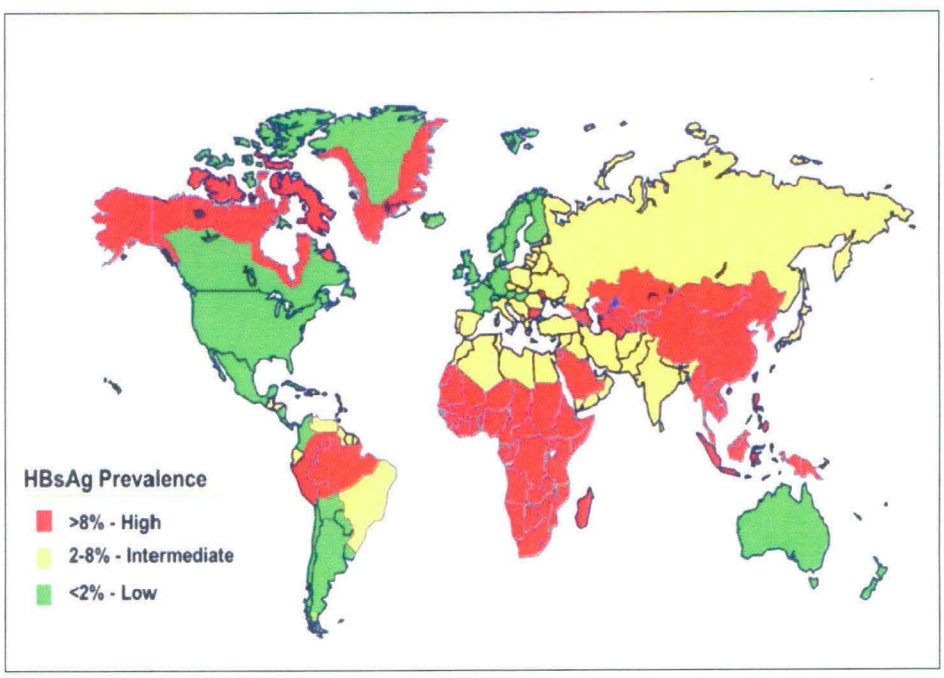

Figure 1.7. Geographic pattern of HBsAg prevalence

1.4.2. The virus

The HBV is a small DNA virus with unusual features similar to retroviruses. It is a prototype virus of the *Hepadnaviridae* family. Related viruses are found in woodchucks, ground squirrels, tree squirrels, Peking ducks, and herons. Based on sequence comparison, HBV is classified into eight genotypes, A to H. Each genotype has a distinct geographic distribution. The infectious HBV virion (Dane particle) has a spherical, double-shelled structure 42 nm in diameter, consisting of a lipid envelope containing hepatitis B surface antigen (HBsAg) that surrounds an inner nucleocapsid composed of hepatitis B core antigen (HBcAg) complexed with virally encoded polymerase and the viral DNA genome [Liang, 2009]. The genome of HBV is a partially double-stranded circular DNA of about 3.2 kilobase (kb) pairs. The partially double-stranded HBV contains four partly overlapping open reading frames (ORFs): '*C*' that encodes for core protein (HBcAg) and 'e' antigen (HBeAg); '*P*' for polymerase (reverse transcriptase) protein (POL), '*S*' for envelope proteins (the three forms of HBsAg known as small (S), middle (M) and large (L)) and '*X*' for a transcriptional *trans*-activator protein (Figure 1.8) [Echevarria and Avellon, 2006; Liang, 2009]

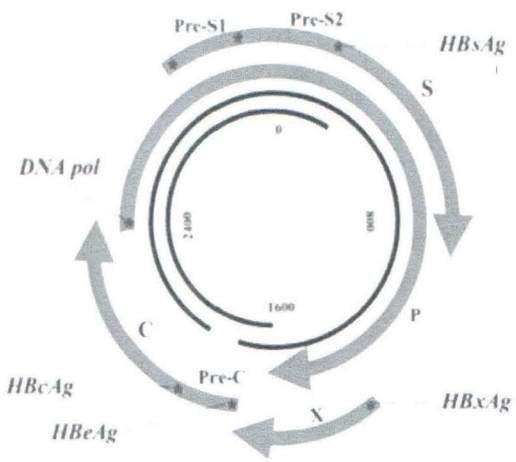

Figure 1.8. Organization of the HBV genome, consisting of four overlapping ORFs - C, P, S and X. *Some of them have two or three codons for initiation of transcription (asterisks) and may synthesize different viral proteins that share common amino acid sequences.*

1.4.3. Genotypes of HBV and its geographical distribution

Eight genotypes of hepatitis B virus (A–H) are currently recognized, and subgenotypes have recently been described in four of these genotypes (A, B, C and F). The genotypes show a distinct geographical distribution between and even within

regions (Figure 1.9) [Kramvis et al., 2005]. Genomic hypervariability can be viewed as a defense mechanism that allows HBV to escape selection pressures imposed by antiviral therapies, vaccines and the host immune system. It is also responsible for the worldwide genetic diversity of HBV strains that separates the virus into genotypes, subgenotypes and subtypes (Table 1.5) [Hollinger, 2007].

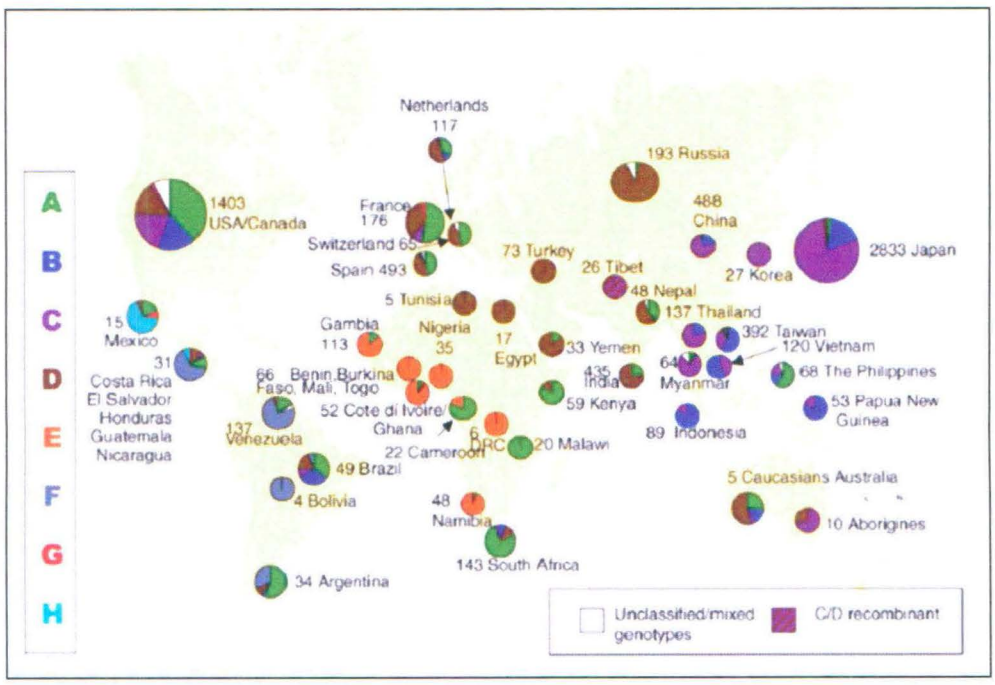

Figure 1.9 Global distribution of the eight genotypes of hepatitis B virus. *The numbers next to the pie charts are the number of isolates genotyped. The size of the pie chart was adjusted so that all the segments of the pie chart are visible.*

Table 1.5. Genotypes and subtypes of HBV and their predominant geographical locations

Genotypes	Subtypes	Geographical locations
A	adw2, ayw1	Europe, India, Africa, N. America
B	adw2, ayw1	Asia
C	adw2, adr, ayr	Asia
D	ayw2, ayw3	Worldwide
E	ayw4	W. Africa, Madagascar
F	adw4	Central America, S. America
G	adw2	S. America, Europe
H	adw4	Central America, US

1.4.4. Disease

1.4.4.1. Transmission

Hepatitis B virus is transmitted by contact with the blood or other body fluids (i.e. semen and vaginal fluid) of an infected person. Modes of transmission are the same for the human immunodeficiency virus (HIV), but HBV is 50 to 100 times more infectious. Unlike HIV, HBV can survive outside the body for at least 7 days. During that time, the virus can still cause infection if it enters the body of an uninfected person. Common modes of transmission in developing countries are perinatal (from mother to baby at birth), early childhood infections (inapparent infection through close interpersonal contact with infected household contacts), unsafe injections practices, blood transfusions and sexual contact [WHO, 2008]. In many developed countries (e.g. those in Western Europe and North America), patterns of transmission are different than those mentioned above. Today, the majority of infections in these countries are transmitted during young adulthood by sexual activity and intravenous drug injections. HBV is a major infectious occupational hazard of health workers. HBV is not spread by contaminated food or water, and cannot be spread casually in the workplace. The virus incubation period is 90 days on average, but can vary from about 30 to 180 days. HBV may be detected 30 to 60 days after infection and persist for widely variable periods of time.

1.4.4.2. Clinical symptoms

Hepatitis B virus can cause an acute illness with symptoms that last several weeks, including yellowing of the skin and eyes (jaundice), dark urine, extreme fatigue, nausea, vomiting and abdominal pain. People can take several months to a year to recover from the symptoms [WHO, 2008]. Infected individuals with acute hepatitis who have persistently (>6 months) high levels of HBsAg or those who maintain HBeAg positivity after symptoms resolve, are likely to develop chronic infection. In patients who develop chronic hepatitis B (CHB), the acute stages of infection are often asymptomatic. The clinical course of chronic infection can be variable, with the activity of the disease fluctuating over time. In a proportion of patients, the disease can be rapidly progressive, and cirrhosis may be established within a decade of initial infection. Patients who fail to clear the virus and continue to have active hepatitis are

at increased risk of developing cirrhosis and hepatocellular carcinoma (HCC) [Bowden, 2006].

1.4.4.3. Therapy

There is no specific treatment for acute hepatitis B. Care is aimed at maintaining comfort and adequate nutritional balance, including replacement of fluids that are lost from vomiting and diarrhoea. Chronic hepatitis B can be treated with drugs, including interferon and anti-viral agents (Table 1.6) [Stürmer et al., 2009].

Table 1.6. Time scale of the availability of antiviral drugs for the treatment of HBV

Year	Polymerase inhibitor of HBV
1992	Interferon α [a]
2000	Lamivudine
2003	Adefovir
2005	Pegylated interferon α
2007	Entecavir, Telbivudine
2008	Tenofovir
2009	Clevudine

[a] *Interferon delays virus particle production in the cell and alters the immune response, but is not involved in the inhibition of the action of the HBV polymerase.*

1.4.4.4. Vaccine

Plasma derived

The first available vaccines were produced by harvesting the hepatitis B surface antigen (HBsAg) from plasma of chronic HBsAg carriers. These plasma-derived hepatitis B vaccines were developed in France and the USA and became commercially available between 1981 and 1982. These immunogenic vaccines contained highly purified ~ 22 nm HBsAg particles inactivated through a combination of urea, pepsin, formaldehyde, and heat. Because of safety concerns, the plasma derived vaccines are no more in use.

Recombinant DNA technology derived

Several vaccine manufacturers used recombinant DNA technology to express HBsAg in HBV transfected yeasts (i.e. *Saccharomyces cerevisiae*). This led to the development of recombinant DNA hepatitis B vaccines, the so-called "second" generation hepatitis B vaccines. These, by now the mostly used recombinant hepatitis B vaccines, were commercialized in 1986. The small envelope S protein is released from the transfected yeasts and purified to eliminate the yeast components by several physical separation techniques (i.e. filtration, chromatography). The expressed HBsAg polypeptides then self assemble into immunogenic spherical particles almost identical to the 22-nm viral-like particles found in serum of hepatitis B carriers. These particles can act as passport to immune recognition [Grgacic and Anderson, 2006]. These can form aggregates of polymeric forms [Tleugabulova et al., 1998]. Currently used yeast-derived hepatitis B vaccines are highly efficacious and safe and widely used in all five continents. Long-term immune memory against the small HBsAg (also termed *S antigen*) was unequivocally demonstrated in vaccines using plasma- and yeast-derived first- and second-generation hepatitis B vaccines, respectively. During the 1990s, the so-called third-generation hepatitis B vaccines were developed in HBV transfected mammalian cells. Third-generation recombinant triple-antigen (pre-S1, pre-S2 and S) vaccines have been reported to be effective for revaccination of people who had an inadequate response to current vaccines [Rendi-Wagner et al., 2006; Zuckerman et al., 2001] or in immuno-suppressed HBV patients after liver transplantation [Lo et al., 2007].

1.4.5. Diagnosis

HBV surface antigen (HBsAg) is the early detection marker that is being used to detect HBV in blood donors [Hollinger, 2007]. The development of serological assays to detect hepatitis B surface antigen (HBsAg) has played a major role in the diagnosis of hepatitis B virus (HBV) infection. With other hepatitis B serological assays, a diagnosis of acute or chronic HBV infection, past infection, or successful vaccination can be determined. However, serological profiles can at times be atypical or ambiguous. Some of these difficulties may be overcome by HBV DNA testing, which may elucidate an individual's true hepatitis B status [Bowden, 2006]. There are a number of antigen and antibody tests used to assess the phase of infection in persons

with chronic HBV. Additionally, the level of virus in the blood can be measured to give a viral load result. These tests are essential in determining if and when to treat and are also used during treatment to determine when treatment can be stopped. The qualitative and quantitative tests for the detection of HBV infection are as follows-

1.4.5.1. Qualitative tests

There are several serologic markers for the detection of HBV infection e.g. HBsAg, HBeAg, Anti-HBs (or HBsAb: antibody against HBsAg), Anti-HBe (or HBeAb: antibody against HBeAg) and Anti-HBc. The envelope protein of the virus, HBsAg (Hepatitis B virus surface antigen), is the primary serological marker for diagnosis of HBV infection. During the typical course of HBV infection, HBsAg increases in concentration during the acute phase of disease, then declines and becomes undetectable as virus-neutralizing antibodies (anti-HBs) appear and infection resolves. In about 5% of infected adults and 90% of childhood cases, the virus establishes a chronic infection marked by continued positivity for HBsAg. The first detectable antibody after infection is the antibody to the HBV core antigen (anti-HBc–IgG and IgM). Anti-HBc IgG remains detectable both in cases where chronic infection is established and after resolution of infection, and thus its presence is indicative of either current acute, current chronic or past infection.

Prevention of transfusion-transmitted HBV has historically relied on serologic screening of blood and plasma donations for HBsAg, beginning with first generation HBsAg assays introduced in 1971. There are >40 commercially available HBsAg assays currently in use around the world and they vary significantly in format and sensitivity. Assays may utilize single or multiple monoclonal antibodies and/or polyclonal antibodies to capture and detect antigen in a variety of formats including enzyme immunoassays (EIAs), agglutination methods, and microparticle assays with fluorescent or chemiluminescent detection systems. Sensitivity of HBsAg assays is expressed in nanograms (ng) of surface antigen per mL. The most sensitive assays detect HBsAg levels ≤0.1 ng/mL [Kuhns and Busch, 2006].

In addition to screening blood donations for HBsAg, some countries (e. g. the US) also screen for anti-HBc to detect anti-HBc reactive chronic carriers with low level viremia who lack detectable HBsAg. Tests for detection of anti-HBc antibodies vary

in format, with some assays constructed as direct, sandwich assays while others use an inhibition assay approach. Total anti-HBc assays typically detect both IgG and IgM antibody classes, in contrast to anti-HBc IgM tests, which are used to identify acute HBV infection in the diagnostic setting. Countries that are highly endemic for HBV typically do not screen for anti-HBc [Kuhns and Busch, 2006].

1.4.5.2. Quantitative tests

New commercial assays with wider dynamic ranges and lower limits of detection have now become available, and a World Health Organization (WHO) international standard for HBV DNA has been established, allowing for comparisons of assay results expressed as international units per milliliter (U/mL). HBV DNA detection and quantification offers several advantages other than being an adjunct to serological testing. Recent data indicate that there is a direct association between HBV viral load and the development of HCC and the risk of HCC increases with increasing HBV load. HBV viral load also provides the most useful parameter for determining eligibility for antiviral therapy and for monitoring the effectiveness of therapy. Quantification is achieved in the AMPLICOR HBV MONITOR test (Roche Diagnostic Systems, Branchburg, NJ) by the simultaneous coamplification of a quantification standard with the HBV DNA in the test sample. The Roche COBAS TaqMan HBV Test is one of the new-generation real-time PCR assays that measures the DNA product in the early exponential phase of amplification when the reaction is at its most efficient. The ability to monitor accumulating PCR product in real time has been brought about by using fluorescent chemistry. The signal is proportional to the amount of PCR product present and greatly increases the dynamic range of the assay. Another real-time assay, the RealArt HBV PCR kit (formerly Artus-Biotech and now marketed through QIAGEN Diagnostics, Hamburg, Germany), contains ready-to-use reagents specifically designed for different real-time platforms. Reagents are provided for use on the ABI Prism instruments (Applied Biosystems, Foster City, CA) employing the TaqMan technology. For the LightCycler instrument (Roche Diagnostics), the assay uses the fluorescence resonance energy transfer (FRET) technology to monitor the fluorescence intensity. Molecular assays for HBV DNA can be useful for resolving ambiguous serological patterns, but the major role of HBV DNA assays is their use in patients undergoing antiviral therapy. HBV load is

important for determining treatment eligibility, monitoring treatment efficacy, and identifying the early development of resistance. In addition, the analysis of viral dynamics on therapy may provide an early indication of therapy outcome and determine which treatments are optimal [Bowden, 2006].

1.4.5.3. Currently available kits

Currently, there are several diagnostic kits available in the market for the detection of HBsAg. These kits use monoclonal and/or polyclonal antibodies for the detection of HBsAg in a sandwich immunoassay format. Some of these kits are: Prism HBsAg (Abbott GmbH & Co, KG), Enzygnost HBsAg 5.0 (Siemens Healthcare Diagnostics Products GmbH), Advia Centaur HBsAg (Siemens Healthcare Diagnostics Inc.), Elecsys HBsAg II (Roche Diagnostics GmbH), Murex HBsAg Version 3 (Abbott Murex Biotech, Ltd.), DS-IFA-HBsAg (RPC Diagnostic Systems), Architect HBsAg (Abbott GmbH & Co, KG) , AxSYM V2 (Abbott GmbH & Co, KG), Hepascan HBsAg Immunoenzyme TS (BioService Borovsk), EIAgen HBsAg Kit (Adaltis Italia S.p.A.), ETI-MAK 4 HBsAg EIA (Diasorin S.p.A.), Monolisa HBsAg Ultra (Bio-Rad Laboratories, Inc.), Vectohep B - HBs-antigen (Vector Best Novosibirsk).

1.5. Overview of the expression systems for production of recombinant protein

The use of recombinant DNA technology to express polypeptides in foreign host cells successfully opened the way for production of large quantities of proteins by genetic engineering. When selecting a protein expression system, a number of considerations must be made, including the intended use, time frame, availability of resources, and the characteristics of the recombinant product. The choice depends on factors such as the need for post-translational processing to obtain a biologically active product, toxicity of the recombinant product on the host and the amount of protein needed. These considerations affect the choice of expression system and type of promoter to be used [AnandaRao et al., 2006; Yang et al., 2004; Yang et al., 2009]. Comparison of the major characteristics of different expression systems is provided in Table 1.7.

Table 1.7. Comparison of expression systems

Desired characteristics	Bacteria	Yeast	Insect	Mammalian Cell culture
Cell growth	Rapid	Rapid	Slow	Slow
Complexity of growth Medium	Minimum	Minimum	Complex	Complex
Cost of the growth medium	Low	Low	High	High
Expression level	High	Low to High	Low to High	Low to Moderate
Extra cellular Expression	Secretion to periplasm	Secretion into medium	Secretion into medium	Secretion into Medium
Post-Translational Modifications				
Protein folding	Refolding usually required	Refolding maybe required	Proper folding	Proper folding
N-linked glycosylation	None	High mannose	Simple, no sialic acid	Complex
O-linked glycosylation	No	Yes	Yes	Yes
Phoshorylation	No	Yes	Yes	Yes
Acetylation	No	Yes	Yes	Yes
Acylation	No	Yes	Yes	Yes
γ-Carboxylation	No	No	No	Yes

Expression in *E. coli* is often used for the production of large quantities of protein because high expression levels can be obtained. Moreover, expression in *E. coli* is inexpensive, can be regulated fairly easily, and some systems are designed to facilitate purification. Among the many systems for protein production, the Gram-negative

bacterium *E. coli* remains most attractive because of its ability to grow rapidly and at high density on inexpensive substrates, its well characterized genetics and the availability of large number of cloning vectors and mutant host strains [Ashraf et al., 2004; Santala and Lamminmäki, 2004]. Most of the *E. coli* expression vectors used for high-level protein production contain regulated promoters that are based on the lac operon. Under optimal conditions, up to 10-40% of the total cellular protein, in an IPTG-induced or heat induced *E. coli* culture, can be the protein product of the cloned gene [Chugh et al., 2006; Talha et al., 2010]. Many types of *E. coli* expression vectors are designed to produce fusion proteins that contain affinity 'tags' at the amino or carboxy terminus of the expressed protein. Examples of commonly used affinity tags in *E. coli* expression systems are shown in Table 1.8.

Table 1.8. Affinity tags used in *E. coli* expression systems

Affinity tag	Size	Affinity ligand for purification
6xHis tag	0.84 kDa	Ni-NTA-Agarose
Glutathione S-transferase	26 kDa	Glutathione-Sepharose
Protein A	30 kDa	IgG-Sepharose
Maltose binding protein	40 kDa	Amylose-Sepharose

1.6. *In vivo* biotinylation of recombinant proteins

The high affinity binding interaction of biotin to avidin or streptavidin has been used widely in biochemistry and molecular biology. Key features of the interaction between biotin and avidin/ streptavidin are its extraordinarily high affinity (i.e. K_d = 10^{-15} M) and extremely slow dissociation rate [Choi-Rhee et al., 2004]. These properties have been exploited by researchers who have attached biotin 'tags' to proteins for protein detection, purification purposes, immobilization of proteins, co-immunoprecipitation studies or to monitor specific protein–protein interactions [Ashraf et al., 2004]. Usually *in vitro* protein biotinylation is performed for these applications, whereby lysine residues of target proteins are modified by chemical agents such as biotin-N-hydroxysuccinimide or similar acylating agents. A limitation of this approach, however, is that such chemical techniques may disrupt the protein conformation and/or its biological function, particularly if critical lysine residues are modified. An alternative site-specific *in vivo* biotinylation procedure would clearly be

superior [Smith et al., 1998]. There are examples, in nature, of proteins which are specifically biotinylated *in vivo*. These proteins typically act as biotin transporters and are involved in metabolic carboxylate transfer processes within the cell. *E. coli* contains only one such biotinylated protein, the biotin carboxyl carrier protein (BCCP), a subunit of acetyl-CoA carboxylase. BCCP is specifically biotinylated by biotin holoenzyme synthetase (BirA), the product of the chromosomal *birA* gene [Santala and Lamminmäki, 2004]. BirA functions by catalyzing activation of biotin by ATP to form biotin-5'-adenylate, with subsequent covalent linkage of the biotin moiety to the ε-NH2 group of a specific BCCP lysine residue [Smith et al., 1998]. BAP (biotin acceptor peptide), a 24 amino acid (aa) sequence, is a part of BCCP and is the minimal peptide substrate for *in vivo* biotinylation by BirA. BirA biotinylates the 13th aa, which is a lysine, of the BAP [Tsao et al., 1996]. Biotinylation with BirA enzyme at a single lysine residue solves several problems associated with traditional chemical-mediated biotinylation method, including random and heterogeneous modification, inactivation of protein biological function, cross-linking, and aggregation after mixing with streptavidin or avidin [Yang et al., 2004]. The chief advantage of this approach is that, unlike chemical reagents, enzymatic biotinylation assures that all molecules will be immobilized in a uniform, bioactive orientation on a streptavin coated microtiter plate [Tsao et al., 1996].

1.7. Streptavidin: highly functional and dense solid phase matrix

The multiplexing objective requires surfaces of high functional capacity and stability while at the same time having insignificant non-specific interactions. Most of the presently used immunoassays utilize binding of a specific antigen or antibody on a solid phase. Commonly used solid phases are 96-well polystyrene microtiter plate. For the immunoassay purposes, the microtitration well surfaces are passively coated with capturing antigen or antibody to allow analyte immobilization. Passive coating of capture antigen/ antibody on the solid surface exhibits several disadvantages, e.g. (i) solid phases could be heterogeneous in their binding behaviour, causing major problems with reproducibility of binding capacity (ii) the binding sites of coated capture molecule could be less accessible than those in solution, due to the coating process (iii) binding avidity of the capturing agent could be decreased causing weaker

binding, leading to faster dissociation of the antigen-antibody bonds and lower sensitivity of the tests [Schetters, 1999].

Direct adsorption of antibodies on the plastic surface may destroy the functional sites to even less than one-tenth of the original activity. Studies have shown that the protein activity could be preserved when the capturing antibody was immobilized as a secondary layer over a primary coated layer such as via streptavidin-biotin linkage. In addition, streptavidin-coated wells provide a universal immobilization surface for any biotinylated molecule and therefore tedious optimization of adsorption conditions for a number of different antibodies or antigens could be avoided [Välimaa et al., 2003].

Avidin and streptavidin have extremely high affinity towards biotin (K_A $1.7x10^{15}$ and $2.5x10^{13}$ M^{-1}, respectively) [Huhtinen et al., 2004]. The formation (affinity) constant of avidin or streptavidin with biotin, is among the highest formation constants reported, which is about 10^3–10^6 times greater than for the interaction of ligands with their specific antibodies. This high affinity ensures that, once formed, the complex is not disturbed by changes in pH, the presence of chaotropes, or manipulations such as multiple washings when the complex is immobilized. Avidin or streptavidin binding to biotin is specific enough to ensure that the binding is directed only to the target of interest. Biotin is a small molecule (244.31 Da) that, when introduced into biologically active macromolecules, in most cases does not affect their biological activity [Diamandis and Christopoulos, 1991]. In addition, avidin and streptavidin permit radical chemical modifications without losing the biotin-binding characteristics [Huhtinen et al., 2004].

Streptavidin-coated plates can be utilized for immobilization of biotinylated capture antibodies, antigens, nucleic acids and even small size biotinylated molecules such as biotinylated peptides and oligonucleotides, which are otherwise difficult to immobilize directly. In addition to these general uses, an advantage of streptavidin-coated surface is the better preserved biological activity of the immobilized molecule compared to direct passive adsorption [Välimaa and Laurikainen, 2006]. Streptavidin has four binding sites for biotin. Because of adsorption of streptavidin to the solid phase or covalent binding to the carrier, one or two of these sites might be lost but still two to three remain active. The binding capacity for the biotinylated antibody or

antigen is at least doubled, resulting in higher surface density of biotinylated capture molecule [Schetters, 1999]. Generally, the streptavidin-biotin technology provides a valuable tool in clinical diagnostics like immobilization of the molecules on the solid phase or signal amplification. Therapeutic applications of biotin-streptavidin use are, e.g., delivery of drug molecules to pre-targeted cancer cells [Välimaa et al., 2003].

1.8. Lanthanide chelates and nanoparticles

Different from organic fluorescence dyes, fluorescent lanthanide complexes of europium (Eu), terbium (Tb), samarium (Sm), and dysprosium (Dy) (Figure 1.10) have the fluorescence properties of long fluorescence lifetime, large Stokes shift and sharp emission profile, which make them favourable to be used as the fluorescent labeling reagents for microsecond time-resolved fluorescence bioassay. Lanthanide complex-based fluorescence labels have been successfully used for highly sensitive time-resolved fluorescence immunoassay, DNA hybridization assay, cell activity assay, and bio-imaging microscopy assay. Since the technique allows easy distinction of the specific fluorescence signal of the long-lived label from short-lived background noises associated with biological samples, scattering lights (Tyndall, Rayleigh and Raman scatterings) and the optical components (cuvettes, filters and lenses), the sensitivity of fluorescence bioassay has been remarkably improved. Lanthanide reagents can take advantage of time-resolved fluorescent technology [Yuan and Wang, 2005]. Further advancement in the field of lanthanide reagents is the use of nanoparticles. These nanoparticles are doped with several thousand lanthanide chelates, which enhance the signal tremendously and thus increase the sensitivity [Valanne et al., 2005; Xia et al., 2009].

58	59	60	61	62	63	64	65	66	67	68	69	70	71
Ce	Pr	Nd	Pm	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu

Figure 1.10. Sm, Eu, Tb and Dy belong to Lanthanide series of Periodic Table. *Atomic no. of each lanthanide is shown at the top in each cell. Complexes of lanthanides allow time-resolved fluorescence measurement, which reduces background fluorescence and increases sensitivity of the assay*

1.8.1. Long lifetime of fluorescence

Compared with organic fluorescence dyes, fluorescent lanthanide complexes have very long fluorescence lifetime (usually over several hundred microseconds or more than 1 ms for Eu(III) and Tb(III) chelates, and 10–100 μs for Sm(III) and Dy(III) chelates (Figure 1.11A) [Yuan and Wang, 2005]. Time-resolved fluorometry (TRF), employing long-lifetime fluorescent lanthanide chelate labels, has been routinely applied in *in vitro* diagnostics for two decades. The benefits of TRF technology are based on the spectral and temporal resolution of label detection allowing highly sensitive assays of biological samples. In addition, the fluorescence has a long lifetime enabling a delayed fluorescence measurement on a scale of microseconds (Figure 1.11B). The delayed measurement increases the sensitivity of the assay since most of the background fluorescence occurring due to biological materials has a short lifetime that decays prior to triggering the detection [Huhtinen et al., 2005].

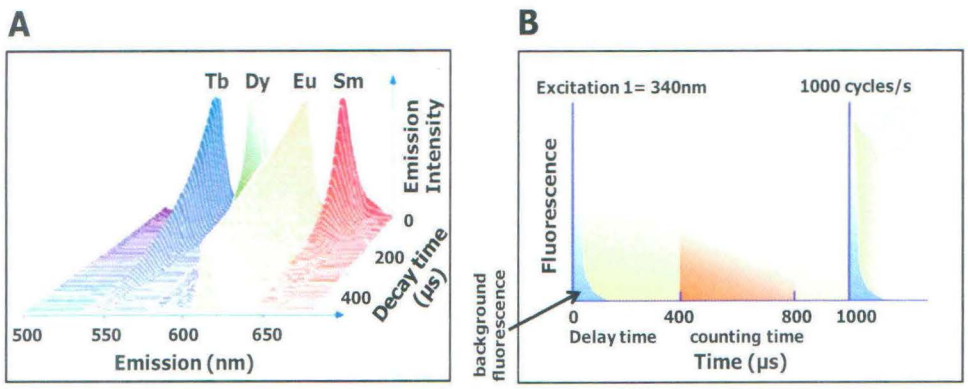

Figure 1.11. Long lifetime of lanthanide chelates and time-resolved fluorometric measurement *(A) Decay of fluorescence of Tb, Dy, Eu and Sm with time is shown, their emission maxima are also shown at respective wavelengths. (B) Principle of the time-resolved fluorometric measurement with a delay time of 400 μs, a counting time of 400 μs, and a cycle time of 1000 μs. In one second 1000 cycles of time-resolved fluorometric measurement can be completed.*

1.8.2. Large stokes shift

Stokes shift is the difference in wavelength between positions of the band maxima of the absorption and emission spectra of the same electronic transition. It is named after Irish physicist George G. Stokes. When a system absorbs a photon, it gains energy and enters an excited state. One way for the system to relax is to emit a photon, thus losing its energy. When the emitted photon has less energy than the absorbed photon, this energy difference is the Stokes shift (Figure 1.12). The fluorescence of lanthanide

chelates features a wide Stokes shift and a narrow emission band [Huhtinen et al., 2005]. Fluorescent lanthanide complexes have absorption maxima generally below 400 nm, and emit fluorescence maximally around 615, 545, 643, and 574 nm for Eu(III), Tb(III), Sm(III), and Dy(III)chelates, respectively. Fluorescent lanthanide complexes have a very sharp emission profile with the full width at half maximum of ~10 nm [Yuan and Wang, 2005].

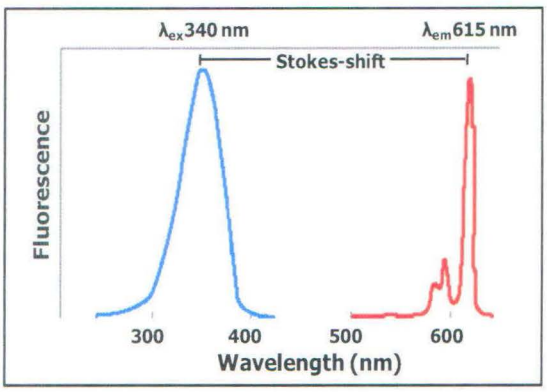

Figure 1.12. Excitation and emission maxima of europium(III) chelate. *The difference in wavelengths of excitation and emission maxima is called stokes shift, which is ~275 nm in case of europium(III) chelate.*

1.8.3. Europium(III) chelate

The intrinsically fluorescent nonadentate europium(III) chelate was used in this study, hereafter this will be referred to as europium(III) chelate. The chelate is highly fluorescent, soluble in water, and provides effective shielding of europium from water. The mentioned chelate forms a nine-coordinate complex with the metal ion (Figure 1.13), thus providing high thermodynamic stability and minimal fluorescence quenching by water. Due to the two sugar side groups incorporated with the chelate structure, the complex is soluble in water which facilitates a gentle and efficient coupling via the isothiocyanate group of the chelate and decreases the tendency of nonspecific binding. The synthesis and full description of fluorescent properties of the europium(III) chelate has been described earlier [Lode et al., 2003; Pettersson et al., 2003]. The use of fluorescent europium(III) chelate enables simplified, rapid and universal test protocols to be constructed for a wide range of analytical applications because of the ease of labeling with europium(III) chelate and easy measurement of fluorescence directly from the dry wells without adding any substrate or stopping the reaction. Europium chelates have a long lifetime of fluorescence, which can be

specifically detected through the time-resolved measurement. Thus, avoiding disturbance from sources of short-lived fluorescence, this gives a high signal to background ratio, making the system highly sensitive [Lode et al., 2003; Pettersson et al., 2003; Talha et al., 2010].

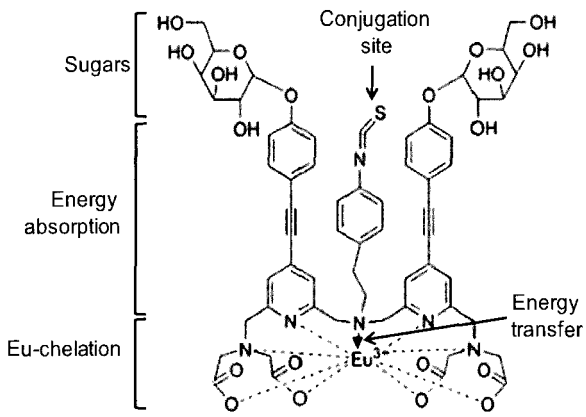

Figure 1.13. Molecular structure of intrinsically fluorescent nonadentate europium(III) chelate. *The different functional parts of the chelates (energy absorption, energy transfer, metal chelation, conjugation site) are indicated in the figure. An isothiocyanate (NCS) group is used for conjugation to primary amino groups of proteins.*

1.8.4. Europium(III) chelate doped nanoparticles

Most common strategies utilized for improvement of the detection limit in an immunoassay have been amplifying labels and multiple labeling [Kricka, 1994]. The use of multiple labeling to maximize the number of labels attached to a biomolecules has been limited due to the interference of the labels to the antigen-antibody binding and increased fractional non-specific binding. 107 nm polystyrene europium(III) nanoparticle, used as a label in this study, is doped with ~30 000 europium(III) chelates, thus the use of europium(III) nanoparticle tremendously enhances the signals and the bulkiness of the label is compensated by the multiple biomolecules covalently linked on the surface of a single nanoparticle [Härmä et al., 2001; Soukka et al., 2001]. The fluorescence properties and standardization of the europium (III) chelate-doped Fluoro-Max^{TM} polystyrene nanoparticles (107 nm in diameter, >30 000 chelates per particle) (Seradyn Inc., Indianapolis, IN) have been fully described in previous publications [Härmä et al., 2001; Soukka et al., 2001]. Europium(III) chelate-doped polystyrene nanoparticles are known to enable high sensitivity of the assay [Härmä et al., 2001; Valanne et al., 2005]. The nanoparticle approach enabled an immediate surface-readout measurement from the excitation area without any

enhancement or amplification step. The use of europium(III) nanoparticle label technology has huge potential because of its simple-to-perform coating protocol and high fluorescence, which makes the system highly sensitive even for samples containing very low titres of analyte, thus enabling the detection of early seroconversion and borderline samples.

1.9. Multiplexed immunoassay

1.9.1. Concept

Currently, blood banks carry out separate tests of variable test formats for HIV, HCV and HBV detection. This greatly increases the cost of transfusion. The detection of HIV and HCV is based on the detection of antibodies to these pathogens using a cocktail of several synthetic peptides and/or full-length recombinant protein antigens; the presence of HBV is identified through the detection of the virus-encoded HBsAg antigen. The multiplexing concept seeks to develop reliable and inexpensive screening test for the detection of multiple analytes, simultaneously. A major and central phase of the multipexing is the combination of the individual, validated test concepts into a single test compartment sharing a common capture phase (homogenous mix). Possible loss of test performance (e.g. sensitivity) and non-specific cross-talk between analytes or reagents are likely to require further optimization to allow the essential specifications of each individual analyte to be reached. In order to avoid redesign of an individual test or binding reagent at this stage it is essential that cross-analyte combinations are included already in the early phase of screening of the different reagent versions.

1.9.2. Current status

Currently, there are no multiplexed immunoassay kits available in the market, though multiplexed nucleic acid tests (NATs) are available, e. g. Chiron Procleix Tigris and Roche Cobas S 201 NAT systems for simultaneous detection of HIV/HCV RNA and HBV DNA. There are some studies where multiplexing of immunoassays have been done in different formats. In one approach, quantum dot-barcodes based immunoassay was developed for the simultaneous detection of antibodies to HIV, HCV and HBV using r-gp41, r-NS4 and r-HBsAg antigens, respectively [Klostranec et al., 2007]. Although this assay showed high sensitivity it was unable to detect antibodies to other antigens of HIV (p24, gp36 of HIV-2 and gp41 of HIV O), HCV (Core, NS3 and

NS5) and the acute biomarker for HBV (HBsAg). In another approach a lateral flow immunochromatography format was developed for the simultaneous detection of antibodies to HIV-1 and -2, *Myobacterium tuberculosis* (TB) and HCV [Corstjens et al., 2007]. The results presented in this study indicated that multiple antibody test lines were feasible. Multiplexed analysis of combined specimens with high loads of antibodies against HIV, HCV and TB did not show relevant interference and the assay showed good detection capability for the detection of antibodies against above mentioned pathogens.

1.9.3. Challenges

The multiplexing procedure demands careful development and optimization of the various capture reagents in order to avoid nonspecific binding and to prevent undesired cross-reactivity of the analytes [Corstjens et al., 2007; Klostranec et al., 2007]. Development of multiplexed immunoassay requires rigorous validation of assay configuration and analytical performance to minimize assay imprecision and inaccuracy [Kellar et al., 2006]. Challenges associated with multiplexed configuration include selection and immobilization of capture ligands, calibration, interference between antibodies and proteins and assay diluents, and compatibility of individual assays. Analytical validation of multiplexed immunoassay performance should be conducted before clinical validation. Proposed analytical performance criteria should include range of linearity, analytical specificity, limits of detection and comparison to a quality reference method. Technical and operational challenges have hindered implementation of multiplexed assays in clinical settings. Formal procedures that guide multiplexed assay configuration, analytical validation, and quality control are needed before broad application of multiplexed arrays can occur in the *in vitro* diagnostic market [Ellington et al., 2010].

2. Aims and Objectives

2. Aims and Objectives

Blood screening is an established, mandatory activity in developed countries, but is frequently insufficient in performance or lacking, in developing and least developed countries. Blood transfusion saves lives and improves health, but millions of patients requiring transfusion do not have timely access to safe blood. While the need for blood is universal, there is a major imbalance between developing and industrialized countries, in access to safe blood. Moreover, with the advances in medical and surgical specialties, the demand for safe blood and blood components has also increased throughout the world. The WHO recommends that all blood for transfusion should be screened for HIV, hepatitis B and hepatitis C and syphilis. Although, developing countries are home to more than 80% of the world's population, yet many of them do not have safe blood supply. As pregnant women are one of the main groups of patients requiring blood transfusion in developing countries, together with children, they are particularly vulnerable to blood transfusion associated infections. Keeping in view the risk of transfusion-transmissible infections in several developing countries, there is an urgent need for developing inexpensive strategies for safe blood transfusion. Currently, blood banks carry out separate tests of variable test formats for HIV, HCV and HBV detection. This greatly increases the cost of transfusion.

The proposed project aims to develop an affordable, robust, simple and sensitive test technology/system for multiplexed testing of HIV, HCV and HBV in blood banks.

Specific aims:

The main objective of the project was to develop a multiplexed assay for the detection of HIV, HCV and HBV using lanthanide chelate as reporter. This proposal aims to provide a comprehensive, robust test technology, of uncompromised performance, for infectious disease testing (HIV, HCV and HBV) for implementation in developing world blood banks. The specific aims of this study can broadly be categorized as follows:-

- Generation of analyte-specific reagents and assay concept building
- Establishing a highly functional and dense solid phase matrix
- Application of lanthanide chelate reporter technology
- Optimizing and validating europium chelate based analyte concepts
- Multiplexing the individual test concept

3. Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1. Bacterial strains, expression plasmids and synthetic genes

3.1.2. Primers for PCR amplification

3.1.3. Restriction endonucleases and DNA modifying enzymes

3.1.4. Molecular biology kits, chromatography columns and molecular markers

3.1.5. Immunochemicals

3.1.6. In-house positive and negative sera

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3.2.2.6.1. Europium(III) chelate and its conjugation to anti-human antibody

3.2.2.6.2. In-house r-HIV-MEP based TRF immunoassay

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3.2.3. r-HCV-MEP and in-house anti-HCV TRF immunoassay

3.2.3.1. Design of r-HCV-MEP

3.2.3.2. Cloning and expression of *in vivo* biotinylated r-HCV-MEP in *E. coli*

3.2.3.3. Purification of biotinylated r-HCV-MEP

3.2.3.4. r-Bio-HCV-MEP based TRF immunoassay for the detection of anti-HCV antibodies using europium(III) chelate as reporter

3.2.4. In-house HBsAg TRF immunoassay

3.2.4.1. Purification of MAbs 5S and 21B specific for HBsAg

3.2.4.2. *In vitro* biotinylation of MAb 21B

3.2.4.3. Enzymatic fragmentation of MAb 5S and coating of 5S $F(ab)_2$ fragments on europium(III) nanoparticles

3.2.4.4. TRF immunoassay for the detection of HBsAg in human sera using europium(III) nanoparticle as reporter

3.2.5. HIV, HCV and HBV multiplexed TRF immunoassay

3.2.6. Other methods

3.2.6.1. Protein estimation and SDS-PAGE

3.2.6.2. Computational methods

3.2.6.3. Statistical methods

3.1. Materials

3.1.1. Bacterial strains, expression plasmids and synthetic genes

E. coli strain DH5α and BL21(DE3) used for all the cloning experiments were purchased from Invitrogen Life Technologies (Carlsbad, USA). Plasmids pET-28b(+), pET-32a(+) and pETDuet-1 were obtained from Novagen (Madison, USA). Plasmid pDW363 was obtained from Addgene Inc. (Cambridge, USA). The synthetic gene, codon optimized for expression in *E. coli*, encoding the r-HIV-MEP antigen was custom-synthesized as a *Bam*HI/*Hin*dIII fragment in pPCRscript by Geneart AG (Regensburg, Germany). The synthetic gene, codon optimized for expression in *E. coli*, encoding the r-HCV-MEP antigen was custom-synthesized as an *Eco*RI/*Hin*dIII fragment in pPCRscript by Geneart AG (Regensburg, Germany).

3.1.2. Primers for PCR amplification

Primers for PCR amplification were procured from Sigma-Aldrich Pvt. Ltd, Bangalore, India. Primers and their sequences are mentioned in Table 3.1.

Table 3.1. Primers for PCR amplification

S. no.	Primer	Sequence*
1	BAP forward (*Nco*I)	5' CATGCCATGGCTGGAGGCCTGAAC 3'
2	BAP reverse (*Bam*HI)	5' CGCGGATCCGCTCGAGCCACCAGTGTC 3'
3	BirA forward (*Nde*I)	5' GGAATTCCATATGAAGGATAACACCGTGCCAC 3'
4	BirA reverse (*Kpn*I)	5' CGGGGTACCTTATTTTTCTGCACTACGCAGGG 3'

** Sequences underlined in black represent the restriction enzyme sites designed within the primers.*

3.1.3. Restriction endonucleases and DNA modifying enzymes

Restriction endonucleases, calf intestine alkaline phosphatase and T4 DNA ligase used in all routine cloning and transformation experiments were procured from MBI Fermentas (Burlington, Canada). Taq- and Pfu-polymerase for PCR screening were in-house preparations.

3.1.4. Molecular biology kits, chromatography columns and molecular markers

Qiagen kits for gel extraction, plasmid mini and midi prep and Ni-NTA super flow for protein purification were purchased from Qiagen (Maryland, USA). HiTrap Protein G HP chromatography column, NAP-5 and NAP-10 columns for gel filtration chromatography were obtained from GE Healthcare (Uppsala, Sweden). Nanosep omega centrifugal filter tubes (300 kDa cut-off) were obtained from Pall Life Sciences (Ann Arbor, USA). DNA size markers (1 kb DNA ladder and 123 bp ladder) were procured from Invitrogen life technologies (Carlsbad, USA). Low molecular weight protein markers for SDS-PAGE were obtained from Amersham Bioscience UK Ltd. (Buckinghamshire, England). Pre-stained protein markers for monitoring Western transfer of SDS-PAGE fractionated proteins were procured from Bio-Rad Laboratories (Hercules, USA).

3.1.5. Immunochemicals

Goat anti-human IgG was purchased from Pierce (Rockford, IL). Nitrocellulose membranes were supplied by Advanced Micro devices Pvt. Ltd. (Ambala Cantt, India). Avidin peroxidase was purchased from Calbiochem-EMD Biosciences (La Jolla, USA). Goat anti-mouse IgG-HRPO and mouse anti-penta-His IgG were procured from Qiagen (Maryland, USA).

3.1.6. In-house positive and negative human sera

A panel of over 70 samples of HBsAg positive and negative human sera was obtained from Turku University Hospital, Turku, Finland. A panel of over 50 negative samples was obtained from local hospitals.

3.1.7. Serum panels from Boston Biomedica Inc. (BBI), now SeraCare Life Sciences Inc. (Milford, USA)

Table 3.2. Serum panels from Boston Biomedica Inc.

Panel ID	Name of panel
PRB 931	HIV-1 seroconversion panel
WWRB 302	Worldwide HIV performance panel
WWHV 301	Worldwide HCV performance panel
PHV 901	HCV seroconversion panel (HCV Genotype 1a)
PCA 201	Viral co-infection performance panel

3.1.8. General Chemicals, media components and other solutions

Table 3.3. General chemicals and their source

Description	Source
Acrylamide	Sigma-Aldrich Co., St. Louis, USA
Ammonium sulfate	Merck Ltd., Mumbai, India
Ammonium per sulfate	Invitrogen Life Technologies, Carlsbad, USA
Agarose DNA grade	Invitrogen Life Technologies, Carlsbad, USA
Ampicillin	Invitrogen Life Technologies, Carlsbad, USA
Arginine	Sigma-Aldrich Co., St. Louis, USA
Bacto Agar	Becton, Dickinson and Company, Sparks, USA
D-Biotin	Shaanxi Sciphar Biotechnology Co., Xi'an, China.
Biotin isothiocyanate	Department of Biotechnology, Turku University, Turku, Finland
Boric acid	Serva, Heidelberg, Germany
Bovine serum albumin	Sigma-Aldrich Co., St. Louis, USA
Brij 35	Sigma-Aldrich Co., St. Louis, USA
Bromophenol blue	Sigma-Aldrich Co., St. Louis, USA
Calcium chloride	Sigma-Aldrich Co., St. Louis, USA
CHAPS	Serva, Heidelberg, Germany
EDC	Fluka, Switzerland
EDTA	USB Corporation, Cleveland, USA
Ethanol	Bengal Chemicals Ltd., Calcutta, India
Ethidium bromide	Boehringer Manheim, GmbH, Germany
Europium(III) chelate	Department of Biotechnology, Turku University, Turku, Finland
Europium(III) chelate-doped Fluoro-Max^{TM} polystyrene nanoparticles	Seradyn Inc., Indianapolis, USA
Kanamycin	Invitrogen Life Technologies, Carlsbad, USA
Gentamycin	Invitrogen Life Technologies, Carlsbad, USA
Glycerol	Sigma-Aldrich Co., St. Louis, USA
Glycine	Serva, Heidelberg, Germany
Hydrochloric acid	Merck Ltd., Mumbai, India
Imidazole	Sigma-Aldrich Co., St. Louis, USA
Isopropyl-β-D thiogalactopyranoside (IPTG)	Calbiochem-EMD Biosciences, La Jolla, USA
Luria Bertani medium	Becton, Dickinson and Company, Sparks, USA
β-Mercaptoethanol	Sigma-Aldrich Co., St. Louis, USA
NHS	Fluka, Switzerland
Polyvinyl pyrrolidone	Sigma Chemical Co.,St. Louis,USA
Propanol	Merck Ltd., Mumbai, India
Potassium Dihydrogen Orthophosphate	Sigma-Aldrich Co., St. Louis, USA
PMSF	Sigma-Aldrich Co., St. Louis, USA
Sodium bicarbonate	Sigma-Aldrich Co., St. Louis, USA

Sodium chloride	Sigma-Aldrich Co., St. Louis, USA
Sodium hydroxide	Sigma-Aldrich Co., St. Louis, USA
Sodium phosphate	Sigma-Aldrich Co., St. Louis, USA
di-Sodium hydrogen phosphate	Serva, Heidelberg, Germany
SDS	Sigma-Aldrich Co., St. Louis, USA
Sulfuric acid	Merck Ltd., Mumbai, India
TEMED	Sigma-Aldrich Co., St. Louis, USA
TMB membrane peroxidase substrate	Amresco, Solon, USA
Tris	USB corporation, Cleveland, USA
Triton X-100	Sigma-Aldrich Co., St. Louis, USA
Tween-20	Sigma-Aldrich Co., St. Louis, USA

Media components and other solutions

LB (Luria-Bertani) Medium (1L): 10 g tryptone, 5 g yeast extract, 10 g NaCl. Sterilized by autoclaving at 15 psi, 121°C, for 20 min.

LB agar: LB medium containing 20 g/l agar. Sterilized by autoclaving at 15 psi, 121°C, for 20 min.

Ampicillin stock solution: 100 mg/ml in water, filter sterilized and stored in aliquots at –20°C until further use.

Kanamycin stock solution: 50 mg/ml in water, filter sterilized and stored in aliquots at –20°C until further use.

Solutions for plasmid midiprep:

Solution 1: 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0); autoclaved and stored at 4°C.

Solution 2: 0.2 N NaOH (freshly diluted from 10 N NaOH), 1% Sodium dodecyl sulphate (SDS).

Solution 3: 5 M potassium acetate (60 ml), glacial acetic acid (11.5 ml), double distilled water (28.5 ml), stored at 4°C.

Delfia enhancement solution: 100 mM Acetic acid, pH 3.2, 6.8 mM potassium hydrogen phthalate, 50 µM tri-n-octylphosphine oxide, 15 µM 4,4,4 trifluoro-1-(naphth-2-yl) butane 1,3-dione, 1 g/l Triton X-100

3.2. Methods

3.2.1. Development of expression system for *in vivo* biotinylation of recombinant MEPs in *E. coli*

Biotinylation takes place with the help of BirA (biotin holoenzyme synthetase), which biotinylates BAP (biotin acceptor peptide), which is the minimal substrate for *in vivo* biotinylation by BirA. Vector pET-32a(+) was digested with restriction enzymes *Xba*I and *Nco*I and the gene encoding -Trx (thioredoxin-6xHis) was cloned into the respective sites of pET-28b(+) vector (Figure 3.2A). The resultant vector pET-28b(+)-Trx was further used to clone BAP gene. The gene encoding BAP was PCR amplified from vector pDW363 (Figure 3.1) using BAP forward primer and BAP reverse primer. PCR amplified BAP gene was digested with restriction enzymes *Nco*I and *Bam*HI and cloned into the respective sites of pET-28b(+)-Trx vector. The resultant expression vector pET-28b(+)-Trx-BAP was further used to clone the gene encoding r-MEP in an open reading frame to express Trx-BAP-r-MEP fusion protein. Recombinant clones were selected on kanamycin (50 µg/ml) containing medium and the inserts were further verified by restriction digestion analysis of the recombinant plasmid DNAs.

BirA gene was PCR amplified from pDW363 using BirA forward primer and BirA reverse primer. PCR amplified BirA gene was digested with restriction enzymes *Nde*I and *Kpn*I and cloned into the respective sites in MCS 2 of pETDuet-1 vector (Figure 3.2B). Recombinant clones were selected on ampicillin (100 µg/ml) containing medium and the insert was verified by restriction digestion analysis of the recombinant plasmid DNA. The resultant expression vector pETDuet-1-BirA was used for co-transformation with expression vector encoding biotinylated MEPs [pET-28b(+)-Trx-BAP-r-MEP].

3.2.2. r-HIV-MEP and in-house anti-HIV TRF immunoassay

3.2.2.1. Design of r-HIV-MEP

To design an r-HIV-**M**ulti-**E**pitope **P**rotein (r-HIV-MEP) that could be of diagnostic utility, linear and conserved immunodominant epitopes, known to elicit anti-HIV antibodies, were selected based on published literature. Multiple sequence alignment of antigens encoded by different HIV types with the selected immunodominant

epitopes of r-HIV-MEP was performed. The selected epitopes were linked together by flexible $(gly)_4$ peptides. The software modeling analysis of the three dimensional structure of the r-HIV-MEP was carried out to check the accessibility of all the epitopes.

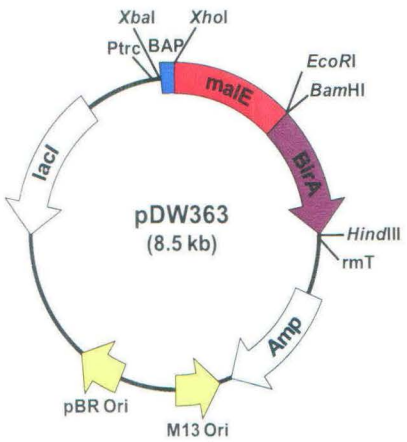


Figure 3.1. *Plasmid vector pDW363 for the production of biotinylated MalE protein in E. coli (Tsao et al, 1996). The Biotin acceptor peptide-BAP (substrate for enzymatic biotinylation by BirA) is followed, in frame, by the ORF of MalE. pDW363 is designed in a way so that when ORFs that terminate with a stop codon are inserted upstream of BirA, it gives rise to dicistronic mRNA, wherein the first reading frame encodes a fusion protein substrate for enzymatic biotinylation and the second reading frame encodes BirA. The vector carries the pBR322 derived ColE1 replicon. The plasmid can be propagated in E. coli using ampicillin as a selection marker.*

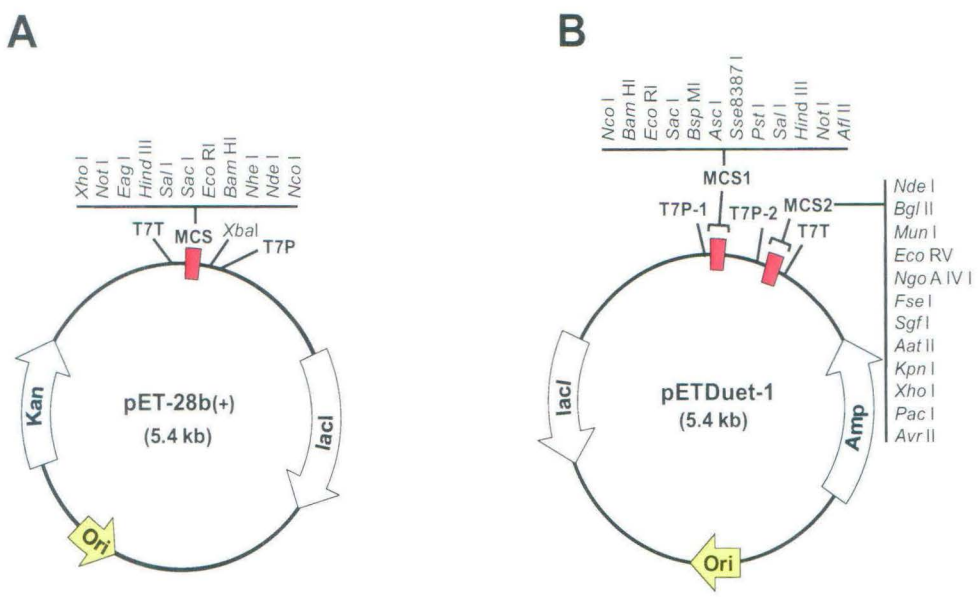


Figure 3.2. Expression vector pET-28b(+) and pETDuet-1. *(A) In pET-28b(+), the multiple cloning site (MCS) is preceded by a T7 promoter. The vector carries the pBR322 derived ColE1 replicon and lacI gene. The vector can be propagated in E. coli using kanamycin as a selection marker. (B) pETDuet-1 is designed for the co-expression of two target genes. The vector contains two MCS, each of which is preceded by a T7 promoter. The vector carries the pBR322 derived ColE1 replicon and lacI gene. The vector can be propagated in E. coli using ampicillin as a selection marker.*

3.2.2.2. Cloning and expression of r-HIV-MEP in *E. coli* expression plasmid

The synthetic gene, encoding the r-HIV-MEP antigen was custom-synthesized as a *Bam*HI/*Hin*dIII fragment in the Geneart vector pPCRscript. The Geneart vector containing r-HIV-MEP gene was digested with restriction enzymes *Bam*HI and *Hin*dIII and cloned into the respective sites of expression vector pET-32a(+) (Figure 3.3), in-frame with the vector-encoded thioredoxin protein and 6x-His tags, under the control of T7 promoter. Recombinant clones were selected on ampicillin (100µg/ml) containing medium and the insert was further verified by restriction digestion analysis of the recombinant plasmid DNA.

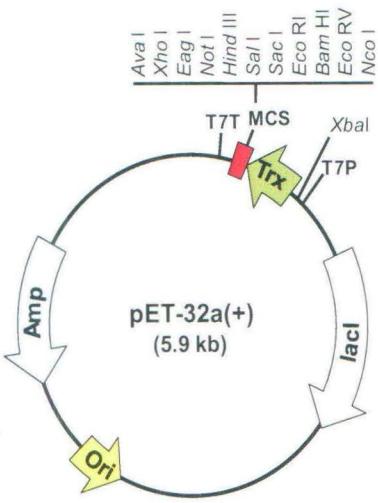


Figure 3.3. Expression vector pET-32a(+). *The vector is designed for high level expression of recombinant proteins fused with Trx (thioredoxin) tag. Trx tag is followed by 6xHis tag and multiple cloning site (MCS), where gene of interest can be cloned. Trx tag is preceded by a T7 promoter. The vector carries the pBR322 derived ColE1 replicon and lacI gene. The vector can be propagated in E. coli using ampicillin as a selection marker.*

The r-HIV-MEP cloned in pET-32a(+) was transformed into the *E. coli* expression host strain BL21(DE3). Transformants were selected on ampicillin containing medium and screened for protein expression as follows: transformed clones were inoculated in 3 ml LB containing 100 µg/ml ampicillin and were allowed to grow overnight at 37°C at 200 rpm. Thereafter, a fresh tube containing 3 ml LB with 100 µg/ml ampicillin was inoculated with 2% overnight grown culture and was allowed to grow at 37°C at 200 rpm till the culture reached logarithmic growth phase [corresponding to an optical density (OD) of ~0.6 at 600 nm]. The culture was then induced with 1 mM IPTG at 37°C for 4 h. After induction, equivalent number of cells from different cultures (normalized on the basis of OD_{600} values) were lysed in Laemmli buffer and analyzed by SDS–PAGE for the expression of r-HIV-MEP. Un-induced cultures were set as controls and analyzed by SDS-PAGE in parallel.

3.2.2.3. Purification of r-HIV-MEP

Cell growth and induction

A pre-inoculum was set up by inoculating 20 ml LB medium containing ampicillin (100µg/ml), with the glycerol stock of BL21(DE3) cells transformed with clone of r-HIV-MEP in pET-32a(+). The culture was grown overnight at 37°C at 200 rpm. Thereafter a fresh 1 litre LB containing 100µg/ml ampicillin in a four litre Haffkine flask was inoculated with 1% of the overnight grown culture and was allowed to grow at 37°C at 200 rpm till the culture reached logarithmic growth phase. The culture was then induced with 1 mM IPTG for 4 h. An un-induced culture was set as control in parallel. After induction, the culture was harvested by centrifugation in a Sorvall SLA3000 rotor at 8000 rpm for 10 min at 4°C. The biomass was washed with an isotonic solution (0.9% NaCl in water). An aliquot of both un-induced and induced cultures were lysed in Laemmli buffer and analyzed by SDS–PAGE for the expression of r-HIV-MEP.

Purification strategy using Ni-NTA column chromatography

Cell biomass (3 g) obtained from 1 litre induced culture was resuspended in 30 ml lysis buffer (6 M Guanidine-HCl, 10 mM Tris–HCl, 100 mM Sodium phosphate, 300 mM NaCl, 1% Tween-20, pH 8) and sonicated in ice using Sonics Vibracell sonicator (amplitude setting of 60), with 20 pulses of 10 seconds each, and 30 seconds off-time in between the pulses. The lysate was clarified by centrifugation at 16000 rpm at 4°C in an SS34 rotor for 45 min and the resultant supernatant was mixed with 5 ml Ni–NTA super flow resin that had been pre-equilibrated with lysis buffer. The suspension was gently shaken for 1 h at room temperature and packed into a column. The flow through was collected and the column was washed with 5 column volumes of lysis buffer, 10 column volumes of buffer 1 (8 M Urea, 10 mM Tris–HCl, 100 mM Sodium phosphate, 150 mM NaCl, 0.2% Tween-20, pH 6.3) and 10 column volumes of buffer 2 (8 M Urea, 10 mM Tris–HCl, 100 mM sodium phosphate, pH 5.9). The protein was eluted with 5 column volumes of elution buffer (8 M Urea, 10 mM Tris–HCl, 100 mM sodium phosphate, pH 4.3). The purity of the protein fractions was further analyzed on 12% SDS–PAGE. Protein in the pooled fractions was estimated spectrophotometrically.

3.2.2.4. Cloning and expression of *in vivo* biotinylated r-HIV-MEP in *E. coli*

The Gencart vector containing r-HIV-MEP gene was digested with restriction enzymes *Bam*HI and *Hin*dIII and cloned into the respective sites in already developed expression vector pET-28b(+)-Trx-BAP, in-frame with the Trx tag and BAP under the control of T7 promoter. Recombinant clones were selected on kanamycin (50 µg/ml) containing medium and the insert was further verified by restriction digestion analysis of the recombinant plasmid DNA.

The r-HIV-MEP in pET-28b(+)-Trx-BAP and BirA in pETDuet-1 were co-transformed into the *E. coli* expression host strain BL21(DE3). Transformants were selected on ampicillin (100 µg/ml) and kanamycin (50 µg/ml) containing medium and screened for protein expression as follows: transformed clones were inoculated in 3 ml LB containing 100 µg/ml ampicillin and 50 µg/ml kanamycin and were allowed to grow overnight at 37°C at 200 rpm. Thereafter, a fresh tube containing 3 ml LB with 100 µg/ml ampicillin, 50 µg/ml kanamycin and 10 µg/ml D-biotin was inoculated with 2% overnight grown culture and was allowed to grow at 37°C at 200 rpm till the culture reached logarithmic growth phase [corresponding to an optical density (OD) of ~0.6 at 600 nm]. The culture was then induced with 1 mM IPTG at 37°C for 4 h. After induction, equivalent number of cells from different cultures (normalized on the basis of OD_{600} values) were lysed in Laemmli buffer and analyzed by SDS–PAGE for the expression of biotinylated r-HIV-MEP (r-Bio-HIV-MEP) and BirA. Un-induced cultures were set as controls and analyzed by SDS-PAGE in parallel.

Western blot analysis

The un-induced and induced cultures of *E. coli* expressing r-Bio-HIV-MEP and BirA proteins were lysed in Laemmli buffer and run on 12% SDS-PAGE. The fractionated proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 2% PVP-40, 5% skim milk, 0.5% Tween-20 in PBS at 4°C overnight. The membrane was washed 6 times with wash buffer (PBS, 0.5% Tween-20) and probed with avidin peroxidase (1:1000) in diluent buffer (2.5% skim milk, 1% PVP, 0.5% Tween-20 in PBS) at RT for 1 h. Membrane was washed 6 times with wash buffer and developed with TMB insoluble.

3.2.2.5. Purification of biotinylated r-HIV-MEP

Cell growth and induction

A pre-inoculum was set up by inoculating 20 ml LB medium containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml), with the glycerol stock of BL21(DE3) cells transformed with r-HIV-MEP in pET-28b(+)-Trx-BAP and BirA in pETDuet-1. The culture was grown overnight at 37°C at 200 rpm. Thereafter a fresh 1 litre LB containing ampicillin (100 µg/ml), kanamycin (50 µg/ml) and D-biotin (10 µg/ml) in a four litre Haffkine flask was inoculated with 1% of the overnight grown culture and was allowed to grow at 37°C at 200 rpm till the culture reached logarithmic growth phase. The culture was then induced with 1 mM IPTG for 4 h. An un-induced culture was set as control in parallel. After induction, the culture was harvested by centrifugation in a Sorvall SLA3000 rotor at 8000 rpm for 10 min at 4°C. The biomass was washed with an isotonic solution (0.9% NaCl in water). An aliquot of both un-induced and induced cultures were lysed in Laemmli buffer and analyzed by SDS–PAGE for the expression of r-Bio-HIV-MEP.

Purification strategy using Ni-NTA column chromatography

Cell biomass (3 g) obtained from 1 litre induced culture was resuspended in 30 ml lysis buffer (6 M Guanidine-HCl, 10 mM Tris–HCl, 100 mM Sodium phosphate, 300 mM NaCl, 1% Tween-20, pH 8) and sonicated in ice using Sonics Vibracell sonicator (amplitude setting of 60), with 20 pulses of 10 seconds each, and 30 seconds off-time in between the pulses. The lysate was clarified by centrifugation at 16000 rpm at 4°C in an SS34 rotor for 45 min and the resultant supernatant was mixed with 5 ml Ni–NTA super flow resin that had been pre-equilibrated with lysis buffer. The suspension was gently shaken for 1 h at room temperature and packed into a column. The flow through was collected and the column was washed with 5 column volumes of lysis buffer, 10 column volumes of buffer 1 (8 M Urea, 10 mM Tris–HCl, 100 mM Sodium phosphate, 150 mM NaCl, 0.2% Tween-20, pH 6.3) and 10 column volumes of buffer 2 (8 M Urea, 10 mM Tris–HCl, 100 mM sodium phosphate, pH 5.9). The protein was eluted with 5 column volumes of elution buffer (8 M Urea, 10 mM Tris–HCl, 100 mM sodium phosphate, pH 4.3). The purity of the protein fractions was further

analyzed on 12% SDS–PAGE. Protein in the pooled fractions was estimated spectrophotometrically.

3.2.2.6. r-HIV-MEP based and r-Bio-HIV-MEP based time-resolved fluorometric (TRF) immunoassays for the detection of anti-HIV antibodies using europium(III) chelate as reporter

3.2.2.6.1. Europium(III) chelate and its conjugation to anti-human antibody

The nonadentate europium(III) chelate, {2,2',2'',2'''-{[2-(4-Isothiocyanatophenyl) ethylimino] bis (methylene)bis {4-{[4-(α-galactopyranoxy)phenyl] ethynyl}pyridine-6,2-diyl}bis (methylene-nitrilo)} tetrakis(acetato)} europium(III), was used in this study. Hereafter, it has been referred to as europium(III) chelate. Europium labeling of amino groups of goat anti-human IgG through a thiourea bond formation was performed in 50 mM Sodium carbonate-bicarbonate buffer, 0.9% NaCl, pH 9.8, with a 40-fold molar excess of the europium(III) chelate. The mixture was incubated overnight (16–20 h) at 4°C. The labeled antibody was separated from excess of free label by changing the buffer into 50 mM Tris, pH 7.75, 150 mM NaCl, 0.01% NaN_3 using gel filtration column (NAP column). The concentration of labeled antibody was estimated by Bradford assay using mouse IgG as standard. The degree of europium(III) chelate labeling was determined using non-conjugated 1 nM europium(III) chelate as standard. Non-conjugated europium(III) chelate was diluted in delfia enhancement solution and 200 µl was added into a 96-well plate in triplicate. Labeled antibody was serially diluted from 10^{-2} to 10^{-8} in delfia enhancement solution and 200 µl of each dilution were added into the same plate in triplicates. The plate was incubated for 45 min at RT. Europium concentration was calculated by comparing the fluorescence of the standard to the fluorescence of the labeled antibody sample. The degree of europium labeling was found to be 2.6 europium(III) chelate per antibody, which was determined by calculating the ratio of no. of moles of europium(III) chelate and labeled antibody. BSA and sodium azide were added into the solution of labeled antibody to a final concentration of 0.1% and 0.01%, respectively The solution was filtered through a 0.22 µm membrane and stored at 4°C until further used.

3.2.2.6.2. In-house r-HIV-MEP based TRF immunoassay

Time-resolved measurement of europium fluorescence was performed using Victor^{3}V 1420 Multilabel counter (Perkin Elmer, Singapore), that allowed the measurement of fluorescence directly from a solid phase. Briefly, 5 µg/ml of r-HIV-MEP was prepared in coating buffer (0.1 M Sodium carbonate-bicarbonate buffer, pH 9.6) and 100 µl of this was added into each well of a 96-well plate and incubated overnight at 37°C. The wells were aspirated and blocked with 300 µl of blocking buffer (37.5 mM Tris-HCl, pH 7.75, 25% goat sera, 115 mM NaCl, 0.05% NaN_3, 0.038% Tween-40, 15 µM EDTA, 1.38% BSA) and incubated for 2 h at room temperature with shaking. The wells were washed 2 times using 96-well plate washer (COLUMBUS Plus-BASIC, TECAN, Grödig, Austria) with wash buffer (10 mM KH_2PO_4, 40 mM K_2HPO_4, pH 7.2, 150 mM NaCl, 0.1% Tween-20, 0.5 M KCl). After washing, 2 µl of each serum sample in 50 µl assay buffer (37.5 mM Tris-HCl, pH 7.75, 25% goat sera, 115 mM NaCl, 0.5 M KCl, 0.05% NaN_3, 0.038% Tween-40, 0.1% Triton X-100, 15 µM EDTA, 0.38% BSA) was incubated in each well for 30 minutes at room temperature with shaking. The wells were washed 4 times with wash buffer. 1 µg/ml of europium(III) chelate labeled anti-human antibody solution was prepared in assay buffer and 50 µl of the prepared solution was added into each well and incubated for 30 minutes at room temperature with shaking. The wells were washed 7 times with wash buffer and europium fluorescence was measured from dry wells by time-resolved fluorometry using Victor^{3}V 1420 Multilabel counter with the following parameters: excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 400 µs; window time, 400 µs; cycling time, 1 ms; measurement time, 1s (i.e., counts resulting from 1000 sequential excitations were integrated for each measurement).

3.2.2.6.3. In-house r-Bio-HIV-MEP based TRF immunoassay

1 µg/ml of r-Bio-HIV-MEP was prepared in assay buffer (37.5 mM Sodium carbonate-bicarbonate buffer, pH 9.6, 18.8 mM NaCl, 0.075% Tween-20, 1.9% BSA, 3.8% D-trehalose, 0.045% γ- globulins from bovine blood, 0.05% NaN_3, 0.5% Mouse sera, 25% Goat sera) and 100 µl of the prepared solution was incubated in each well of streptavidin plate (Kaivogen Oy, Finland) for 1h at RT with shaking. The wells were washed twice using 96-well plate washer with wash buffer (10 mM KH_2PO_4, 40

mM K_2HPO_4, pH 7.2, 0.85% NaCl, 0.1% Tween-20, 0.5 M KCl). After washing, 2 µl of each serum sample in 50 µl assay buffer was incubated in each well for 30 min at RT with shaking. The wells were washed 4 times with wash buffer. 1 µg/ml of europium(III) chelate labeled anti-human antibody solution was prepared in assay buffer and 50 µl of the prepared solution was added into each well and incubated for 30 minutes at RT with shaking. The wells were washed 7 times with wash buffer and europium fluorescence was measured from dry wells by time-resolved fluorometry using the same parameters as mentioned for r-HIV-MEP based TRF immunoassay.

3.2.3. r-HCV-MEP and in-house anti-HCV TRF immunoassay

3.2.3.1. Design of r-HCV-MEP

To design an r-HCV-**M**ulti-**E**pitope **P**rotein (MEP) that could be of diagnostic utility, linear and conserved immunodominant epitopes, known to elicit anti-HIV antibodies, were selected based on published literature. HCV-F-MEP Version1 (HCV-F-MEP V1) was modified to HCV-MEP V2 (r-HCV-MEP) by incorporation of additional epitopes and deletion of a pre-existing epitope. The epitopes were selected from structural and non-structural proteins of HCV polyprotein. The selected epitopes were linked together by flexible $(gly)_4$ peptides. The software modeling analysis of the three dimensional structure of the r-HCV-MEP was carried out to check the accessibility of all epitopes.

3.2.3.2. Cloning and expression of *in vivo* biotinylated r-HCV-MEP in *E. coli*

The synthetic gene, encoding the r-HCV-MEP antigen was custom-synthesized as a EcoRI/*Hin*dIII fragment in the Geneart vector pPCRscript. The Geneart vector containing r-HCV-MEP gene was digested with restriction enzymes *Eco*RI and *Hin*dIII and cloned into the respective sites in already developed expression vector pET-28b(+)-Trx-BAP, in-frame with the Trx tag and BAP under the control of T7 promoter. Recombinant clones were selected on kanamycin (50 µg/ml) containing medium and the insert was further verified by restriction digestion analysis of the recombinant plasmid DNA.

The r-HCV-MEP in pET-28b(+)-Trx-BAP and BirA in pETDuet-1 were co-transformed into the *E. coli* expression host strain BL21(DE3). Transformants were

selected on ampicillin (100 µg/ml) and kanamycin (50 µg/ml) containing medium and screened for protein expression as follows: transformed clones were inoculated in 3 ml LB containing 100 µg/ml ampicillin and 50 µg/ml kanamycin and were allowed to grow overnight at 37°C at 200 rpm. Thereafter, a fresh tube containing 3 ml LB with 100 µg/ml ampicillin, 50 µg/ml kanamycin and 10 µg/ml D-biotin was inoculated with 2% overnight grown culture and was allowed to grow at 37°C at 200 rpm till the culture reached logarithmic growth phase [corresponding to an optical density (OD) of ~0.6 at 600 nm]. The culture was then induced with 1 mM IPTG at 37°C for 4 h. After induction, equivalent number of cells from different cultures (normalized on the basis of OD_{600} values) were lysed in Laemmli buffer and analyzed by SDS–PAGE for the expression of biotinylated r-HCV-MEP (r-Bio-HCV-MEP) and BirA. Un-induced cultures were set as controls and analyzed by SDS-PAGE in parallel.

Western blot analysis

The un-induced and induced cultures of *E. coli* expressing r-Bio-HCV-MEP and BirA proteins were lysed in Laemmli buffer and run on 12% SDS-PAGE. The fractionated proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 2% PVP-40, 5% skim milk, 0.5% Tween-20 in PBS at 4°C overnight. The membrane was washed 6 times with wash buffer (PBS, 0.5% Tween-20) and probed with avidin peroxidase (1:1000) in diluent buffer (2.5% skim milk, 1% PVP, 0.5% Tween-20 in PBS) at RT for 1 h. Membrane was washed 6 times with wash buffer and developed with TMB insoluble.

3.2.3.3. Purification of biotinylated r-HCV-MEP

Cell growth and induction

A pre-inoculum was set up by inoculating 20 ml LB medium containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml), with the glycerol stock of BL21(DE3) cells transformed with r-HCV-MEP in pET-28b(+)-Trx-BAP and BirA in pETDuet-1. The culture was grown overnight at 37°C at 200 rpm. Thereafter a fresh 1 litre LB containing ampicillin (100 µg/ml), kanamycin (50 µg/ml) and D-biotin (10 µg/ml) in a four litre Haffkine flask was inoculated with 1% of the overnight grown culture and was allowed to grow at 37°C at 200 rpm till the culture reached logarithmic growth phase. The culture was then induced with 1 mM IPTG for 4 h. An un-induced culture

was set as control in parallel. After induction, the culture was harvested by centrifugation in a Sorvall SLA3000 rotor at 8000 rpm for 10 min at 4°C. The biomass was washed with an isotonic solution (0.9% NaCl in water). An aliquot of both un-induced and induced cultures were lysed in Laemmli buffer and analyzed by SDS–PAGE for the expression of r-Bio-HCV-MEP.

Purification strategy using Ni-NTA column chromatography

Cell biomass (3 g) obtained from 1 litre induced culture was resuspended in 30 ml lysis buffer (6 M Guanidine-HCl, 10 mM Tris–HCl, 100 mM Sodium phosphate, 300 mM NaCl, 1% Tween-20, pH 8) and sonicated in ice using Sonics Vibracell sonicator (amplitude setting of 60), with 20 pulses of 10 seconds each, and 30 seconds off-time in between the pulses. The lysate was clarified by centrifugation at 16000 rpm at 4°C in an SS34 rotor for 45 min and the resultant supernatant was mixed with 5 ml Ni–NTA super flow resin that had been pre-equilibrated with lysis buffer. The suspension was gently shaken for 1 h at room temperature and packed into a column. The flow through was collected and the column was washed with 5 column volumes of lysis buffer, 10 column volumes of buffer 1(8 M Urea, 10 mM Tris–HCl, 100 mM Sodium phosphate, 150 mM NaCl, 0.2% Tween-20, pH 6.3) and 10 column volumes of buffer 2 (8 M Urea, 10 mM Tris–HCl, 100 mM sodium phosphate, pH 5.9). The protein was eluted with 5 column volumes of elution buffer (8 M Urea, 10 mM Tris–HCl, 100 mM sodium phosphate, pH 4.3). The purity of the protein fractions was further analyzed on 12% SDS–PAGE. Protein in the pooled fractions was estimated spectrophotometrically.

3.2.3.4. r-Bio-HCV-MEP based TRF immunoassay for the detection of anti-HCV antibodies using europium(III) chelate as reporter

1 µg/ml of r-Bio-HCV-MEP was prepared in assay buffer (37.5 mM Sodium carbonate-bicarbonate buffer, pH 9.6, 18.8 mM NaCl, 0.075% Tween-20, 1.9% BSA, 3.8% D-trehalose, 0.045% γ- globulins from bovine blood, 0.05% NaN_3, 0.5% Mouse sera, 25% Goat sera) and 100 µl of this was incubated in each well of streptavidin plate (Kaivogen Oy, Finland) for 1h at RT with shaking. The wells were washed twice using 96-well plate washer with wash buffer (10 mM KH_2PO_4, 40 mM K_2HPO_4, pH 7.2, 0.85% NaCl, 0.1% Tween-20, 0.5 M KCl). After washing, 2 µl of each serum

sample in 50 µl assay buffer was incubated in each well for 30 min at RT with shaking. The wells were washed 4 times with wash buffer. 1 µg/ml of europium(III) chelate labeled anti-human antibody solution was prepared in assay buffer and 50 µl of this solution was added into each well and incubated for 30 minutes at RT with shaking. The wells were washed 6 times with wash buffer and europium fluorescence was measured from dry wells by time-resolved fluorometry using Victor^{3}V 1420 Multilabel counter with the following parameters: excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 400 µs; window time, 400 µs; cycling time, 1 ms; measurement time, 1s (i.e., counts resulting from 1000 sequential excitations were integrated for each measurement).

3.2.4. In-house HBsAg TRF immunoassay

3.2.4.1. Purification of MAbs 5S and 21B specific for HBsAg

The hybridoma clones producing MAb 21B (50 000 cells) and MAb 5S (200 000 cells) specific for HBsAg were inoculated into 2 different mice and ascitic fluid was collected in between 10 to 15 days post inoculation. Ascitic fluid was diluted 1:1 (v/v) with buffer (1 M glycine, 0.3 M NaCl, pH 8.6), incubated at 4°C for 24 h, centrifuged at 13000 rpm for 15 min at 4°C in SS-34 rotor. The supernatant was further diluted in equal volume binding buffer (1 M glycine, 0.15 M NaCl, pH 8.6) and filtered with 0.22 µm syringe filter. The filtered ascitic fluid was loaded on 1 ml prosep-VA ultra (protein A) column equilibrated with binding buffer. The column was washed extensively with 50 column volumes of binding buffer and the MAb was eluted with 10 column volumes of elution buffer (0.1 M Citric acid-NaOH buffer, pH 3.0). The pH of the eluted fractions was adjusted to ~7 by addition of 2 M unbuffered Tris. The purity of the MAbs was analysed on 12% SDS-PAGE.

3.2.4.2. *In vitro* biotinylation of MAb 21B

The antibody to be biotinylated should be in a buffer free from any reactive -NH_2 group. The storage buffer of antibody was changed from 0.1 M Citric acid-Tris buffer, pH ~7 to 0.9% NaCl with gel filtration column (NAP-10 column). The pH of the antibody solution was adjusted to 9.8 with 1/10th volume of 0.5 M carbonate buffer, pH 9.8. The concentration of antibody was kept close to 2 mg/ml, as much as possible. Biotin isothiocyanate (BITC) was dissolved in absolute ethanol to a final

concentration of 10 mM. The solution was centrifuged at 13000 rpm for 15 min, to pellet undissolved impurities. A 40-fold molar excess of BITC was used in the biotinylation reaction of MAb 21B. The reaction mixture was incubated for 4 h at RT. The unreacted BITC was removed by passing through gel filtration column (NAP-5 and NAP-10 columns sequentially). The concentration of biotinylated MAb was estimated with Bradford assay and BSA and sodium azide were added into the solution to a final concentration of 0.1% and 0.01%, respectively

3.2.4.3. Enzymatic fragmentation of MAb 5S and coating of 5S F(ab)$_2$ fragments on europium(III) nanoparticles

MAb 5S was enzymatically fragmented into F(ab)$_2$ and Fc regions. Fragmented 5S F(ab)$_2$ was purified to coat it on europium(III) nanoparticles. For the fragmentation, 100 mM L-cysteine and 10 mg/ml bromelain was prepared in fragmentation buffer (50 mM Tris-HCl pH 7.0, 2 mM EDTA). The fragmentation reaction was set up by mixing the following components - MAb 5S (at a final conc. of 1 mg/ml), 10x fragmentation buffer (1/10th volume of Ab solution), bromelain (0.2 U/ml), L-cysteine (1 mM). The reaction was incubated for 4 h at 37°C. The reaction was stopped by adding 1/10th volume of fresh 100 nM solution of N-Ethylmaleimide (NEM) (made in fragmentation buffer). The reaction was mixed and incubated for 10 min at RT. The sample was filtered through 0.22 µm filter. The HiTrap Protein G HP chromatography column was equilibrated with wash buffer (20 mM Sodium phosphate buffer, pH 7.0). The fragmentation reaction product was injected into the column. The column was washed with 5 column volumes of wash buffer and eluted with 10 column volumes of elution buffer (0.1 M Glycine-HCl, pH 2.7). The pH of the eluted fractions was adjusted to ~7 by addition of 2 M unbuffered Tris. The purity of the 5S F(ab)$_2$ was analysed on 12% SDS-PAGE. The buffer of purified 5S F(ab)$_2$ was changed to 0.9% NaCl by NAP columns.

1.23x10^{12} europium(III) nanoparticles were added into Nanosep omega filter tubes (300 kDa cut-off), centrifuged at 8000 rpm for 8 min. Nanoparticles were washed with 400 µl of Sodium phosphate buffer by centrifuging at 8000 rpm for 8 min. 150 µl of phosphate buffer was added into the filter tube and nanoparticles were recovered from the filter by sonication. 10 µl of 72 mM EDC and 90 µl of 60 mM NHS solution prepared in sodium phosphate buffer were mixed together. The

nanoparticles were activated by mixing 150 µl nanoparticles and 30 µl EDC-NHS solution (final concentrations: EDC 1.2 mM, NHS 9 mM) in a fresh tube. The reaction was vortexed for 15 min. The solution was transferred to filter tube and centrifuged at 8000 rpm for 8 min. The filter was washed twice with 150 µl 20 mM MES by centrifuging at 8000 rpm for 5 min. 150 µl of 20 mM MES was added to the tubes and nanoparticles were recovered from the filter by sonication. For the conjugation of 5S $F(ab)_2$ with the nanoparticles, 300 µg of 5S $F(ab)_2$ (pH adjusted with $1/10^{th}$ vol of 200 mM MES) was added to nanoparticle solution in a fresh tube and vortexed for 2 h. For blocking free binding sites on the nanoparticles 0.1 % Brij 35 and 0.1% BSA were added into the reaction and incubated overnight at RT in slow rotation. The nanoparticle solution was transferred to the fresh filter tubes and centrifuged at 8000 rpm for 5–10 min (until the filter was dry). The nanoparticles were washed 5 times with 400 µl of storage buffer (2 mM Tris-HCl, pH 9.0, 0.01 % Tween-20) by centrifuging at 8000 rpm for 5–10 min. 200 µl of storage buffer was added into the filter tube and the nanoparticles were recovered from the filter by sonication. The nanoparticles were transferred into a fresh tube and BSA and sodium azide were added into this solution to a final concentration of 0.1% and 0.01%, respectively. The particles were stored at +4°C in dark. The concentration of the 5S $F(ab)_2$ coated nanoparticles was determined by using non-conjugated europium nanoparticles as standard. 5S $F(ab)_2$ coated nanoparticle solution (sample) was diluted 1:10 in 0.1% Triton-X-100 and 2 µl of this and undiluted sample were added to 700 µl 0.1% Triton X-100 separately. Standard (non-conjugated 107 nm nanoparticles were diluted in 0.1% Triton X-100 to a concentration of $1x10^8$ pcs/µl) was mixed and 2 µl of it was added into 700 µl of TritonX-100. The standard and sample dilutions were added into BSA blocked wells in 3 replicates of 200 µl/well each. After 5 min the fluorescence was measured with Victor^{3}V 1420 Multilabel counter. The concentration of nanoparticles was calculated by comparing the fluorescence of the standard to the fluorescence of the sample.

3.2.4.4. TRF immunoassay for the detection of HBsAg in human sera using europium(III) nanoparticle as reporter

3 µg/ml of Bio-MAb 21B was prepared in assay buffer (50 mM Sodium carbonate-bicarbonate buffer, pH 9.6, 25 mM NaCl, 0.1% Tween-20, 0.1% Triton X-100, 2.5%

BSA, 5% D-trehalose, 0.06% γ- globulins from bovine blood, 0.05% NaN_3, 0.01% native mouse IgG, 0.005% denatured mouse IgG, $2x10^{-4}$ M $CaCl_2$) and 50 µl of this solution was incubated in each well of streptavidin plate for 1h at RT with shaking. The wells were washed twice using 96-well plate washer with wash buffer (10 mM KH_2PO_4, 40 mM K_2HPO_4, pH 7.2, 0.85% NaCl, 0.1% Tween-20, 0.5 M KCl). After washing, 10 µl of each serum sample in 40 µl assay buffer was incubated in each well for 1 h at RT with shaking. The wells were washed 4 times with wash buffer. $2x10^9$ 5S $F(ab)_2$-nanoparticles/ml solution was made in assay buffer and 50 µl of this solution was added into each well and incubated for 1h at RT with shaking. The wells were washed 6 times with wash buffer and europium fluorescence was measured from dry wells by time-resolved fluorometry using Victor^{3}V 1420 Multilabel counter with the following parameters: excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 400 µs; window time, 400 µs; cycling time, 1 ms; measurement time, 1s (i.e., counts resulting from 1000 sequential excitations were integrated for each measurement).

3.2.5. HIV, HCV and HBV multiplexed TRF immunoassay

1 µg/ml of Bio-MAb 21B, 1 µg/ml of r-Bio-HIV-MEP and 1 µg/ml of r-Bio-HCV-MEP were prepared in assay buffer (37.5 mM Sodium carbonate-bicarbonate buffer, pH 9.6, 18.8 mM NaCl, 0.075% Tween-20, 0.075% Triton X-100, 1.9% BSA, 3.8% D-trehalose, 0.045% γ- globulins from bovine blood, 0.05% NaN_3, 0.5% Mouse sera, 25% Goat sera) and 50 µl of this solution was incubated in each well of streptavidin plate (Kaivogen Oy, Finland) for 1h at RT with shaking. The wells were washed twice using 96-well plate washer with wash buffer (10 mM KH_2PO_4, 40 mM K_2HPO_4, pH 7.2, 0.85% NaCl, 0.1% Tween-20, 0.5 M KCl). After washing, 5 µl of each serum sample and 45 µl assay buffer was incubated in each well for 30 min at RT with shaking. The wells were washed 4 times with wash buffer. $2x10^9$ 5S $F(ab)_2$ nanoparticles/ml and 1 µg/ml of europium(III) chelate labeled anti-human antibody solution was prepared in assay buffer and 50 µl of this solution was added into each well and incubated for 1h at RT with shaking. The wells were washed 6 times with wash buffer and europium fluorescence was measured from dry wells by time-resolved fluorometry using Victor^{3}V 1420 Multilabel counter with the following parameters: excitation wavelength, 340 nm; emission wavelength, 615 nm; delay

time, 400 μs; window time, 400 μs; cycling time, 1 ms; measurement time, 1s (i.e., counts resulting from 1000 sequential excitations were integrated for each measurement).

3.2.6. Other Methods

3.2.6.1. Protein Estimation and SDS-PAGE

Protein estimation was done by either spectrophotometrically or as described by Bradford [Bradford, 1976]. **S**odium **D**odecyl**S**ulphate-**P**oly**A**crylamide **G**el **E**lectrophoresis (SDS-PAGE) of proteins was carried out using standard protocol described by Laemmli [Laemmli, 1970].

3.2.6.2. Computational methods

The amino acids sequences of the r-HIV-MEP and r-HCV-MEP were submitted to the websites http://www.sbg.bio.ic.ac.uk/~3dpssm and http://zhanglab.ccmb.med.umich.edu/I-TASSER/, respectively and 3-dimentional structures were predicted for both the MEPs. The predicted 3-dimentional structures of the MEPs were further polished by using the softwares Accelrys ViewerLite 5.0 and Pov-Ray for Windows v3.62. The amino acid sequences of the proteins were submitted to the website http://www.expasy.ch/tools/protparam.html and physico-chemical parameters of a protein sequence like isoelectric point (pI), molar extinction coefficient and molecular weight were computed.

3.2.6.3. Statistical Methods

The sensitivity and specificity of the TRF immunoassay were calculated as follows [Constantine and Lana, 2003] -

Sensitivity = a / (a + c) * 100

Specificity = d / (d + b) * 100

Where, a = no. of true positive sera, b = no. of false positive sera, c = no. of false negative sera, d = no. of true negative sera.

To designate sera as either positive or negative, a stringent cut-off value was used for TRF immunoassay, which was obtained by adding either three or five times the standard deviation to the mean of the negative serum samples [Leland, 2000].

4. Results

4. Results

4.1. Expression system for *in vivo* biotinylation of recombinant MEPs in *E. coli*

4.2. Establishment of highly functional and dense solid phase matrix

4.3. r-HIV-MEP and in house anti-HIV TRF immunoassay

4.3.1. Design of r-HIV-MEP

4.3.2. Cloning, expression and purification of r-HIV-MEP

4.3.3. Cloning, expression and purification of *in vivo* biotinylated r-HIV-MEP

4.3.4. r-HIV-MEP based and r-Bio-HIV-MEP based TRF immunoassays for the detection of anti-HIV antibodies using europium(III) chelate as reporter

4.3.5. Evaluation of r-HIV-MEP based and r-Bio-HIV-MEP based TRF immunoassays

4.3.6. Conclusion

4.4. r-HCV-MEP and in house anti-HCV TRF immunoassay

4.4.1. Design of r-HCV-MEP

4.4.2. Cloning, expression and purification of *in vivo* biotinylated r-HCV-MEP

4.4.3. r-Bio-HCV-MEP based TRF immunoassay for the detection of anti-HCV antibodies using europium(III) chelate as reporter

4.4.4. Evaluation of r-HCV-MEP based TRF immunoassay

4.4.5. Conclusion

4.5. In house HBsAg TRF immunoassay

4.5.1. Purification of MAbs 5S and 21B specific for HBsAg

4.5.2. *In vitro* biotinylation of MAb 21B

4.5.3. Enzymatic fragmentation of MAb 5S and coating of 5S F(ab)2 fragments on europium(III) nanoparticles

4.5.4. TRF immunoassay for the detection of HBsAg in human sera using europium(III) nanoparticles as reporter

4.5.5. Evaluation of HBsAg TRF immunoassay

4.5.6. Conclusion

4.6. HIV, HCV and HBV multiplexed TRF immunoassay

4.6.1. Design of HIV, HCV and HBV multiplexed TRF immunoassay

4.6.2. Evaluation of HIV, HCV and HBV multiplexed TRF immunoassay

4.6.3. Conclusion

The r-HIV-MEP and r-HCV-MEP were designed, *in vivo* biotinylated, using cloning strategies in *E. coli* and purified to homogeneity. In-house TRF immunoassays were developed for the detection of anti-HIV antibodies and anti-HCV antibodies in infected human sera using these MEPs. Monoclonal antibodies (MAbs) 21B and 5S specific for HBsAg, were purified to homogeneity and in-house HBsAg TRF immunoassay was developed using these MAbs. The three individual immunoassays mentioned above were combined to develop an HIV, HCV and HBV multiplexed TRF immunoassay. The visualization of experimental section is shown in Figure 4.1.

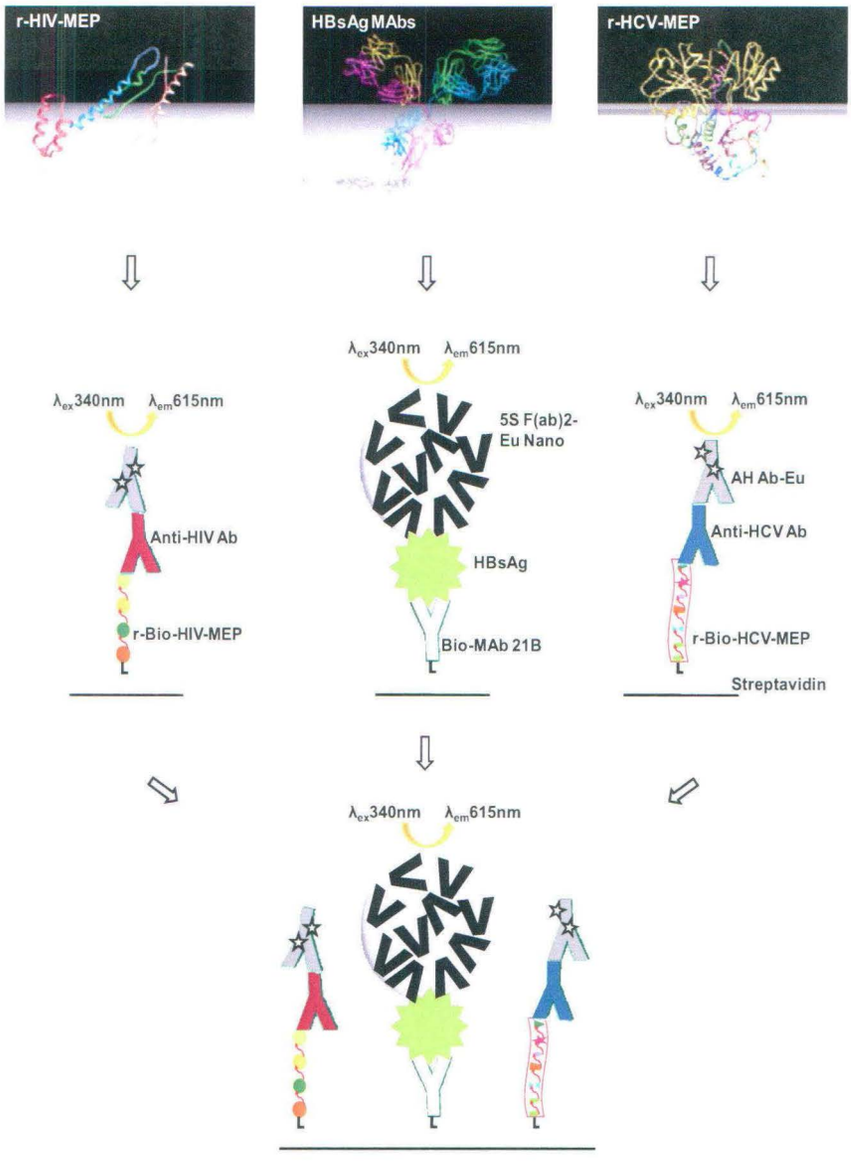


Figure 4.1. Visualization of experimental section

4.1. Expression system for *in vivo* biotinylation of recombinant MEPs in *E. coli*

A specific plasmid expression system was developed for *in vivo* synthesis of biotinylated recombinant MEPs in *E. coli.* Biotinylation takes place with the help of BirA (biotin holoenzyme synthetase), which biotinylates the 13th amino acid (aa), which is a lysine, of the 24 aa BAP (biotin acceptor peptide). BAP is the minimal substrate for biotinylation by BirA. Gene encoding Trx (Trx-6xHis) from pET-32a(+) vector was cloned into the *Xba*I and *Nco*I restriction sites of pET-28b(+) vector. The gene encoding BAP was PCR amplified from vector pDW363 and cloned into the MCS of pET-28b(+)-Trx at *Nco*I and *Bam*HI sites (Figure 4.2). The gene encoding r-MEP was cloned in the MCS of expression vector pET-28b(+)-Trx-BAP in an open reading frame to express Trx-BAP-r-MEP fusion protein.

Earlier, an attempt was made where Trx-BAP-r-MEP and BirA were cloned in the same vector (pETDuet-1) in two different MCS, but the former did not express, to circumvent this problem the Trx-BAP-r-MEP was cloned in pET-28b(+).

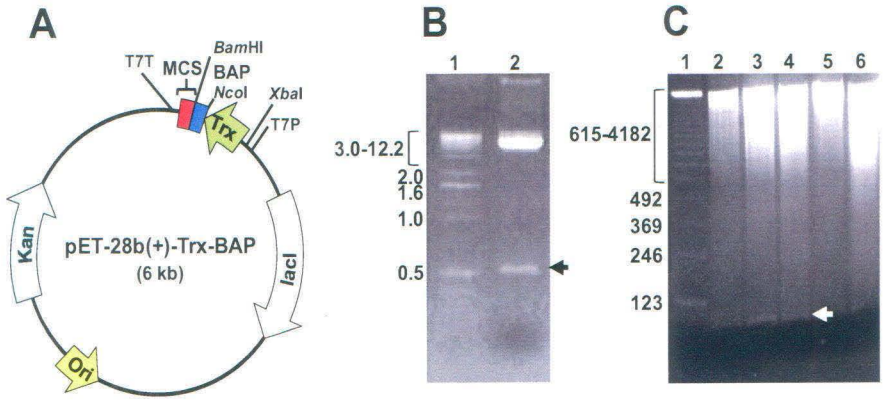


Figure 4.2. Cloning of genes encoding Trx and BAP in vector pET-28b(+). *(A) Map of expression vector pET-28b(+) with gene encoding Trx, inserted into the unique XbaI and NcoI sites and gene encoding BAP, inserted into the unique NcoI and BamHI sites. (B) Trx gene in pET-32a(+) was digested with XbaI and NcoI enzymes and cloned in pET-28b(+) at respective sites. Putative clone was confirmed by digestion with XbaI and NcoI. The resultant digested product was analyzed on 1% agarose gel. Lane 2 shows the positive clone with the expected insert fall-out of Trx gene at 0.52 kb. DNA size markers are shown in lane 1 with relevant sizes (in kb) indicated on the left. Arrow on right indicates position of Trx gene insert. (C) BAP gene was PCR amplified from vector pDW363, digested with NcoI and BamHI enzymes and cloned in pET-28b(+)-Trx at respective sites. Putative clones were confirmed by digestion with NcoI and BamHI. The resultant digested products were analyzed on 2.5% agarose gel. Lanes 2, 3 and 4 show the positive clones with expected insert fall-out of BAP gene at 0.08 kb. DNA size markers are shown in lane 1 with relevant sizes (in kb) indicated on the left. Arrow on right indicates position of BAP gene insert.*

BirA gene was PCR amplified from pDW363 and cloned into the MCS-2 of pETDuet-1 vector at *Nde*I and *Kpn*I sites (Figure 4.3).

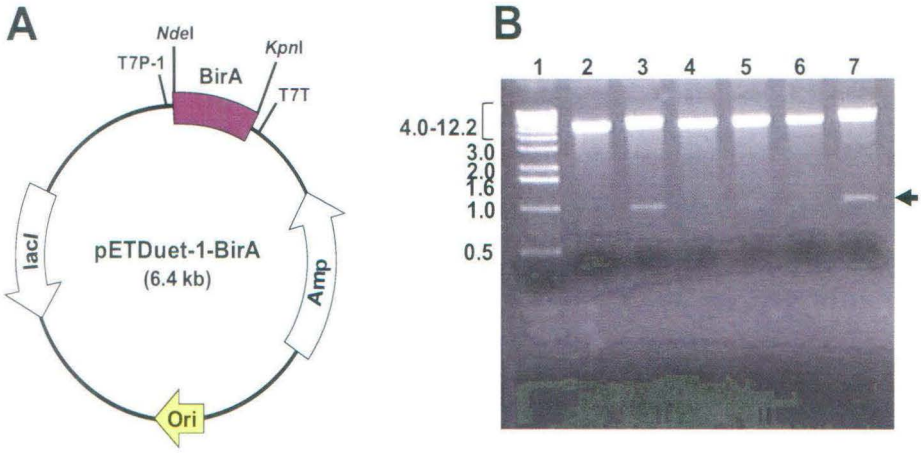


Figure 4.3. Cloning of gene encoding BirA in MCS2 of vector pETDuet-1. *(A) Map of expression vector pETDuet-1 with gene encoding BirA, inserted into the unique NdeI and KpnI sites in MCS2. (B) BirA gene was PCR amplified from vector pDW363, digested with NdeI and KpnI enzymes and cloned in MCS2 of pETDuet-1 at respective sites. Putative clones were confirmed by digestion with NdeI and KpnI. The resultant digested products were analyzed on 1% agarose gel. Lanes 3 and 7 show the positive clones with the expected insert fall out of BirA gene at 0.97 kb. DNA size markers are shown in lane 1 with relevant sizes (in kb) indicated on the left. Arrow on right indicates position of BirA gene insert.*

pET-28b(+) plasmid containing the fusion gene expression cassette Trx-BAP-r-MEP and pETDuet-1 plasmid containing BirA were used to co-transform the *E. coli* strain BL21(DE3). Transformants harboring the Trx-BAP-r-MEP along with BirA expressing plasmids were selected in presence of ampicillin and kanamycin containing medium. Positive clones were induced to express *in vivo* biotinylated r-MEP (Trx-BAP-r-MEP) and BirA. Design of expression system for *in vivo* biotinylation of recombinant MEPs in *E. coli* is shown in Figure 4.4.

4.2. Establishment of highly functional and dense solid phase matrix

The objective of multiplexing requires surfaces of high functional capacity and stability while at the same time having insignificant non-specific interactions. Passive coating of capture antigen on the solid surface exhibits several disadvantages. Therefore, streptavidin-coated microtitration plates are widely used for immobilization of capturing molecules. Streptavidin is a tetrameric molecule, so four biotinylated molecules can bind to a single streptavidin molecule, thus it provides a generic surface for immobilization of any biotinylated molecule and preserves

biomolecule activity much better than direct passive adsorption. Biotinylated molecules are spatially organized on streptavidin coated well in such a way that their epitopes are accessible in solution phase maximally. In our study, streptavidin was selected as the generic capture molecule to which the secondary binders were immobilized. Biotinylated r-MEPs or MAb were immobilized on streptavidin-coated plate for immunoassays. The saturating concentration of each of the biotinylated molecules was optimized to obtain an optimal signal to noise ratio in individual test concepts for HIV, HCV and HBV. Hypothetical visualization of streptavidin coated and passively coated 96-well plate for the immobilization of recombinant MEPs is shown in Figure 4.5. Streptavidin coated plates provide higher surface density and better display of epitopes of immobilized antigen as compared to direct passive coating.

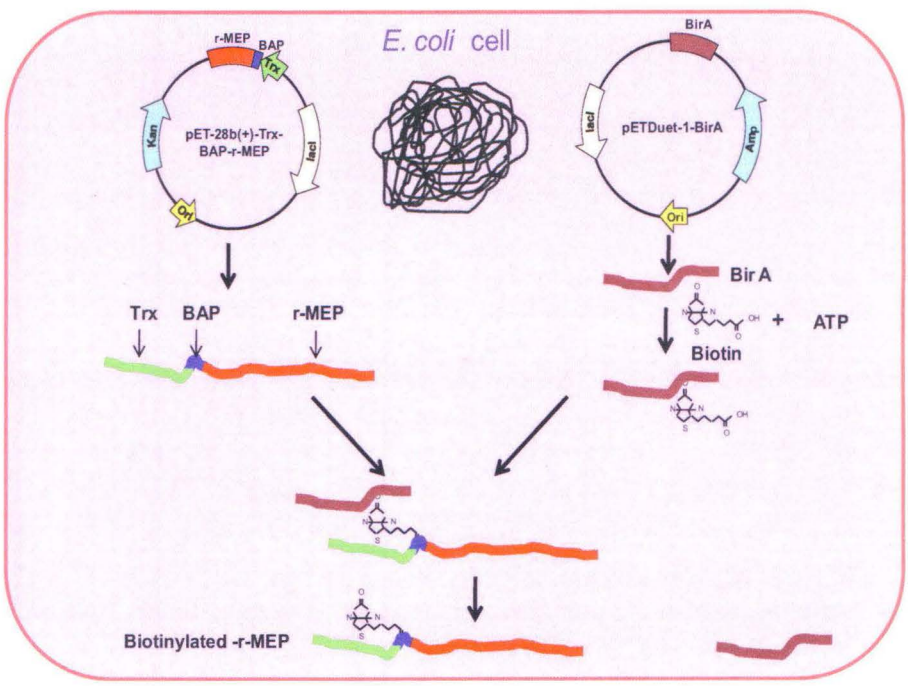


Figure 4.4. Design of expression system for in vivo biotinylation of recombinant MEPs in E. coli. *A specific plasmid expression system was developed for site specific in vivo biotinylation of recombinant MEPs in E. coli. Biotinylation takes place with the help of BirA (biotin holoenzyme synthetase), which biotinylates the 13th aa, which is a lysine, of the 24 aa BAP (biotin acceptor peptide) (MAGGLNDIFEAQKIEWHEDTGGSS). BAP is a part of Acetyl CoA carboxylase and is the minimal substrate for biotinylation by BirA. The gene encoding r-MEP was cloned into the bacterial expression vector pET-28b(+)-Trx-BAP under the control of T7 promoter. The BirA gene was cloned into the bacterial expression vector pETDuet-1. Trx-BAP-r-MEP in pET-28b(+) along with BirA in pETDuet-1 were co-transformed into the E. coli strain BL21(DE3) and co-expressed as biotinylated fusion protein and BirA. Biotin was supplemented into the medium for efficient biotinylation.*

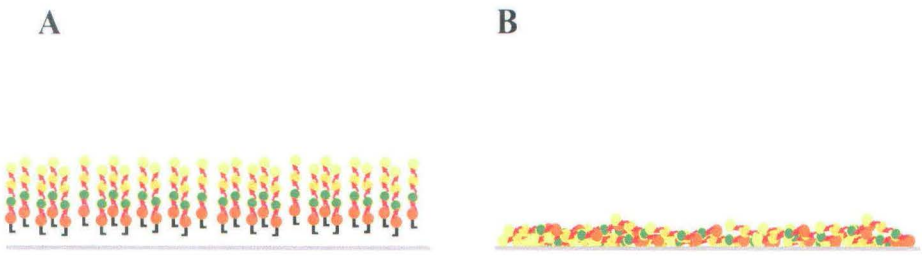


Figure 4.5. Hypothetical visualization of streptavidin coated and passively coated 96-well plate for the immobilization of recombinant MEPs. *(A) Side-view of streptavidin coated well, r-biotinylated MEP is immobilized on streptavidin coated well by strong and unique biotin-streptavidin interaction. Biotinylated r-MEPs are spatially organized on streptavidin coated well in such a way that their epitopes are accessible in solution phase maximally. (B) Side-view of passively coated well, r-MEP is adsorbed at the surface of the well. In passive coating some of the epitopes may be destroyed or become inaccessible.*

4.3. r-HIV-MEP and in-house anti-HIV TRF immunoassay

A novel recombinant multiepitope diagnostic intermediate, r-HIV-MEP was designed by fusing four virus specific, immuno-dominant, linear and phylogenetically conserved epitopes from core and envelope proteins of HIV. r-HIV-MEP was *in vivo* biotinylated using cloning strategies in *E. coli.* Non-biotinylated (r-HIV-MEP) as well as *in vivo* biotinylated (r-Bio-HIV-MEP) antigens were purified to homogeneity using Ni-NTA affinity chromatography. In-house TRF immunoassays were developed based on r-HIV-MEP and r-Bio-HIV-MEP as captures for the detection of anti-HIV antibodies in infected human serum samples. The assays were evaluated using commercially available and well-characterized serum panels from Boston Biomedica Inc. Overview of the experimental section is shown in Figure 4.6.

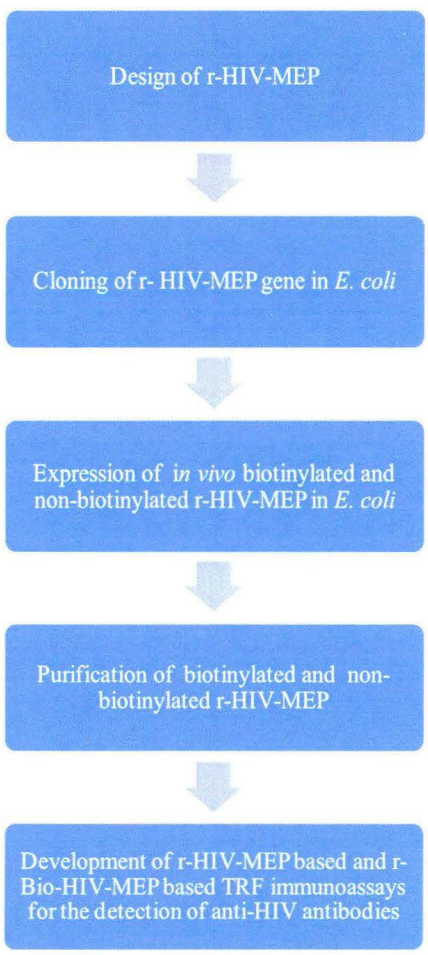


Figure 4.6. Overview of experimental section for r-HIV-MEP and in-house anti-HIV TRF immunoassay

4.3.1. Design of r-HIV-MEP

HIV has several sub-types and recombinant forms throughout the world. The major factor which prevents universal screening of anti-HIV antibodies in blood samples, in a blood-bank setting, is the high cost of currently available anti-HIV EIA kits. The high cost is due to requirement of multiple diagnostic intermediates, for the production of these kits. To address this issue, we explored an alternative approach of designing a single recombinant HIV **M**ulti-**E**pitope **P**rotein (r-HIV-MEP), as a suitable inexpensive diagnostic intermediate for anti-HIV immunoassay kits (Talha et al, 2010).

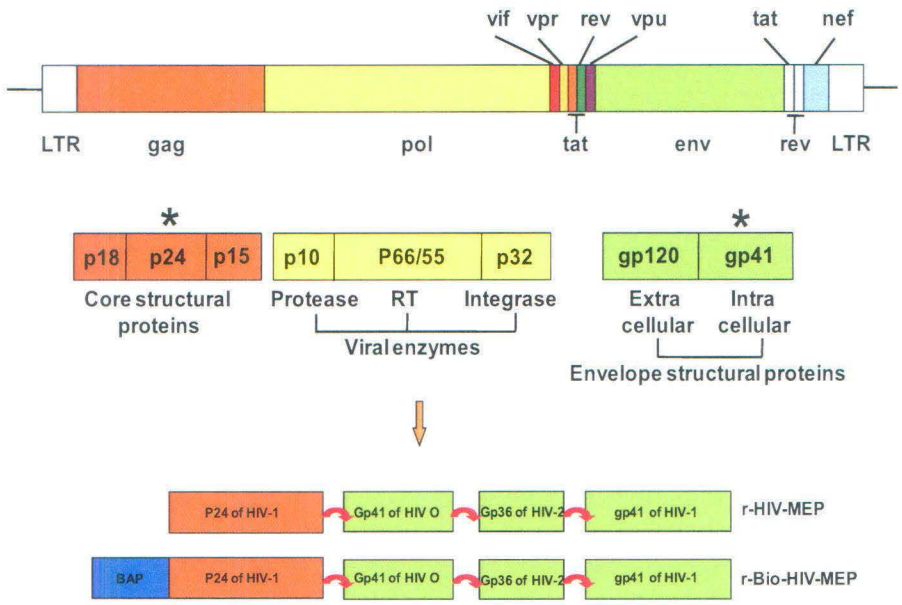


Figure 4.7. Schematic representation of genome, virus encoded proteins and design of synthetic non-biotinylated and biotinylated multiepitope proteins of HIV. *The integrated form of HIV-1, also known as the provirus, is approximately 9.8 kilobases in length. Both ends of the provirus are flanked by a repeated sequence known as the long terminal repeats (LTRs). The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins. These proteins are divided into three classes, the major structural proteins (Gag, Pol, and Env), the regulatory proteins (Tat and Rev) and the accessory proteins (Vpu, Vpr, Vif, and Nef). Four conserved and immunodominant epitopes from P24 of HIV-1, gp41 of HIV O, gp36 of HIV-2 and gp41 of HIV-1 were selected and stitched with flexible linkers to form r-HIV-MEP. In vivo biotinylation of r-HIV-MEP was further done to immobilize it on a streptavidin coated plate.*

To design a MEP that could be of diagnostic utility, linear and conserved immunodominant epitopes, known to elicit anti-HIV antibodies, were selected based on published literature (Table 4.1). These epitopes were from HIV-1 Core (p24) and the envelope proteins of HIV-1 and HIV-2 (Figure 4.7).

Multiple sequence alignment of antigens encoded by different HIV types with the selected immunodominant epitopes of r-HIV-MEP is shown in Figure 4.8. It showed that the selected immunodominant HIV epitopes manifested a considerable level of conservation among the specific types and groups of HIV. This suggested that r-HIV-MEP may exhibit immunoreactivity to antibodies specific to different types and groups of HIV.

```
HIV-1 p24     NKIVRMYSPT SILDIRQGPK EPFRDYVDRF YKTLRAEQAS QEYKNWMTET L
HIV-1 grM_A   .........V .......... .......... F.........T ..V....... .
HIV-1 grM_B   .......... .......... .......... .......... ..V....... .
HIV-1 grM_C   .........V .....K.... .......... F.........T .DV.....D. .
HIV-1 grM_D   .........V .......... .......... .......... .DV....... .
HIV-1 grM_F   .........V .....K.... .......... F.V........ .DV.G...D. .
HIV-1 grM_G   .........V .......... .......... F.C........ .DV.G...D. .
HIV-1 grM_H   .........V .....K.... .......... F.........T .DV....... .
HIV-1 grM_J   .........V .......... .......... F.A.......T .DV.....D. .
HIV-1 grN     .R........V ...E.K.... .......... .........T .DV....... .
HIV-1 grO     ..M.K....V .....K.... .......... .........T ..V....... .
HIV-2_A       Q.C....N.. N....K.... ...QS..... ..S.....TD PAV.....Q. .
HIV-2_B       Q.C....N.. N....K.... ...QS..... ..S.....TD PAV.....Q. .

HIV-1 grO gp41       WGIRQLRARL LALETLLQNQ QLLSLWGCKG KLVCYTS
HIV-1 grO ANT70      ..........  ..........  ..........  .......
HIV-1 grO MVP-5180   ..........  Q.....I...  .R.N......  ..I....
HIV-1 grO 99CMU4122  ..........  Q.....I...  ...N......  R.I....
HIV-1 grO 99USTWLA   ..........  Q.....M...  ...N......  .SI....
HIV-1 grO VAU        ..........  .....FI...  ..HN.....N  R.I....

HIV-2 gp36 QDQARLNSWG SAFRQVCHTT VPWVNDSL
HIV-2_A    ..........  C.........  ........
HIV-2_B    K...Q.....  C.........  ...P.ET.
HIV-2_A/B  K...Q.....  C.........  ........
HIV-2_U    K...S..A..  C.........  ...I..T.
HIV-2_G    K.........  C.........  ...DALGA

HIV-1 grM gp41  WGIKQLQARI LAVERYLKDQ QLLGIWGCSG KLICTTAVPW NASWSN
HIV-1 grM_A     .........V .......Q.. R...M.....  .H....F...  .S....
HIV-1 grM_B     .........V .......R..  ..........  ......T...  .T....
HIV-1 grM_C     .......T.V ..I..H.R..  ..........  ..........  .S....
HIV-1 grM_D     ..........  ..........  ..........  .H....N...  .S....
HIV-1 grM_F     .........V ..........  ..........  ......N...  .S....
HIV-1 grM_G     .......S.V ..I.......  ..........  ......N...  .T....
HIV-1 grM_H     .........V .......R..  ..........  ......N...  .S....
HIV-1 grM_J     .........V ..........  ..........  ......N...  ......
```

Figure 4.8. Multiple sequence alignment of the four r-HIV-MEP epitopes (in red) with the corresponding epitopes of different HIV types, groups, and subtypes (in black) *(http://bioinfo.genotoul.fr/multalin/multalin. html). The black dots indicate identical residues. Variants are indicated by the standard single-letter amino acid code. Letters in the virus names indicate subtypes. In the case of HIV-1 group O gp41, the alignment has been done with different isolates within the group.*

The r-HIV-MEP was designed by linking selected epitopes in tandem using $(gly)_4$ peptides. The tetra glycyl linkers are considered to be one of the preferred linkers

while designing flexible chimeric proteins (Robinson and Sauer, 1998). The DNA and predicted amino acid sequence of r-HIV-MEP is shown in Figure 4.9.

P24 of HIV-1

aacaaaatcgttcgcatgtatagcccgaccagcattctggatattcgtcagggtccgaaa
N K I V R M Y S P T S I L D I R Q G P K
gaaccgtttcgcgattacgttgatcgcttctacaaaaccctgcgtgcggaacaggcgagc
E P F R D Y V D R F Y K T L R A E Q A S

Gp41 of HIV-1 grp. O

caggaatacaaaaactggatgaccgaaaccctgggcggtggtggttggggtattcgtcag
Q E Y K N W M T E T L G G G G W G I R Q
ctgcgtgcgcgtctgctggcgctggaaaccctgctgcagaatcaacagctgctgtctctg
L R A R L L A L E T L L Q N Q Q L L S L

Gp36 of HIV-2

tggggttgtaaaggcaaactggtttgctataccagcggtggtggtggtcaggatcaggcg
W G C K G K L V C Y T S G G G G Q D Q A
cgtctgaatagctggggtagcgcgtttcgtcaggtttgtcataccaccgtgccgtgggtt
R L N S W G S A F R Q V C H T T V P W V

Gp41 of HIV-1 grp. M

aatgatagcctggggtggcggcggttggggcattaaacagctgcaggcgcgtattctggcg
N D S L G G G G W G I K Q L Q A R I L A
gttgaacgctatctgaaagatcagcaactgctgggtatttggggttgtagcggtaaactg
V E R Y L K D Q Q L L G I W G C S G K L
atttgtaccaccgcggttccgtggaatgcgagctggagcaattaa
I C T T A V P W N A S W S N *

Figure 4.9. Complete nucleotide (small case alphabets) and predicted aa (capital case alphabets) sequences of r-HIV-MEP gene showing four epitopes *[aa 1-51: p24 of HIV-1; aa 56-92: gp41 of HIV-1 group O; aa 97-124: gp36 of HIV-2; and aa 129-174: gp41 of HIV-1 group M)] linked together with flexible tetra glycyl linkers (underlined). The asterisk indicates the engineered stop codon.*

Table 4.1. List of HIV-specific immunodominant epitopes selected from the literature in designing the r-HIV-MEP antigen

Viral protein	Position of epitopes in HIV proteins[a]	% Positivity with patient sera	Reference
HIV-1 p24	aa 272-322 of p55 (505)	ND[b]	[Janvier et al., 1996]
HIV-1 group O gp41	aa 580-616 of gp160 (863)	84	[Dorn et al., 2000]
HIV-2 gp36	aa 587-614 of gp160 (858)	100[c]	[Gupta and Chaudhary, 2003]
HIV-1 group M gp41	aa 580-625 of gp160 (853)	100	[Dorn et al., 2000]

[a] *Numbers in parentheses indicate the total numbers of amino acid residues of the corresponding full-length proteins.*
[b] *ND, not done.*
[c] *In combination with HIV-1 gp41 (31 aa) and p24 (146 aa).*

The software modeling analysis of the three dimensional structure of the r-HIV-MEP is shown in Figure 4.10. The analysis suggested that the designed MEP permitted easy accessibility of all its constituent epitopes. All these epitopes may therefore be freely

available for interaction with their cognate antibodies and may contribute significantly to their overall sensitivity and specificity for patient sera.

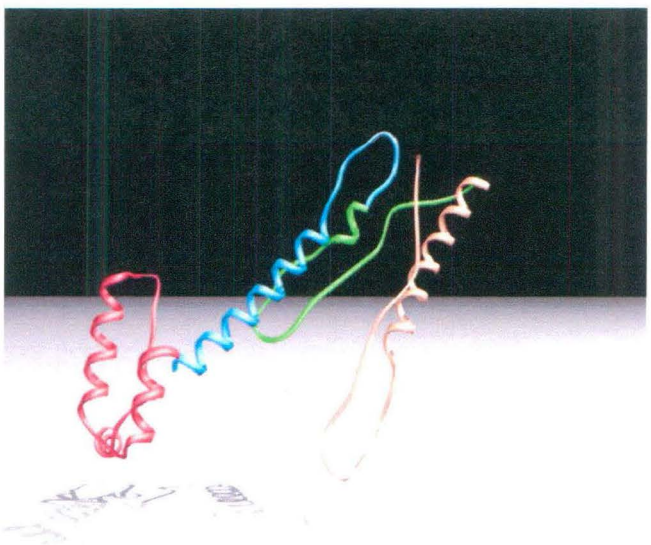


Figure 4.10. Software-generated graphic visualization (http://www.sbg.bio.ic.ac.uk/~3dpssm) of ribbon model of r-HIV-MEP. All four epitopes are shown in different colours. The colour of each epitope corresponds to the amino acid sequence shown in the same colour in figure 4.9.

4.3.2. Cloning, expression and purification of r-HIV-MEP

The r-HIV-MEP gene was cloned into the bacterial expression vector pET-32a(+) at unique *Bam*HI and *Hin*dIII sites, in-frame with the 5' thioredoxin- and 6x His tag-encoding sequences of the vector, under the control of T7 promoter (Figure 4.11). Vector pET-32a(+)-r-HIV-MEP was transformed into the *E. coli* strain BL21(DE3) and transformants were selected in presence of ampicillin containing medium. The recombinants were induced with 1 mM IPTG and were analyzed for expression. Figure 4.12A shows the polypeptide profile of a typical r-HIV-MEP clone. The synthesis of the predicted ~41 kDa protein was evident in the induced cells (Figure 4.12A, lane 2) and the protein is absent in un-induced cells (Figure 4.12A, lane 1).

Lysis of the induced cells by sonication under native conditions showed that the protein was not present in the soluble fraction. Therefore, guanidinium-HCl was used to solubilize the protein. Subsequently, its purification was carried out under denaturing condition in presence of 8 M urea using Ni-NTA affinity chromatography. The fractions of eluted protein were analysed by SDS-PAGE (Figure 4.12B, lane 1-7). The crude lysate (loaded on the column) containing the r-HIV-MEP is shown in lane

'L' and the flow through after binding is shown in lane 'F'. Extensive washing at pH 6.3 and 5.9 ensured that no non-specific contaminants remained bound to the column. Elution at pH 4.3 yielded highly pure r-HIV-MEP from the column (lanes 1-7). From a comparison of the protein profiles of the elution with crude lysate, it was evident that >95% purity was achieved. Starting from 3g induced cell biomass from 1L *E. coli* culture, ~21 mg of purified recombinant protein (pooled fractions in lanes 1-7) was obtained.

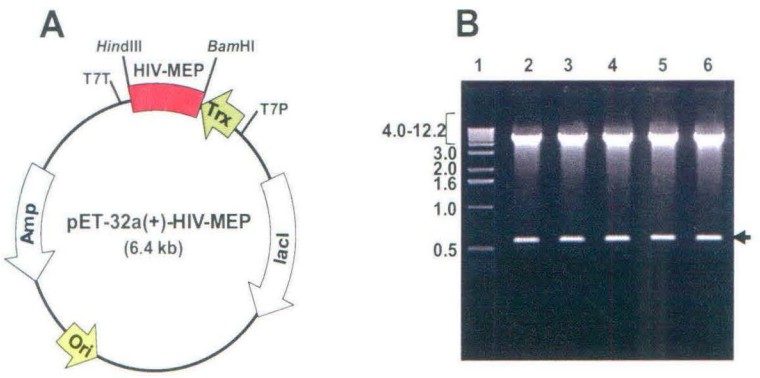


Figure 4.11. Cloning of r-HIV-MEP gene in vector pET-32a(+). *(A) Map of expression vector pET-32a(+) with gene encoding r-HIV-MEP, inserted into the unique BamHI and HindIII sites. (B) Synthetic gene encoding r-HIV-MEP was digested with BamHI and HindIII enzymes and cloned in pET-32a(+) at respective sites. Putative clones were confirmed by digestion with BamHI and HindIII. The resultant digested products were analyzed on 1% agarose gel. Lanes 2, 3, 4, 5 and 6 show the positive clones with the expected insert fall out of r-HIV-MEP gene at 0.54 kb. DNA size markers are shown in lane 1 with relevant sizes (in kb) indicated on the left. Arrow on right indicates position of r-HIV-MEP gene insert.*

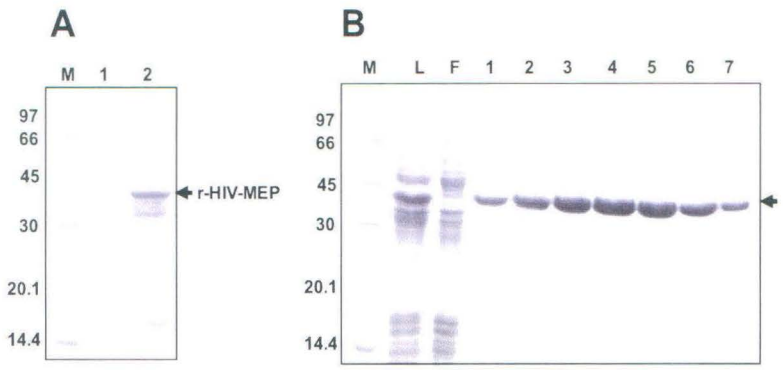


Figure 4.12. Expression and purification of r-HIV-MEP. *(A) SDS-PAGE analysis of Un-induced and induced cultures of E. coli expressing the r-HIV-MEP. Low molecular weight protein markers were analyzed in lane M. Their sizes in kDa are shown on the left. Lane 1 and 2 show Un-induced and Induced cultures of E. coli expressing r-HIV-MEP (~41 kDa) respectively. (B) SDS- PAGE analysis of the Ni-NTA eluted fraction of r-HIV-MEP. Fractions from the affinity column were assayed on a SDS-PAGE. Low molecular weight protein markers were analyzed in lane M. Their molecular weights, in kDa, are shown on the left. Lanes L and F show the load and flow-through fractions of the column. Lanes 1-7 show the purified fractions under denaturing conditions in presence of 8M urea. Protein bands were visualized by Coomassie Blue staining. Arrow on right indicates position of r-HIV-MEP.*

4.3.3. Cloning, expression and purification of *in vivo* biotinylated r-HIV-MEP

The r-HIV-MEP gene was cloned into the *in vivo* biotinylation expression vector pET-28b(+)-Trx-BAP at unique *Bam*HI and *Hind*III sites in an open reading frame under the control of T7 promoter (Figure 4.13).

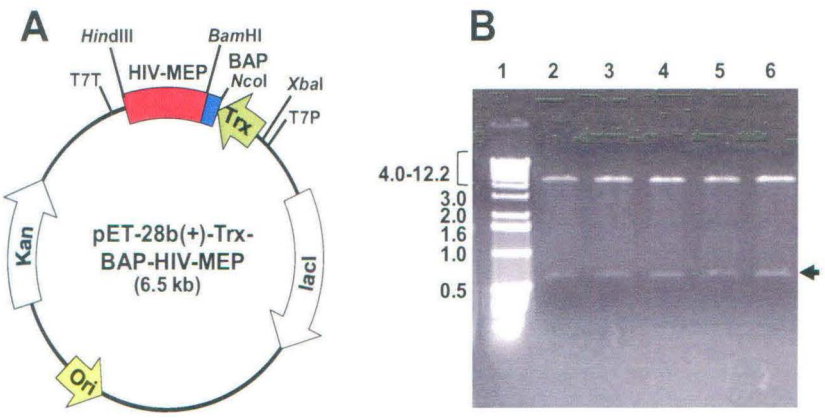


Figure 4.13. Cloning of r-HIV-MEP gene in in vivo biotinylation expression vector pET-28b(+)-Trx-BAP . *(A) Map of expression vector pET-28b(+)-Trx-BAP with gene encoding r-HIV-MEP, inserted into the unique BamHI and HindIII sites. (B) Synthetic gene of r-HIV-MEP was digested with BamHI and HindIII enzymes and cloned in pET-28b(+)-Trx-BAP at respective sites. Putative clones were confirmed by digestion with BamHI and HindIII. The resultant digested products were analyzed on 1% agarose gel. Lane 2, 3, 4, 5 and 6 show the positive clones with the expected insert fall out of r-HIV-MEP gene at 0.54 kb. DNA size markers are shown in lane 1 with relevant sizes (in kb) indicated on the left. Arrow on right indicates position of r-HIV-MEP gene insert.*

The pET-28b(+)-Trx-BAP-r-HIV-MEP plasmid and pETDuet-1-BirA plasmid were co-transformed into the *E. coli* strain BL21(DE3) and transformants were selected in presence of ampicillin and kanamycin containing medium. The recombinants were induced with 1 mM IPTG and were analyzed for expression. Figure 4.14A shows the polypeptide profile of r-Trx-BAP-HIV-MEP (r-Bio-HIV-MEP) clone. The synthesis of the predicted ~41 kDa r-Bio-HIV-MEP and 34 kDa BirA protein was evident in the induced cells (Figure 4.14A, lane 2) and both the proteins were absent in un-induced cells (Figure 4.14A, lane 1). The expression and *in vivo* biotinylation of r-Bio-HIV-MEP was confirmed by western blot. The un-induced and induced samples were transferred onto nitrocellulose membrane after SDS–PAGE and probed with avidin peroxidase (Figure 4.14B). It indicated the presence of ~41 kDa biotinylated protein (r-Bio-HIV-MEP) in induced cells (Figure 4.14B, lane 2) and un-induced cells did not express any biotinylated protein (Figure 4.14B, lane 1).

Lysis of the induced cells by sonication under native conditions showed that the protein was not present in the soluble fraction. Therefore, guanidinium-HCl was

used to solubilize the protein. Subsequently, its purification was carried out under denaturing condition in presence of 8 M urea using Ni-NTA affinity chromatography. The fractions of eluted protein were analysed by SDS-PAGE (Figure 4.15, lane 1-7). The crude lysate (loaded on the column) containing the r-Bio-HIV-MEP is shown in lane 'L' and the flow through after binding is shown in lane 'F'. Extensive washing at pH 6.3 and 5.9 ensured that no non-specific contaminants remained bound to the column. Elution at pH 4.3 yielded highly pure r-Bio-HIV-MEP from the column (lanes 1-7). From a comparison of the protein profiles of the elution with crude lysate, it was evident that >95% purity was achieved. Starting from 3g induced cell biomass from 1L *E. coli* culture, ~20 mg of purified r-Bio-HIV-MEP (pooled fractions in lanes 1-7) was obtained.

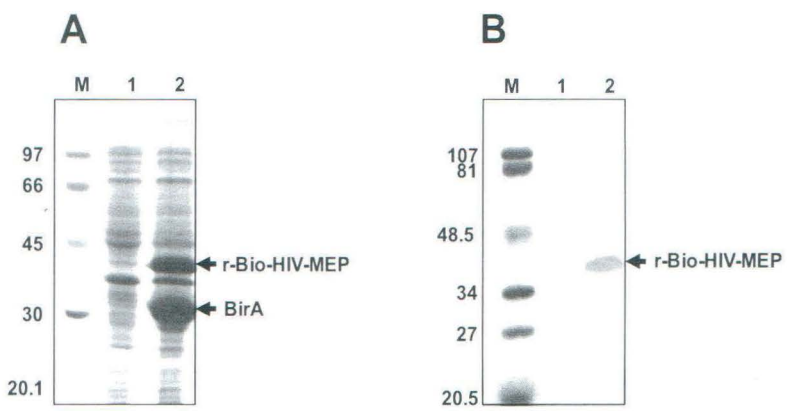


Figure 4.14. Expression and western blot analysis of r-Bio-HIV-MEP. *(A) SDS-PAGE analysis of Un-induced and induced cultures of E. coli expressing the r-Bio-HIV-MEP. Low molecular weight protein markers were analyzed in lane M. Their sizes in kDa are shown on the left. Lanes 1 and 2 show Un-induced and Induced cultures of E. coli expressing r-Bio-HIV-MEP (41 kDa) respectively. BirA (34 kDa) is co-expressed along with r-Bio-HIV-MEP in lane 2. (B) Western blot analysis of r-Bio-HIV-MEP with avidin peroxidase. Prestained protein markers were analyzed in lane M. Their sizes in (kDa) are shown on the left. Lanes 1 and 2 show Un-induced and Induced cultures of E. coli expressing r-Bio-HIV-MEP (41 kDa) respectively. r-Bio-HIV-MEP is recognized by avidin peroxidase in lane 2.*

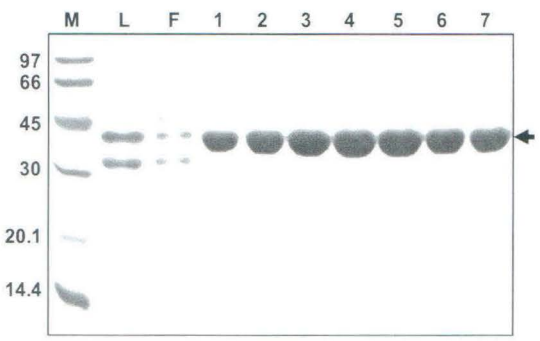


Figure 4.15. SDS- PAGE analysis of the Ni-NTA eluted fraction of r-Bio-HIV-MEP. *Fractions from the affinity column were assayed on a SDS-PAGE. Low molecular weight protein markers were analyzed in lane M. Their molecular weights, in kDa, are shown on the left. Lanes L and F show the load and flow-through fractions of the column. Lanes 1-7 show the purified fractions under denaturing conditions in presence of 8M urea. Protein bands were visualized by Coomassie Blue staining. Arrow on right indicates position of r-Bio-HIV-MEP.*

4.3.4. r-HIV-MEP based and r-Bio-HIV-MEP based TRF immunoassays for the detection of anti-HIV antibodies using europium(III) chelate as reporter

The purified r-HIV-MEP and r-Bio-HIV-MEP were used to establish in-house anti-HIV **T**ime-**R**esolved **F**luorometric (TRF) immunoassays. In these assays, the purified r-HIV-MEP (500 ng/well), passively coated on a microtiter plate, and r-Bio-HIV-MEP (100 ng/well), immobilized on a streptavidin coated plate, were used to capture anti-HIV antibodies in human sera. Bound anti-HIV antibodies were detected by 50 ng/well anti-human antibodies labeled with europium(III) chelate and europium fluorescence was measured by time-resolved fluorometry. The formats of these assays are schematically represented in Figure 4.16.

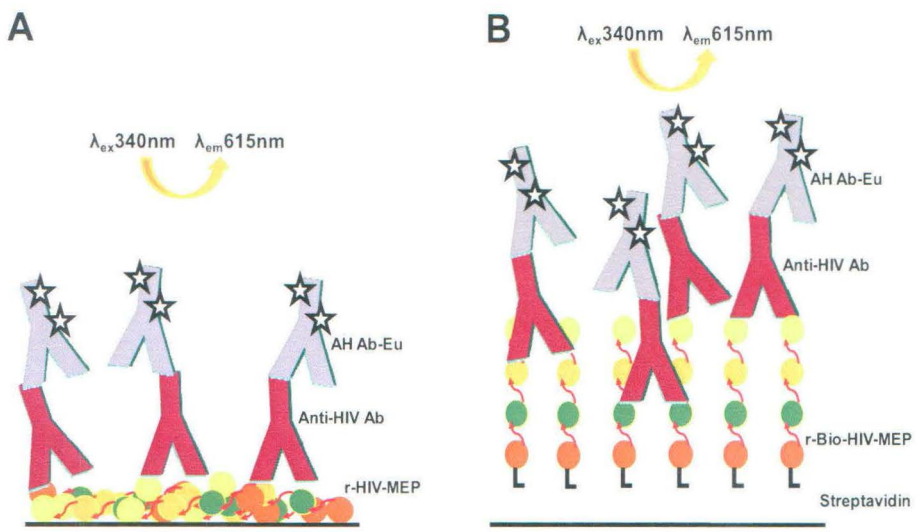


Figure 4.16. Diagrammatic representation of r-HIV-MEP based and r-Bio-HIV-MEP based TRF immunoassays *(A) r-HIV-MEP passively coated on a 96-well plate and blocked. Step-1, addition of human sera containing antibodies to HIV. Step-2, addition of europium(III) chelate-labeled anti-human antibody and detection by TRF. (B) r-Bio-HIV-MEP immobilized on streptavidin coated wells. Step-1, addition of human sera containing antibodies to HIV. Step-2, addition of europium(III) chelate-labeled anti-human antibody and detection by TRF.*

4.3.5. Evaluation of r-HIV-MEP based and r-Bio-HIV-MEP based TRF immunoassays

To establish the specificity of r-HIV-MEP and r-Bio-HIV-MEP as intermediate for anti-HIV immunoassays, more than 50 HIV-negative human sera samples were evaluated. To designate sera as either positive or negative, we used a stringent cut-off value of 6500 counts/sec for r-HIV-MEP based- and 6000 counts/sec for r-Bio-HIV-MEP based-TRF immunoassay, which was obtained by adding three times the

standard deviation to the mean of HIV-negative sera [Leland, 2000]. Sera with a 'signal to cut-off' (S/Co) ratio <1.0 were designated as Negative (-) while those with S/Co ≥ 1.0 were designated as Positive (+). Results demonstrated that r-HIV-MEP and r-Bio-HIV-MEP did not exhibit any false-positivity with normal human serum samples. These results unequivocally established the high degree of specificity of these antigens for the detection of anti-HIV antibodies in infected human sera.

Next, fifty seven serum samples from various well-characterized BBI panels (PRB 931, WWRB 302 and PCA 201) were used to evaluate the in-house r-HIV-MEP-based TRF immunoassay and 9 samples from BBI panel (PRB 931) were used to evaluate the in-house r-Bio-HIV-MEP-based TRF immunoassay. Table 4.2 compares the ability of our in-house r-HIV-MEP-based and r-Bio-HIV-MEP-based TRF immunoassays to detect early seroconversion, with those of other commercial EIA kits using a set of 9 sera constituting the HIV-1 Seroconversion Panel (PRB 931). The earliest time point at which seroconversion was detected in this panel was at 28 days, using both the in-house assay formats, represented by panel member #6. It was found that out of the five commercial kits tested using PRB 931, this member was detected by only Abbott HIV-1/2 EIA kit and our in-house assays. The sensitivity of the r-HIV-MEP antigen to detect anti-HIV antibodies was assessed further by testing it against BBI's Worldwide HIV Performance Panel (WWRB 302) consisting of 25 sera. Of this, 21 sera were HIV-1 positive based on commercial EIA kits, representing genotypes A, B, C, D, E, F, G and O, from diverse geographical locations such as USA, Spain, and from several countries in Asia and Africa. Of the remaining four sera in this panel, two were HIV-2 positive and two were HIV-negative (Table 4.3). Interestingly, the in-house immunoassay using r-HIV-MEP identified all 21 HIV-1 and the two HIV-2 samples. Further, the two sera that were HIV seronegative using 5 different commercial EIA kits were also found to be seronegative in the in-house assay.

In order to examine the specificity of the in-house assay in the background of other infections, BBI's Viral Co-infection Performance Panel consisting of 25 sera (PCA 201) was used. Of these, 9 were HIV-seronegative while the rest (n=16) were HIV-seropositive. Many of these samples were also seropositive for HBV, HCV and/ or HTLV, based on commercial assays. We tested 7 of the HIV-seronegative, all 16 of

the HIV-seropositive samples using the r-HIV-MEP-based TRF immunoassay. The results are summarized in Table 4.4. Significantly, regardless of the presence or absence of antibodies to HBV, HTLV or HCV, the results of the in-house immunoassay for HIV antibodies closely matched the results obtained with the commercial assays. The lone exception was provided by panel member #20. This serum which scored as HIV-positive with the commercial kits was assigned as HIV-negative using the r-HIV-MEP-based assay. A closer examination revealed that this discrepancy is attributable to this sample being a borderline specimen. The S/Co ratio which must be ≥ 1.0 to designate a sample as seropositive was 1.1 for the commercial kit and 0.9 using the in-house assay.

Overall, the data showed that the performance of the single antigen (r-HIV-MEP) -based TRF immunoassay was in near total agreement with the commercially available multi antigen based anti-HIV EIA kits, namely, Abbott HIV-1, Abbott HIV-1/2, Genetic Systems HIV-1, Genetic Systems HIV-1/2 and Organon Teknika HIV-1. The sensitivity and specificity of r-HIV-MEP based TRF immunoassay were calculated to be 97.7% and 100%, respectively.

Table 4.2. Evaluation of r-HIV-MEP based and r-Bio-HIV-MEP based TRF immunoassays using HIV-1 Seroconversion Panel (PRB 931, Boston Biomedica Inc.)

PRB 931 Member ID#	Days since first bleed[a]	Abbott[b]		Gen. Sys.[b]		Org. Tek. HIV[b]	r-HIV-MEP TRF immuno assay[c]	r-Bio-HIV-MEP TRF Immuno assay[d]
		HIV1	HIV1/2	HIV1	HIV1/2			
		2 Ag[e]	3 Ag[e]	1 Ag[e] + Viral lysate	2 Ag[e]	1 Ag[e] + Viral lysate	This study* 1 Ag[e]	This study 1 Ag[e]
1	0	0.2	0.1	0.2	0.1	0.3	**0.1 (-)**	**0.2 (-)**
2	2	0.2	0.1	0.1	0.1	0.3	**0.2 (-)**	**0.1 (-)**
3	7	0.2	0.1	0.2	0.1	0.4	**0.3 (-)**	**0.1 (-)**
4	9	0.2	0.1	0.2	0.1	0.3	**0.3 (-)**	**0.2 (-)**
5	15	0.2	0.1	0.2	0.1	0.3	**0.3 (-)**	**0.3 (-)**
6	28	0.9	6	0.3	0.4	0.6	**1.2 (+)**	**1.3 (+)**
7	33	3.9	>18.7	0.8	1.1	2.3	**5.6 (+)**	**5.9 (+)**
8	35	5.7	>18.7	1.3	1.9	3.1	**9.2 (+)**	**9.3 (+)**
9	42	10.5	>18.7	2.9	4	4.6	**10.6 (+)**	**11.0 (+)**

[a] Bleed dates for member IDs 01 to 05 were in August 1995; those for IDs 06 to 09 were in September 1995.

[b] Values indicate 'signal-to-cut-off'(S/Co) ratios, provided by the panel supplier (BBI) using the indicated commercial EIA kits. Gen. Sys., Genetic Systems; Org. Tek., Organon Teknika. S/Co values ≥ 1.0 are considered as positive.

[c] and [d] Values indicate 'signal-to-cut-off' ratios obtained using the in-house r-HIV-MEP based and r-Bio-HIV-MEP based TRF immunoassays. The results using the in-house assays are indicated in parentheses. Samples with S/Co values < 1.0 are designated as negative (-) and those with values ≥ 1.0 are designated as positive (+).

[e] Ag indicates no. of antigens used in the corresponding immunoassay.

* Talha et al, 2010.

Table 4.3. Evaluation of r-HIV-MEP based TRF immunoassay using Worldwide HIV Performance Panel (WWRB 302, Boston Biomedica Inc.)

WWRB 302 Member ID#[a]	Origin	Genotype	Abbott[b]		Gen. Sys.[b]		Org. Tek. HIV1[b]	r-HIV-MEP TRF Immunoassay[c]
			HIV1	HIV1/2	HIV1	HIV1/2		
			2 Ag[d]	3 Ag[d]	1 Ag[d] + Viral lysate	2 Ag[d]	1 Ag[d] + Viral lysate	This study* 1 Ag[d]
01	Spain	O	1.1	1.8	0.8	5.6	1.3	**10.8** (+)
02	Ghana	A	>11.5	>16.1	6.9	8.7	7.0	**11.8** (+)
03	Ghana	G	>11.5	>16.1	7.1	8.8	7.2	**12.3** (+)
04	Ghana	G	>11.5	>16.1	7.1	8.8	6.5	**11.7** (+)
05	Ghana	A	>11.5	>16.1	7.1	8.7	7.0	**1.8** (+)
06	Ghana	G	>11.5	>16.1	6.9	8.8	7.1	**9.2** (+)
08	Ivory Coast	G	>11.5	>16.1	6.9	8.7	6.7	**11.8** (+)
09	Ivory Coast	A	>11.5	>16.1	6.9	8.6	6.5	**12.5** (+)
10	Ivory Coast	Neg	0.4	0.2	0.1	0.4	0.4	**0.5** (-)
11	Mozambique	HIV-2	1.2	14.6	0.6	9.7	3.0	**1.8** (+)
12	Mozambique	C	>11.5	>16.1	7.1	8.9	6.9	**12.9** (+)
14	Uganda	D	>11.5	>16.1	4.5	8.5	6.2	**6.4** (+)
15	Uganda	D	>11.5	>16.1	6.3	8.1	7.2	**3.5** (+)
16	Uganda	D	>11.5	>16.1	7.0	8.8	6.9	**8.8** (+)
17	Uganda	D	>11.5	>16.1	6.8	9.8	7.0	**2.7** (+)
19	Zimbabwe	C	>11.5	>16.1	6.0	9.9	7.0	**10.4** (+)
21	China	B	>11.5	>16.1	6.7	8.8	7.0	**12.7** (+)
22	Thailand	E	>11.5	>16.1	7.3	9.8	7.0	**12.5** (+)
24	Thailand	E	>11.5	>16.1	7.4	9.8	6.9	**12.2** (+)
25	India	HIV-2	0.4	15.4	3.8	10	2.1	**11.5** (+)
26	USA	D	>11.5	>16.1	7.4	9.8	7.1	**11.0** (+)
27	USA	B/D	>11.5	>16.1	7.0	9.8	7.2	**13.3** (+)
28	Argentina	F	>11.5	>16.1	7.0	8.9	6.8	**12.0** (+)
29	Argentina	B	>11.5	>16.1	6.9	8.5	6.6	**12.3** (+)
30	Argentina	Neg	0.3	0.2	0.2	0.2	0.4	**0.3** (-)

[a] Panel comprised of 25 members. Sample # 7, 13, 18, 20, 23 were not provided by the panel supplier (BBI).

[b] Values indicate 'signal-to-cut-off'(S/Co) ratios, provided by the panel supplier (BBI) using the indicated commercial EIA kits. Gen. Sys., Genetic Systems; Org. Tek., Organon Teknika. S/Co values ≥ 1.0 are considered as positive.

[c] Values indicate 'signal-to-cut-off' ratios obtained using the in-house r-HIV-MEP based TRF immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values < 1.0 are designated as negative (-) and those with values ≥ 1.0 are designated as positive (+).

[d] Ag indicates no. of antigens used in the corresponding immunoassay.

* Talha et al, 2010.

Table 4.4. Evaluation of r-HIV-MEP based TRF immunoassay using Viral Co-infection Performance Panel (PCA 201, Boston Biomedica Inc.)

PCA 201 Member ID#	Abbott EIA HBsAg[a]	Org.Tek. Anti HBc[a]	Abbott Anti-HTLV[a]	Genetic systems HTLV Blots	Result[b]	Abbott Anti-HIV1[a]	Dupont HIV Western Blots	Result[c]	Ortho anti-HCV[a]	r-HIV-MEP TRF immuno assay[d]
	2 MAbs + 1 PAb	1 Ag	Inactivated virus			2 Ag			3 Ag	This study* 1 Ag
01	53.4	9.0	2.1	68/61,55/53,42,36,29.24	P	0.2	Not done	-	6.3	**0.3** (-)
02	51.6	8.8	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.7	**10.8** (+)
03	49.3	8.7	2.1	68/61,55/53,42,36,29.24	P	0.1	Not done	-	6.3	**0.2** (-)
04	26.4	7.7	2.1	68/61,55/53,42,36,34,29,24,19	P	0.1	Not done	-	0.4	**ND**
05	0.4	7.4	2.1	68/61,55/53,46,36,34,29,24,19	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**12.3** (+)
06	0.5	0.5	0.1	N/A	-	0.1	Not done	-	0.5	**ND**
07	0.7	0.2	2.1	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**12.3** (+)
08	38.9	57.9	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	2.1	**10.5** (+)
09	1.0	0.6	2.1	68/61,55/53,42,36,29.24	P	13.5	15,24,31,41,53,55,64,120,160	P	2.2	**12.0** (+)
10	42.8	7.8	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.2	**12.1** (+)
11	48.6	8.5	2.1	68/61,55/53,42,36,29.24,19	P	0.1	Not done	-	6.3	**0.5** (-)
12	38.7	8.5	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**14.0** (+)
13	33.63	9.2	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.7	**10.8**(+)
14	51.8	8.2	2.1	68/61,55/53,42,36,29.24,19	P	0.32	Not done	-	0.5	**0.1** (-)
15	64.5	2.1	1.6	55/53,42,36,29.24	P	0.2	Not done	-	6.3	**0.7** (-)
16	0.5	5.4	2.1	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**13.0** (+)
17	42.3	8.1	0.1	N/A	-	13.5	15,24,31,41,53,64,120,160	P	1.1	**12.1** (+)
18	55.1	0.4	2.3	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**12.3** (+)
19	45.6	2.9	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.4	**12.2** (+)
20	12.2	6.2	0.1	N/A	-	1.1	15,24,vf31, vf41, vf53,vf55,f16	P	0.1	**0.9** (-) ?
21	42.2	2.7	0.2	N/A	-	13.5	15,24,31,41,53,64,120,160	P	0.9	**12.5** (+)
22	41.5	7.2	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**13.7** (+)
23	46	8.1	2.1	68/61,55/53,42,32,24	P	0.2	Not done	-	6.3	**0.6** (-)
24	0.5	0.5	0.2	N/A	-	0.1	Not done	-	0.4	**0.4** (-)
25	50.1	8.5	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.9	**9.7** (+)

.....Continued footnotes, Table 4.4

[a] Values indicate 'signal-to-cut-off'(S/Co) ratios, provided by the panel supplier (BBI) using the indicated commercial EIA kits. Org. Tek., Organon Teknika. S/Co values ≥ 1.0 are considered as positive.

[b] and [c] result of HTLV and HIV Western blot, respectively, provided by the panel supplier (BBI). 'P' and '-' indicate the presence and absence, respectively, of antigen bands in the blot assays.

[d] Values indicate 'signal-to-cut-off' ratios obtained using the in-house r-HIV-MEP based TRF immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values < 1.0 are designated as negative (-) and those with values ≥ 1.0 are designated as positive (+). 'ND' indicates 'not determined' due to lack of samples. '?' indicates a borderline result in the in-house assay.

* Talha et al, 2010.

4.3.6. Conclusion

- The r-HIV-MEP was designed using phylogenetically conserved epitopes, because they would facilitate the recognition of multiple HIV types and groups. The selected epitopes were fused in frame using flexible tetra glycyl linkers between adjacent epitopes.
- The synthetic gene encoding r-HIV-MEP was cloned, *in vivo* biotinylated and expressed in *E. coli.* r-HIV-MEP and r-Bio-HIV-MEP were purified using Ni-NTA affinity chromatography and evaluated as single antigen diagnostic intermediates for the detection of anti-HIV antibodies using commercially available and well-characterized serum panels from Boston Biomedica Inc.
- r-HIV-MEP based TRF immunoassay detected HIV infection from diverse geographical locations, with high specificity and sensitivity.
- r-HIV-MEP based and r-Bio-HIV-MEP based TRF immunoassays showed high sensitivity in monitoring seroconversion, which was comparable with the commercially available EIA kits.
- The sensitivity and specificity of r-HIV-MEP based TRF immunoassay were calculated to be 97.7% and 100%, respectively.
- The use of fluorescent europium(III) chelate labeled antibody as tracer further simplified the immunoassay by easy measurement of time-resolved fluorescence directly from the dry wells of microtiter plate without addition of any substrate and stopping the reaction.

4.4. r-HCV-MEP and in-house anti-HCV TRF immunoassay

A novel recombinant multiepitope diagnostic intermediate, r-HCV-MEP was designed by fusing seven virus specific, immuno-dominant, linear and phylogenetically conserved epitopes from structural and non-structural proteins of HCV. r-HCV-MEP was *in vivo* biotinylated using cloning strategies in *E. coli*. *In vivo* biotinylated r-HCV-MEP (r-Bio-HCV-MEP) was purified to homogeneity using Ni-NTA affinity chromatography. In-house TRF immunoassay was developed based on r-Bio-HCV-MEP as capture antigen for the detection of anti-HCV antibodies in infected human serum samples. The assay was evaluated using commercially available and well-characterized serum panels from Boston Biomedica Inc. Overview of the experimental section is shown in Figure 4.17.

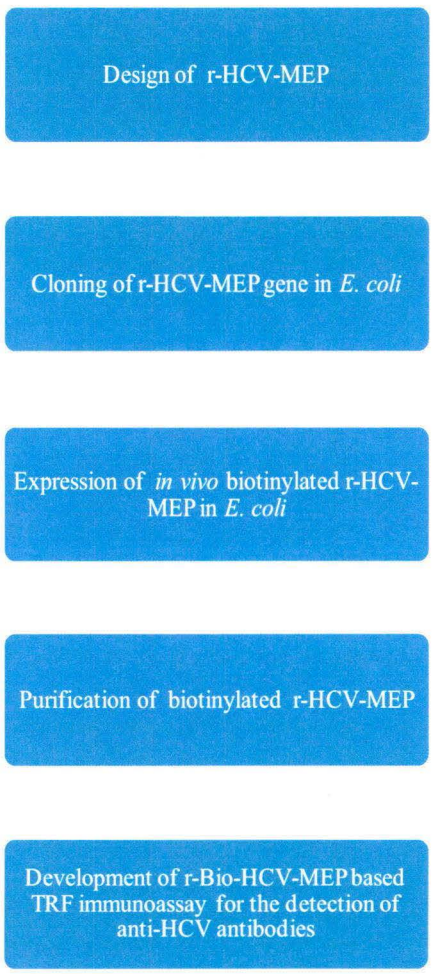


Figure 4.17. Overview of experimental section for r-HCV-MEP and in-house anti-HCV TRF immunoassay

4.4.1. Design of r-HCV-MEP

The two major factors, which prevent universal screening of anti-HCV antibodies in blood samples, in a blood-bank setting, are the high costs and unreliable worldwide performance of the currently available anti-HCV EIA kits. The high cost is due to requirement of multiple diagnostic intermediates, for the production of these kits. The diverse geographical distribution of HCV genotypes and subtypes, results in the unreliable performance of these kits. To address these issues, an alternative approach was explored to design a single recombinant HCV **M**ulti-**E**pitope **P**rotein, HCV-MEP Version2 (HCV-MEP V2), as a suitable inexpensive diagnostic intermediate for anti-HCV EIA kits. For the designing of HCV-F-MEP Version1 (HCV-F-MEP V1) six immuno-dominant and linear epitopes, conserved in most genotypes of HCV worldwide, were selected from the literature, as described by Chugh et al., 2006. The following modifications were made in HCV-F-MEP V1 to generate HCV-MEP V2: additional epitopes from non-structural proteins of HCV, namely, NS4A (3a) and NS4A (2b), a longer region of NS3 (1b), slightly longer region of NS4B (1b) and NS5A (1a) were incorporated and a core epitope (subtype 3g) was omitted in the design of HCV-MEP V2 (Figure 4.18). These immunodominant epitopes, known to elicit anti-HCV antibodies, were selected based on published literature and are summarized in Table 4.5.

Table 4.5: List of HCV-specific immunodominant epitopes selected from the literature to design the r-HCV-MEP V2 antigen

Viral protein (Subtype)	Position of epitopes in HCV Polyprotein (aa) #	% Positivity with patient sera	Reference
NS3 (1b)*	1192-1457 (266)	15	[Park et al., 1995]
NS4A (3a)	1681-1735 (55)	52.4	[Park et al., 1995]
NS4A (2b)	1681-1735 (55)	52.4	[Park et al., 1995]
NS4A (1b)*	1681-1736 (56)	52.4	[Park et al., 1995]
NS4B (1b)*	1920-1941 (22)	82.1	[Park et al., 1995]
NS5A (1a)*	2273-2307 (35)	46.6	[Duo et al., 2002]
Core (1b)*	1-44 (44)	92.6	[Park et al., 1995]

** Indicates that the same or partial epitopes were used in the design of HCV-F-MEP V1 [Chugh et al., 2006].*

 # Values in parentheses represent the total no. of amino acid residues taken of the corresponding epitope.

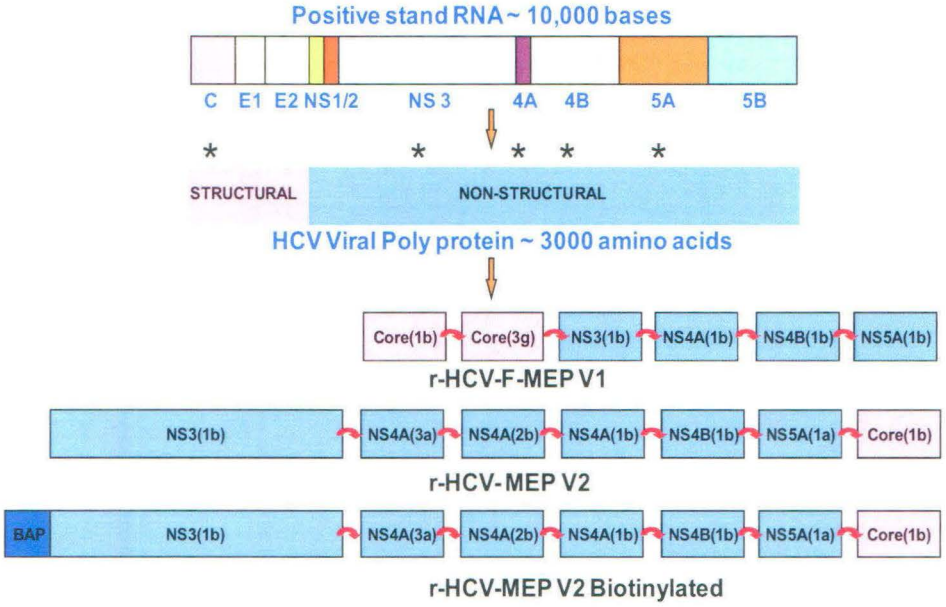
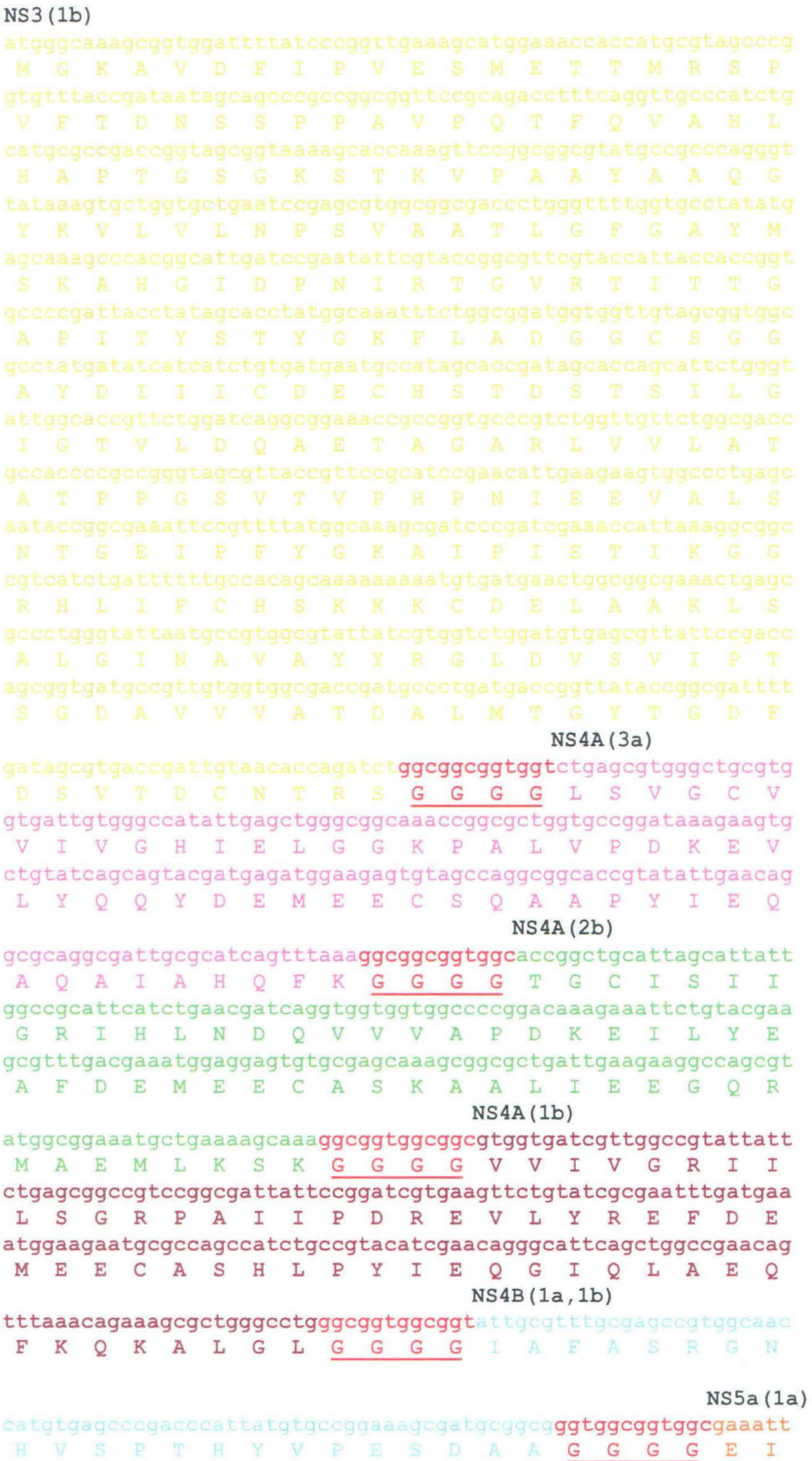


Figure 4.18. Schematic representation of genome, poly-protein and design of synthetic non-biotinylated and biotinylated multiepitope proteins of HCV. *The HCV genome is a single-stranded, positive-sense RNA molecule approximately 10 kilo bases (kb) in length with a single open reading frame encoding a polyprotein of 3,010 to 3,033 depending upon the strain. The poly-protein is processed into several structural and non-structural polypeptides by host cell and virally encoded proteases. There is a non-coding region (NCR) of 324-341 nucleotides at the 5' end & a 3' NCR of variable length including a poly (U) tract. The structural proteins are the core and two envelope polypeptides. The several non-structural proteins have various enzymatic functions. Five conserved and immunodominant epitopes from the structural [Core (1b and 3g)] and non-structural [NS3 (1b), NS4A (1b), NS4B (1b) and NS5A (1b)] proteins were selected and stitched with flexible linkers to form r-HCV-F-MEP V1. Seven conserved and immunodominant epitopes from the non-structural [(NS3 (1b), NS4A (3a, 2b and 1b), NS4B (1b) and NS5A (1a)] and structural [Core (1b)] proteins were selected and stitched with flexible linkers to form r-HCV-MEP V2. The asterisks indicate the regions of the HCV viral polyprotein from where the epitopes have been selected. In vivo biotinylation of r-HCV-MEP V2 was further done to immobilize it on streptavidin plate.*

The r-HCV-MEP V2 (hereafter referred to as r-HCV-MEP) was designed by linking the selected epitopes in tandem using $(gly)_4$ peptides. The DNA and predicted amino acid sequence of r-HCV-MEP is shown in Figure 4.19.

The software modeling analysis of the three dimensional structure of the r-HCV-MEP is shown in Figure 4.20. The analysis suggested that the designed MEP permitted easy accessibility of all its constituent epitopes. All these epitopes may therefore be freely available for interaction with their cognate antibodies and may contribute significantly to their overall sensitivity and specificity for patient sera.


NS3 (1b)
NS4A (3a)
NS4A (2b)
NS4A (1b)
NS4B (1a, 1b)
NS5a (1a)

```
ctgcgtaaaagccgtcgttttgcgcaggcgctgccggtctgggcgcgtccggattacaat
 L  R  K  S  R  R  F  A  Q  A  L  P  V  W  A  R  P  D  Y  N
                                                  Core(1a,1b)
ccgccgctggttgaaacctggaaaaaaccggattatgaaggcggtggtggcatgagcacc
 P  P  L  V  E  T  W  K  K  P  D  Y  E  G  G  G  G  M  S  T
aatccgaaaccgcagcgtaaaaccaaacgtaacaccaaccgtcgtccgcaggatgtgaaa
 N  P  K  P  Q  R  K  T  K  R  N  T  N  R  R  P  Q  D  V  K
tttccgggcggtggccagattgtgggcggcgtgtatctgctgccgcgtcgtggtccgcgt
 F  P  G  G  G  Q  I  V  G  G  V  Y  L  L  P  R  R  G  P  R
ctgacggtggccagattgtgggcggcgtgtatctgctgccgcgtcgtggtccgcgtctga
 L  T  V  A  R  L  W  A  A  C  I  C  C  R  V  V  V  R  V  *
```

Figure 4.19. Complete nucleotide (small case alphabets) and predicted aa (capital case alphabets) sequences of r-HCV-MEP gene showing seven epitopes *[aa 1-270: NS3(1b); aa 275-329: NS4A (3a); aa 334-388: NS4A (2b); aa 393-448: NS4A (1b); aa 453-474: NS4B (1a, 1b); aa 479-513: NS5A (1a); aa 518-561: Core (1a, 1b)] linked together with flexible tetra glycyl linkers (underlined). The asterisk indicates the engineered stop codon.*

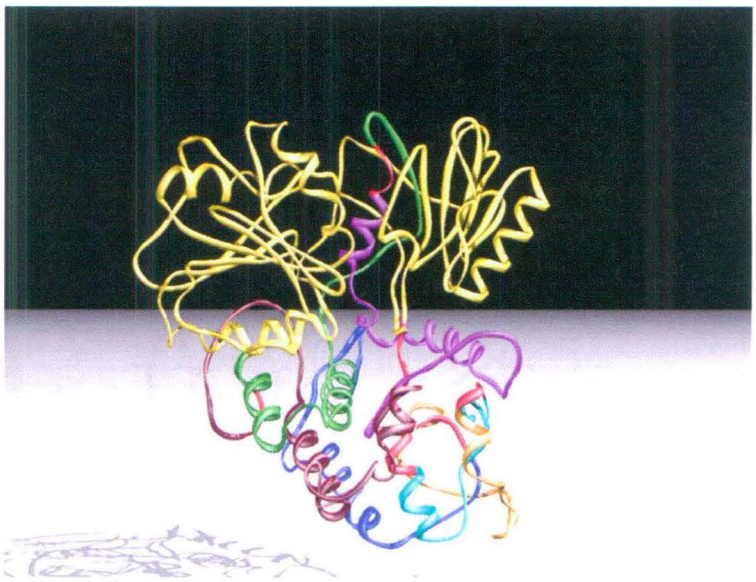


Figure 4.20. Software-generated graphic visualization (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) of ribbon model of r-HCV-MEP. All seven epitopes are shown in different colours. The colour of each epitope corresponds to the amino acid sequence shown in the same colour in figure 4.19.

4.4.2. Cloning, expression and purification of *in vivo* biotinylated r-HCV-MEP

The r-HCV-MEP gene was cloned into the *in vivo* biotinylation expression vector pET-28b(+)-Trx-BAP at unique *Eco*RI and *Hin*dIII sites in an open reading frame under the control of T7 promoter (Figure 4.21).

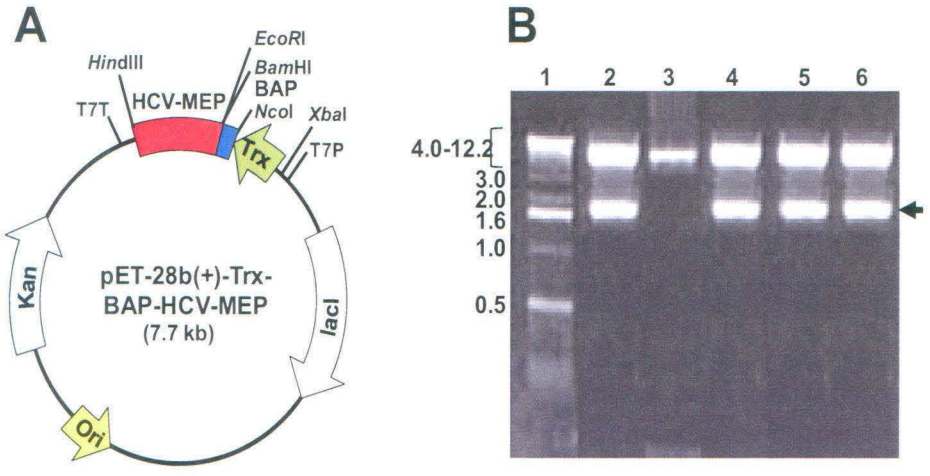


Figure 4.21. Cloning of r-HCV-MEP gene in in vivo biotinylation vector pET-28b(+)-Trx-BAP . *(A) Map of expression vector pET-28b(+)-Trx-BAP with gene encoding r-HCV-MEP, inserted into the unique EcoRI and HindIII sites. (B) Synthetic gene of r-HCV-MEP was digested with EcoRI and HindIII enzymes and cloned in pET-28b(+)-Trx-BAP at respective sites. Putative clones were confirmed by digestion with EcoRI and HindIII. The resultant digested products were analyzed on 1% agarose gel. Lane 2, 4, 5 and 6 show the positive clones with the expected insert fall out of r-HCV-MEP gene at 1.7 kb. DNA size markers are shown in lane 1 with relevant sizes (in kb) indicated on the left. Arrow on right indicates position of r-HCV-MEP gene insert.*

pET-28b(+)-Trx-BAP-r-HCV-MEP plasmid and pETDuet-1-BirA plasmid were co-transformed into the *E. coli* strain BL21(DE3) and transformants were selected in presence of ampicillin and kanamycin containing medium. The recombinants were induced with 1 mM IPTG and were analyzed for expression. Figure 4.22A shows the polypeptide profile of r-Trx-BAP-HCV-MEP (r-Bio-HCV-MEP) clone. The synthesis of the predicted ~79 kDa r-Bio-HCV-MEP and 34 kDa BirA protein was evident in the induced cells (Figure 4.22A, lane 2) and both the proteins were absent in un-induced cells (Figure 4.22A, lane 1). The expression and *in vivo* biotinylation of r-Bio-HCV-MEP was confirmed by western blot. The un-induced and induced samples were transferred onto nitrocellulose membrane after SDS–PAGE and probed with avidin peroxidase (Figure 4.22B). It indicated the presence of ~79 kDa biotinylated protein (r-Bio-HCV-MEP) in induced cells (Figure 4.22B, lane 2) whereas the un-induced cells did not express any biotinylated protein (Figure 4.22B, lane 1).

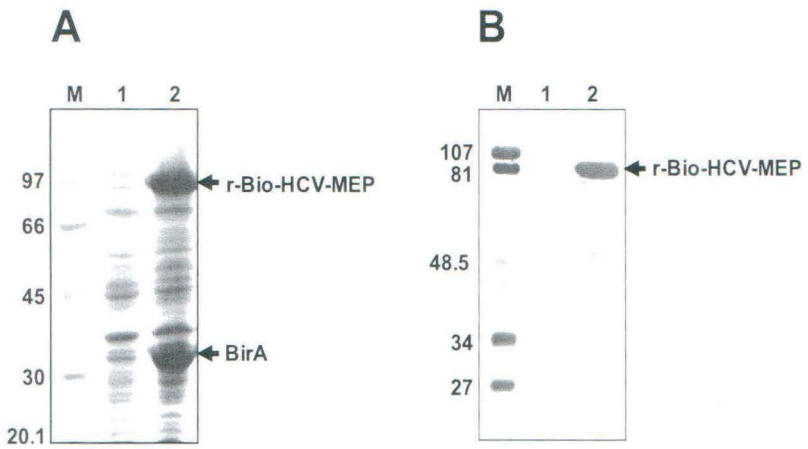


Figure 4.22. Expression and western blot analysis of r-Bio-HCV-MEP. *(A) SDS-PAGE analysis of Un-induced and induced cultures of E. coli expressing the r-Bio-HCV-MEP. Low molecular weight protein markers were analyzed in lane M. Their sizes in (kDa) are shown on the left. Lanes 1 and 2 show Un-induced and Induced cultures of E. coli expressing r-Bio-HCV-MEP (79 kDa) respectively. BirA (34 kDa) is co-expressed along with r-Bio-HCV-MEP in lane 2. (B) Western blot analysis of r-Bio-HCV-MEP with avidin peroxidase. Prestained protein markers were analyzed in lane M. Their sizes in (kDa) are shown on the left. Lanes 1 and 2 show Un-induced and Induced cultures of E. coli expressing r-Bio-HCV-MEP, respectively. r-Bio-HCV-MEP is recognized by avidin peroxidase in lane 2.*

Lysis of the induced cells by sonication under native conditions showed that the protein was not present in the soluble fraction. Therefore, guanidinium-HCl was used to solubilize the protein. Subsequently, its purification was carried out under denaturing condition in presence of 8 M urea using Ni-NTA affinity chromatography. The fractions of eluted protein were analysed by SDS-PAGE (Figure 4.23, lane 1-7). The crude lysate (loaded on the column) containing the r-Bio-HCV-MEP is shown in lane 'L' and the flow through after binding is shown in lane 'F'. Extensive washing at pH 6.3 and 5.9 ensured that no non-specific contaminants remained bound to the column. Elution at pH 4.3 yielded highly pure r-Bio-HCV-MEP from the column (lanes 1-7). From a comparison of the protein profiles of the elution with crude lysate, it was evident that >95% purity was achieved. Starting from 3g induced cell biomass from 1L *E. coli* culture, ~30 mg of purified r-Bio-HCV-MEP (pooled fractions in lanes 1-7) was obtained.

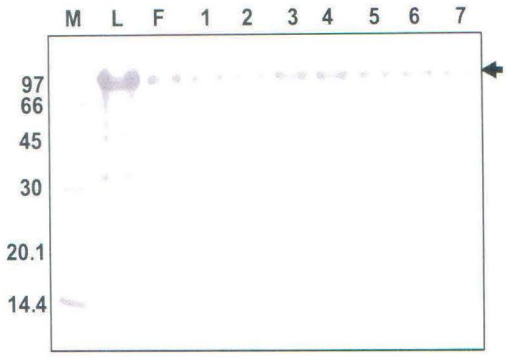


Figure 4.23. SDS- PAGE analysis of the Ni-NTA eluted fraction of r-Bio-HCV-MEP. *Fractions from the affinity column were assayed on a SDS-PAGE. Low molecular weight protein markers were loaded in lane M. Their molecular weights, in kDa, are shown on the left. Lanes L and F show the load and flow-through fractions of the column. Lanes 1-7 show the purified fractions under denaturing conditions in presence of 8M urea. Protein bands were visualized by Coomassie Blue staining. Arrow on right indicates position of r-Bio-HCV-MEP.*

4.4.3. r-Bio-HCV-MEP based TRF immunoassay for the detection of anti-HCV antibodies using europium(III) chelate as reporter

The purified r-Bio-HCV-MEP was used to establish in-house anti-HCV **T**ime-**R**esolved **F**luorometric (TRF) immunoassay. In this assay, the purified r-Bio-HCV-MEP (100 ng/well) immobilized on a streptavidin coated plate was used to capture anti-HCV antibodies in human sera. Bound anti-HCV antibodies were detected by 50 ng/well anti-human antibodies labeled with europium(III) chelate and europium fluorescence was measured by time-resolved fluorometry. The format of this assay is schematically represented in Figure 4.24.

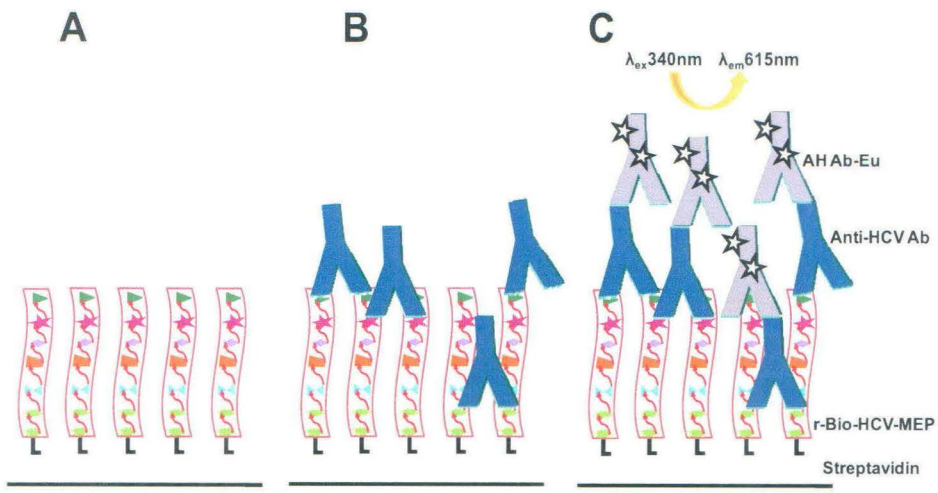


Figure 4.24. Diagrammatic representation of r-Bio-HCV-MEP based TRF immunoassay *(A) r-Bio-HCV-MEP immobilized on streptavidin coated plate (B) Step-1, addition of human sera containing antibodies to HCV. (C) Step-2, addition of europium(III) chelate-labeled anti-human antibody and detection by TRF.*

4.4.4. Evaluation of r-Bio-HCV-MEP based TRF immunoassay

To establish the specificity of r-Bio-HCV-MEP as an intermediate for anti-HCV TRF immunoassay, more than 50 HCV-negative human sera samples were evaluated. To designate sera as either positive or negative, we used a stringent cut-off value of 7400 counts/sec for r-Bio-HCV-MEP based TRF immunoassay, which was obtained by adding three times the standard deviation to the mean of HCV-negative sera [Leland, 2000]. Sera with a 'signal to cut-off' (S/Co) ratio <1.0 were designated as Negative (-) while those with S/Co $\geq$ 1.0 were designated as Positive (+). Results demonstrated that r-Bio-HCV-MEP did not exhibit any false-positivity with normal human serum samples. These results unequivocally established the high degree of specificity of r-HCV-MEP protein for the detection of anti-HCV antibodies in infected human sera.

Next, fifty four serum samples from various well-characterized BBI panels (PHV 901, WWHV 301 and PCA 201) were used to evaluate in-house r-Bio-HCV-MEP based TRF immunoassay. Table 4.6 compares the ability of the in-house immunoassay to detect early seroconversion, with those of other commercial kits using a set of 11 sera constituting the HCV Seroconversion Panel (PHV 901). The earliest time point at which seroconversion was detected in this panel was at 97 days, using the in-house assay, represented by panel member #3. It was found that both the commercial kits tested, Abbott HCV 2.0 EIA and Ortho HCV 2.0 EIA and our in-house assay, detected this member from PHV 901, but the signal-to-cut-off ratios obtained with the in-house assay were several folds higher than the commercially available kits tested. The sensitivity of the r-Bio-HCV-MEP to detect anti-HCV antibodies was further assessed by testing it against BBI's Worldwide HCV Performance Panel (WWHV 301) consisting of 20 sera. Of these 18 sera were HCV positive based on commercial EIA kits, representing genotypes 1-4 and different subtypes, from diverse geographical locations such as Argentina, Uganda, Ghana, China, Egypt and US. The remaining two sera in this panel were HCV-negative (Table 4.7). The in-house immunoassay using r-Bio-HCV-MEP identified all 18 HCV positive samples. Further, the two sera that were HCV seronegative using 3 different commercial kits were also found to be seronegative in the in-house assay.

In order to determine the specificity of the in-house assay in the background of other infections, BBI's Viral Co-infection Performance Panel consisting of 25 sera

(PCA 201) was used. Of these, 11 were HCV-seronegative while the rest (n=14) were HCV-seropositive and many of these samples were also seropositive for HBV, HIV and/ or HTLV, based on commercial assays. We tested 9 of the HCV-seronegative and all 14 of the HCV-seropositive samples using the r-Bio-HCV-MEP-based TRF immunoassay. The results are summarized in Table 4.8. Significantly, regardless of the presence or absence of antibodies to HBV, HIV or HTLV, the results of the in-house immunoassay for HCV antibodies closely matched the results obtained with the commercial assays. Overall, the data showed that the performance of our single r-Bio-HCV-MEP-based immunoassay was in total agreement with the commercially available multi antigen based anti-HCV EIA kits, namely, Abbott HCV 2.0, Abbott HCV 3.0 and Ortho HCV 3.0. The sensitivity and specificity both, of r-Bio-HCV-MEP based TRF immunoassay were calculated to be 100%.

Table 4.6. Evaluation of r-Bio-HCV-MEP based TRF immunoassay using HCV Seroconversion Panel (PHV 901, Boston Biomedica Inc.)

PHV 901 Member ID#	Bleed dates	Days since first bleed	Abbott HCV 2.0[a]	Ortho HCV 2.0[a]	r-Bio-HCV-MEP TRF immunoassay[b]
			3 Ag[c]	2 Ag[c]	This study 1 Ag[c]
1	23 SEP 93	0	0.2	0.0	**0.7** (-)
2	27 NOV 93	65	0.2	0.0	**0.8** (-)
3	29 DEC 93	97	1.6	1.2	**4.0** (+)
4	31 DEC 93	99	1.7	1.2	**3.7** (+)
5	05 JAN 94	104	1.7	1.4	**3.7** (+)
6	07 JAN 94	106	1.6	1.8	**5.6** (+)
7	01 FEB 94	131	3.8	>3.6	**6.8** (+)
8	09 FEB 94	139	3.6	>3.6	**10.8** (+)
9	01 MAR 94	159	>5.0	>3.6	**11.0** (+)
10	08 MAR 94	166	>5.0	>3.6	**11.2** (+)
11	14 APR 94	203	>5.0	>3.6	**10.9** (+)

[a] Values indicate 'signal-to-cut-off' (S/Co) ratios, provided by the panel supplier (BBI) using the indicated commercial EIA kits. S/Co values ≥ 1.0 are considered as positive.
[b] Values indicate 'signal-to-cut-off' ratios obtained using the in-house r-Bio-HCV-MEP based TRF immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values < 1.0 are designated as negative (-) and those with values ≥ 1.0 are designated as positive (+).
[c] Ag indicates no. of antigens used in the corresponding immunoassay.

Table 4.7. Evaluation of r-Bio-HCV-MEP based TRF immunoassay using Worldwide HCV Performance Panel (WWHV 301, Boston Biomedica Inc.)

WWHV 301 Member ID#	origin	Murex HCV serotyping[a]	Innogenetics INNO-LIPA[a]	Abbott HCV 2.0[b]	Abbott HCV 3.0[b]	Ortho HCV 3.0[b]	r-Bio-HCV-MEP TRF immunoassay[c]
				3 Ag[d]	3 Ag[d]	3 Ag[d]	This study 1 Ag[d]
1	Argentina	1	1b	>5.0	>5.6	>5.0	**8.6** (+)
2	Argentina	Not typable	1b	>5.0	>5.6	>5.0	**9.5** (+)
3	Argentina	3	3a/b	>5.0	>5.6	>5.0	**9.5** (+)
4	Argentina	2	2a/c	>5.0	>5.6	>5.0	**10.0** (+)
5	Argentina	Neg	Not tested	0.2	0.1	0.0	**0.2** (-)
6	Uganda	4	4c/d	4.7	>5.6	>5.0	**9.0** (+)
7	Uganda	2	Not typable	2.1	4.4	>5.0	**2.5** (+)
8	Ghana	Neg	Not tested	0.3	0.4	0.1	**0.2** (-)
9	China	1	1b, 2a/c	>5.0	>5.6	>5.0	**3.9** (+)
10	China	1	2	>5.0	>5.6	>5.0	**10.5** (+)
11	China	1	1b	2.4	>5.6	>5.0	**2.4** (+)
12	China	2	2	>5.0	>5.6	>5.0	**10.2** (+)
13	China	1,2	1a/b, 2a/c	>5.0	>5.6	>5.0	**9.7** (+)
14	Egypt	(3)	3a	>5.0	>5.6	>5.0	**3.7** (+)
15	Egypt	(4)	4	3.8	>5.6	>5.0	**10.1** (+)
16	Egypt	(4)	4h	>5.0	>5.6	>5.0	**5.6** (+)
17	Egypt	(4)	Not typable	>5.0	>5.6	>5.0	**2.4** (+)
18	US	1	1b	3.5	>5.6	>5.0	**1.3** (+)
19	US	Not typable	1a	0.8	3.2	>5.0	**1.7** (+)
20	US	1	1a	4.7	>5.6	>5.0	**3.3** (+)

[a] ( ) = stronger reactivity with the peptide indicated, but does not meet all of manufacturer's criteria; type confirmed by research amplification method. Results provided by the panel supplier (BBI).
[b] Values indicate 'signal-to-cut-off'(S/Co) ratios, provided by the panel supplier (BBI) using the indicated commercial EIA kits. S/Co values ≥ 1.0 are considered as positive.
[c] Values indicate 'signal-to-cut-off' ratios obtained using the in-house r-Bio-HCV-MEP based TRF immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values < 1.0 are designated as negative (-) and those with values ≥ 1.0 are designated as positive (+).
[d] Ag indicates no. of antigens used in the corresponding immunoassay.

Table 4.8. Evaluation of r-Bio-HCV-MEP based TRF immunoassay using Viral Co-infection Performance Panel (PCA 201, Boston Biomedica Inc.)

PCA 201 Member ID#	Abbott EIA HBsAg[a]	Org. Tek. Anti HBc[a]	Abbott Anti-HTLV[a]	Genetic systems HTLV Blots	Result[b]	Abbott Anti-HIV1[a]	Dupont HIV Western Blots	Result[c]	Ortho anti-HCV[a]	r-Bio-HCV-MEP TRF immunoassay[d]
	2 MAbs + 1 PAb	1 Ag	Inactivated virus			2 Ag			3 Ag	This study 1 Ag
01	53.4	9.0	2.1	68/61,55/53,42,36,29.24	P	0.2	Not done	-	6.3	**12.5** (+)
02	51.6	8.8	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.7	**0.7** (-)
03	49.3	8.7	2.1	68/61,55/53,42,36,29.24	P	0.1	Not done	-	6.3	**11.0** (+)
04	26.4	7.7	2.1	68/61,55/53,42,36,34,29.24,19	P	0.1	Not done	-	0.4	**ND**
05	0.4	7.4	2.1	68/61,55/53,46,36,34,29.24,19	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**10.9** (+)
06	0.5	0.5	0.1	N/A	-	0.1	Not done	-	0.5	**ND**
07	0.7	0.2	2.1	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**13.6** (+)
08	38.9	57.9	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	2.1	**5.4** (+)
09	1.0	0.6	2.1	68/61,55/53,42,36,29.24	P	13.5	15,24,31,41,53,55,64,120,160	P	2.2	**8.9** (+)
10	42.8	7.8	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.2	**0.2** (-)
11	48.6	8.5	2.1	68/61,55/53,42,36,29.24,19	P	0.1	Not done	-	6.3	**12.3** (+)
12	38.7	8.5	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**13.8** (+)
13	33.63	9.2	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.7	**0.7** (-)
14	51.8	8.2	2.1	68/61,55/53,42,36,29.24,19	P	0.32	Not done	-	0.5	**0.6** (-)
15	64.5	2.1	1.6	55/53,42,36,29.24	P	0.2	Not done	-	6.3	**13.1** (+)
16	0.5	5.4	2.1	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**11.8** (+)
17	42.3	8.1	0.1	N/A	-	13.5	15,24,31,41,53,64,120,160	P	1.1	**1.4** (+)
18	55.1	0.4	2.3	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**11.7** (+)
19	45.6	2.9	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.4	**0.8** (-)
20	12.2	6.2	0.1	N/A	-	1.1	15,24,vf31, vf41, vf53,vf55,f16	P	0.1	**0.6** (-)
21	42.2	2.7	0.2	N/A	-	13.5	15,24,31,41,53,64,120,160	P	0.9	**0.6** (-)
22	41.5	7.2	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**10.0** (+)
23	46	8.1	2.1	68/61,55/53,42,32,24	P	0.2	Not done	-	6.3	**11.6** (+)
24	0.5	0.5	0.2	N/A	-	0.1	Not done	-	0.4	**0.8** (-)
25	50.1	8.5	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.9	**0.3** (-)

.....Continued footnotes, Table 4.8

[a] Values indicate 'signal-to-cut-off'(S/Co) ratios, provided by the panel supplier (BBI) using the indicated commercial EIA kits. Org. Tek., Organon Teknika. S/Co values ≥ 1.0 are considered as positive.

[b] and [c] result of HTLV and HIV Western blot, respectively, provided by the panel supplier (BBI). 'P' and '-' indicate the presence and absence, respectively, of antigen bands in the blot assays.

[d] Values indicate 'signal-to-cut-off' ratios obtained using the in-house r-Bio-HCV-MEP based TRF immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values < 1.0 are designated as negative (-) and those with values ≥ 1.0 are designated as positive (+). 'ND' indicates 'not determined' due to lack of samples.

4.4.5. Conclusion

- To enhance the sensitivity of ant-HCV immunoassay, the r-HCV-F-MEP V1 was modified to design r-HCV-MEP V2 by addition of longer regions of NS3, NS4B and NS5A, and two new NS4A immunodominant epitopes from different subtypes. The selected epitopes were fused in frame using flexible tetra glycyl linkers between adjacent epitopes.
- The synthetic gene encoding r-HCV-MEP was cloned, *in vivo* biotinylated and expressed in *E. coli.* r-Bio-HCV-MEP was purified using Ni-NTA affinity chromatography and evaluated as a single antigen diagnostic intermediate for the detection of anti-HCV antibodies using commercially available and well-characterized serum panels from Boston Biomedica Inc.
- r-Bio-HCV-MEP based TRF immunoassay detected HCV infection from diverse geographical locations, with high specificity and sensitivity. It was also useful in monitoring seroconversion.
- The sensitivity and specificity both, of r-Bio-HCV-MEP based TRF immunoassay were calculated to be 100%.
- The use of fluorescent europium(III) chelate labeled antibody as tracer further simplified the immunoassay by easy measurement of time-resolved fluorescence directly from the dry wells of microtiter plate without addition of any substrate and stopping the reaction.

4.5. In-house HBsAg TRF immunoassay

Monoclonal antibodies (MAbs) 21B and 5S specific for HBsAg were purified to homogeneity. An in-house HBsAg TRF immunoassay was developed using the two purified MAbs. Purified and chemically biotinylated-MAb 21B, immobilized on streptavidin-coated wells, was used to capture HBsAg in human serum samples. The bound HBsAg were detected using 5S $F(ab)_2$ fragments coated on europium(III) nanoparticles. Time-resolved fluorescence of europium was measured directly from dry wells. The HBsAg TRF immunoassay was evaluated by an in-house Finnish human serum panel and a commercially available and well-characterized serum panel from Boston Biomedica Inc. Overview of the experimental section is shown in Figure 4.25.

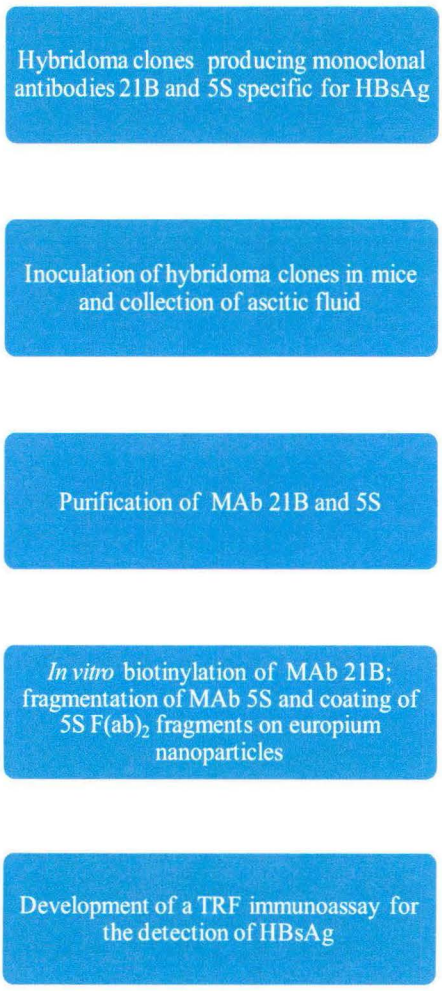


Figure 4.25. Overview of experimental section for in-house HBsAg TRF immunoassay

4.5.1. Purification of MAbs 5S and 21B specific for HBsAg

The hybridoma clones producing monoclonal antibodies (MAbs) 5S and 21B, specific for HBsAg, were inoculated in mice to induce ascitic fluid formation. The ascitic fluid was collected and MAbs 5S and 21B were purified using prosep-VA ultra (protein A) column. Purified MAbs were analysed on SDS-PAGE (Figure 4.26). Both the MAbs were obtained in highly pure form as is clearly evident.

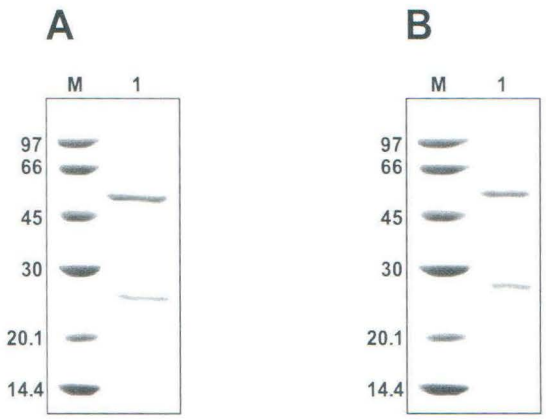


Figure 4.26. Purification of MAb 5S and 21B. *Panel A shows the purified MAb 21B (Lane 1). Panel B shows the purified MAb 5S (Lane 1). Protein markers were analyzed in lane M. Their sizes in kDa are shown on the left.*

4.5.2. *In vitro* biotinylation of MAb 21B

HBsAg specific MAb 21B was biotinylated with 40-fold molar excess of biotin isothiocyanate, as described in materials and methods. Biotinylated MAb was segregated from non-reacted free biotin. Non-biotinylated and biotinylated MAb were analysed on SDS-PAGE (Figure 4.27).

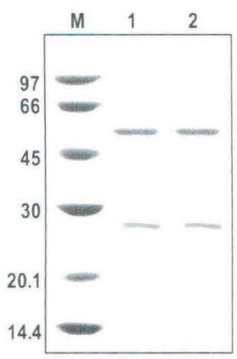


Figure 4.27. Biotinylation of MAb 21B. *Lane1 shows the purified MAb 21B. Lane 2 shows the biotinylated MAb 21B (Bio-MAb21B). Protein markers were analyzed in lane M. Their sizes in kDa are shown on the left.*

4.5.3. Enzymatic fragmentation of MAb 5S and coating of 5S F(ab)$_2$ fragments on europium(III) nanoparticles

5S F(ab)$_2$ fragments were produced by enzymatic fragmentation of 5S MAb using bromelain. The fragmentation product was purified on HiTrap Protein G HP chromatography column. Removal of Fc region of MAb 5S resulted in lower background in subsequent TRF immunoassays (data not shown). The intact MAb 5S and 5S F(ab)$_2$ fragments were analysed on SDS-PAGE (Figure 4.28). Amino groups of 5S F(ab)$_2$ were covalently coupled to activated carboxyl groups of europium(III) nanoparticles. Activation of carboxyl groups was performed using NHS- and EDC-chemistry, as described in materials and methods.

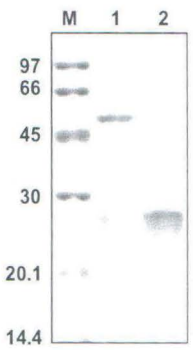


Figure 4.28. Fragmentation of MAb 5S. *Lane 1 shows the purified MAb 5S. Lane 2 shows the purified 5S F(ab)$_2$ fragments. Protein markers were analyzed in lane M. Their sizes in kDa are shown on the left.*

4.5.4. TRF immunoassay for the detection of HBsAg in human sera using europium(III) nanoparticles as reporter

The purified Bio-MAb 21B and 5S F(ab)$_2$ coated on europium nanoparticles were used to establish in-house HBsAg **T**ime-**R**esolved **F**luorometric (TRF) immunoassay. In this assay, the Bio-MAb 21B (150 ng/well) immobilized on a streptavidin coated plate was used to capture HBsAg in human sera. Bound HBsAg were detected by using 5S F(ab)$_2$ coated on europium(III) nanoparticles (10^8 nanoparticles/well) as tracer and europium fluorescence was measured by time-resolved fluorometry. The format of this assay is schematically represented in Figure 4.29.

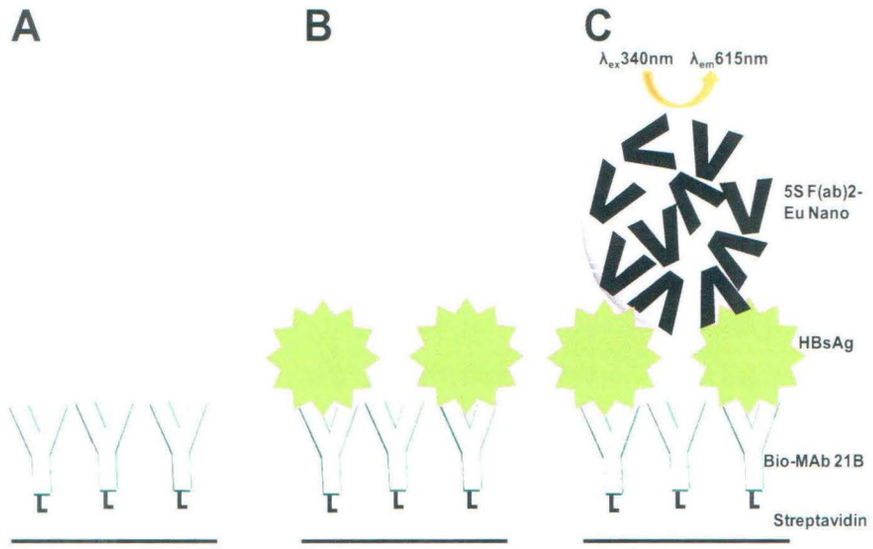


Figure 4.29. Diagrammatic representation of HBsAg TRF immunoassay *(A) Bio-MAb 21B immobilized on streptavidin coated plate (B) Step-1, addition of human serum containing HBsAg (C) Step-2, addition of 5S F(ab)$_2$ coated europium(III) nanoparticles and detection by TRF.*

4.5.5. Evaluation of HBsAg TRF immunoassay

In order to establish the specificity of HBsAg immunoassay, more than 50 HBV negative human serum samples were evaluated. To designate sera as either positive or negative, we used a stringent cut-off value of 1145 counts/sec for HBsAg TRF immunoassay, which was obtained by adding five times the standard deviation to the mean of HBsAg-negative sera [Leland, 2000]. Sera with a 'signal to cut-off' (S/Co) ratio <1.0 were designated as Negative (-) while those with S/Co $\geq$ 1.0 were designated as Positive (+). Results demonstrated that in-house HBsAg TRF immunoassay did not exhibit any false-positivity with normal human serum samples (Figure 4.30). These results unequivocally established the high degree of specificity of monoclonal antibodies (MAb 21B and 5S) for the detection of HBsAg in infected human sera. The assay was further evaluated with an in-house Finnish human serum panel consisting of 25 HBV positive samples, out of which 23 were efficiently detected by the HBsAg TRF immunoassay, while one was undetectable and one was on borderline (Figure 4.30). To evaluate the sensitivity of HBsAg TRF immunoassay to detect HBsAg, dilution series of r-HBsAg were analysed with HBsAg TRF immunoassay. Calibration curve is shown in Figure 4.31. The lowest detectable concentration of r-HBsAg was 0.02 ng/ml and the dynamic linear range was 0.02–200 ng/ml.

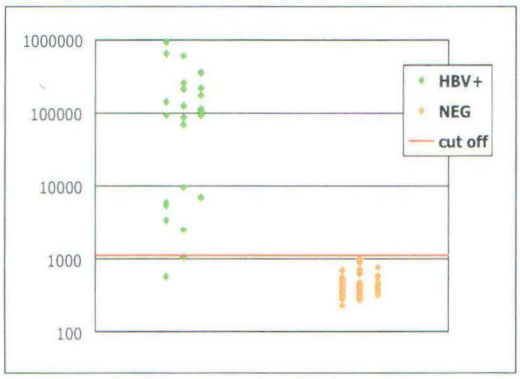


Figure 4.30. HBsAg TRF immunoassay with in-house Finnish human serum panel consisting of normal and HBV infected serum samples.

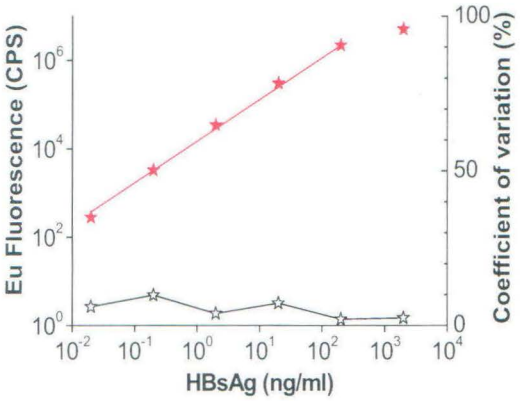


Figure 4.31. Calibration curve for HBsAg detection in HBsAg TRF immunoassay. *Red star represents HBsAg detection. Un-filled star represents coefficient of variation of the assay.*

In order to examine specificity of the in-house assay, in the background of other infections, BBI's Viral Co-infection Performance Panel consisting of 25 sera (PCA 201), was used. Of these, 5 were HBsAg-seronegative while the rest (n=20) were HBsAg-seropositive and many of these samples were also seropositive for HIV, HCV and/ or HTLV, based on commercial assays. We tested 4 of the HBsAg-seronegative and 19 of the HBsAg-seropositive samples using the HBsAg TRF immunoassay. The results are summarized in Table 4.9. Out of the 19 HBsAg-seropositive samples, 3 were not detected by HBsAg TRF immunoassay. The three samples were PCA201 #9, #18, #20. These three samples were tested with Hepanostika HBsAg ultra kit (bioMérieux SA, Marcy l'Etoile, France) and it was found that sample #9 and #18 were borderline cases, while #20 was a clear positive sample. The samples were further tested in a format where Hepanostika kit's coated wells were used as capture and 5S $F(ab)_2$ coated on europium nanoparticles was used as tracer. In this format sample #20 was clear positive, while #9 and #18 remained undetectable. The sensitivity and specificity of HBsAg TRF immunoassay were calculated to be 91.6% and 100%, respectively.

Table 4.9. Evaluation of HBsAg TRF immunoassay using Viral Co-infection Performance panel (PCA 201, Boston Biomedica Inc.)

PCA 201 Member ID#	Abbott EIA HBsAg[a]	Org. Tek. Anti HBc[a]	Abbott Anti-HTLV[a]	Genetic systems HTLV Blots	Result[b]	Abbott Anti-HIV1[a]	Dupont HIV Western Blots	Result[c]	Ortho anti-HCV[a]	HBsAg TRF immuno assay[d]
	2 MAbs + 1 PAb	1 Ag	Inactivated virus			2 Ag			3 Ag	This study 2 MAbs
01	53.4	9.0	2.1	68/61,55/53,42,36,29.24	P	0.2	Not done	-	6.3	**352.4 (+)**
02	51.6	8.8	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.7	**676.4 (+)**
03	49.3	8.7	2.1	68/61,55/53,42,36,29.24	P	0.1	Not done	-	6.3	**168.8 (+)**
04	26.4	7.7	2.1	68/61,55/53,42,36,34,29,24,19	P	0.1	Not done	-	0.4	**ND**
05	0.4	7.4	2.1	68/61,55/53,46,36,34,29,24,19	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**0.3 (-)**
06	0.5	0.5	0.1	N/A	-	0.1	Not done	-	0.5	**ND**
07	0.7	0.2	2.1	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**0.4 (-)**
08	38.9	57.9	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	2.1	**700.6 (+)**
09	1.0	0.6	2.1	68/61,55/53,42,36,29.24	P	13.5	15,24,31,41,53,55,64,120,160	P	2.2	**0.4 (-) ?**
10	42.8	7.8	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.2	**624.0 (+)**
11	48.6	8.5	2.1	68/61,55/53,42,36,29.24,19	P	0.1	Not done	-	6.3	**1.3 (+)**
12	38.7	8.5	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**182.9 (+)**
13	33.63	9.2	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.7	**719.8 (+)**
14	51.8	8.2	2.1	68/61,55/53,42,36,29.24,19	P	0.32	Not done	-	0.5	**276.7 (+)**
15	64.5	2.1	1.6	55/53,42,36,29.24	P	0.2	Not done	-	6.3	**184.2 (+)**
16	0.5	5.4	2.1	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**0.5 (-)**
17	42.3	8.1	0.1	N/A	-	13.5	15,24,31,41,53,64,120,160	P	1.1	**115.6 (+)**
18	55.1	0.4	2.3	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**0.4 (-) ?**
19	45.6	2.9	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.4	**909.5 (+)**
20	12.2	6.2	0.1	N/A	-	1.1	15.24.vf31, vf41, vf53,vf55,f16	P	0.1	**0.5 (-) ?**
21	42.2	2.7	0.2	N/A	-	13.5	15,24,31,41,53,64,120,160	P	0.9	**727.4 (+)**
22	41.5	7.2	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**774.8 (+)**
23	46	8.1	2.1	68/61,55/53,42,32,24	P	0.2	Not done	-	6.3	**255.7 (+)**
24	0.5	0.5	0.2	N/A	-	0.1	Not done	-	0.4	**0.3 (-)**
25	50.1	8.5	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.9	**638.4 (+)**

[a] Values indicate 'signal-to-cut-off'(S/Co) ratios, provided by the panel supplier (BBI) using the indicated commercial EIA kits. Org. Tek., Organon Teknika. S/Co values ≥ 1.0 are considered as positive.

[b] and [c] result of HTLV and HIV Western blot, respectively, provided by the panel supplier (BBI). 'P' and '-' indicate the presence and absence, respectively, of antigen bands in the blot assays.

[d] Values indicate 'signal-to-cut-off' ratios obtained using the in-house HBsAg TRF immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values < 1.0 are designated as negative (-) and those with values ≥ 1.0 are designated as positive (+). 'ND' indicates 'not determined' due to lack of samples. '?' indicates negative result with HBsAg positive samples in in-house immunoassay

4.5.6. Conclusion

- Monoclonal antibodies 5S and 21B, specific for HBsAg, were purified using respective hybridoma clones already prepared in our lab.
- Purified MAb 21B was chemically biotinylated, immobilized on streptavidin-coated wells and was used to capture HBsAg in human serum samples. Bound HBsAg was detected using 5S $F(ab)_2$ coated on europium(III) nanoparticles.
- To establish the specificity of HBsAg TRF immunoassay more than 50 HBV negative human serum samples were evaluated. Results demonstrated that in-house immunoassay did not exhibit any false positivity with normal human serum samples.
- The assay was further evaluated by an in-house Finnish human serum panel and a commercially available and well-characterized serum panel from Boston Biomedica Inc. A total of 44 HBV positive samples were tested, out of which one sample was on borderline and 4 samples remained undetectable with our in-house assay.
- In HBsAg TRF immunoassay, the lowest detectable concentration of r-HBsAg was 0.02 ng/ml.
- The sensitivity and specificity of HBsAg TRF immunoassay were calculated to be 91.6% and 100%, respectively.

4.6. HIV, HCV and HBV multiplexed TRF immunoassay

Individual in-house r-Bio-HIV-MEP based-, r-Bio-HCV-MEP based- and HBsAg - TRF immunoassays were combined to develop an HIV, HCV and HBV multiplexed TRF immunoassay for the simultaneous detection of one or more of the following analytes in human serum samples from the same well – anti-HIV antibody, anti-HCV antibody and HBsAg. The multiplexed assay was evaluated with BBI viral co-infection performance panel. The overview of the experimental section is shown in Figure 4.32.

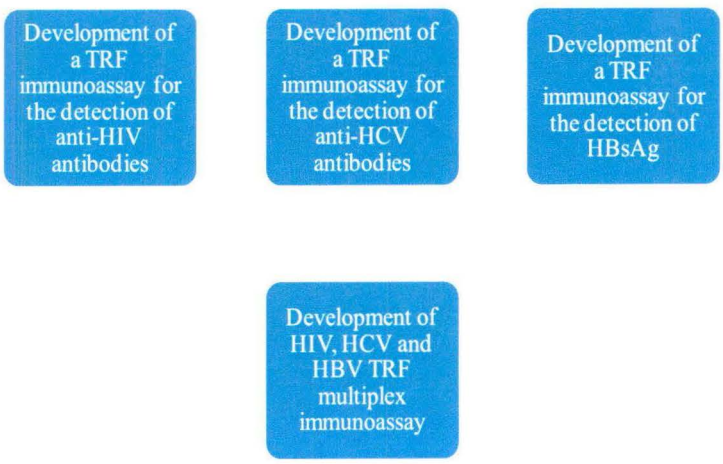


Figure 4.32. Overview of experimental section for HIV, HCV and HBV multiplexed TRF immunoassay

4.6.1. Design of HIV, HCV and HBV multiplexed TRF immunoassay

A major and central objective of multiplexing was the combination of already developed and validated TRF immunoassay concepts for HIV, HCV and HBV into a single test compartment in order to capture all the three analytes (anti-HIV antibody, anti-HCV antibody and HBsAg) from the sera samples, simultaneously. In the multiplexed assay r-Bio-HIV-MEP, r-Bio-HCV-MEP and Bio-MAb 21B (50 ng/well each) were used as capture on streptavidin coated plate and europium(III) chelate labeled anti-human antibody (50 ng/well) and 5S F(ab)2 coated onto europium nanoparticles (10^8 nanoparticles/well) were used as tracer. Anti-HIV antibody, if present, in the human serum was captured in between r-Bio-HIV-MEP and europium(III) chelate labeled anti-human antibody. Anti-HCV antibody, if present, in the human serum was captured in between r-Bio-HCV-MEP and europium(III)

chelate labeled anti-human antibody. The HBsAg, if present, in the human serum was captured in between biotinylated MAb 21B and 5S $F(ab)_2$ fragments coated onto the europium(III) nanoparticles. Europium signals were measured from dry wells using time-resolved fluorometry. Format of the assay is represented in Figure 4.33A. The presence of europium signals indicated the presence of one or more of the following analytes: Anti-HIV antibody, Anti-HCV antibody and HBsAg.

4.6.2. Evaluation of HIV, HCV and HBV multiplexed TRF immunoassay

More than 50 normal human sera were tested to evaluate the specificity of the multiplexed assay and no false positive results were obtained. To designate sera as either positive or negative, a stringent cut-off value of 7500 counts/sec was used, which was obtained by adding three times the standard deviation to the mean of negative sera [Leland, 2000]. Sera with a 'signal to cut-off' (S/Co) ratio <1.0 were designated as Negative (-) while those with S/Co ≥ 1.0 were designated as Positive (+). The performance of multiplexed assay was first evaluated with serum samples obtained by artificial mixing of HIV-, HCV-, and HBV-infected individual human sera in all possible combinations (Figure 4.33B) and then with BBI viral co-infection panel PCA 201 (Table 4.10). TRF multiplexed assay did not show any false positive or false negative results. The lone exception was provided by one sample (PCA201#20), which was characterized by commercial assays as borderline sample for HIV and positive for HBsAg. This sample was also not detected with individual r-HIV-MEP based TRF immunoassay and HBsAg TRF immunoassay. All the samples that were positive by individual in-house immunoassays (r-HIV-MEP based TRF immunoassay, r-Bio-HCV-MEP based TRF immunoassay and HBsAg TRF immunoassay) were detected by the multiplexed assay also. The results suggested that the objective of multiplexing was achieved successfully.

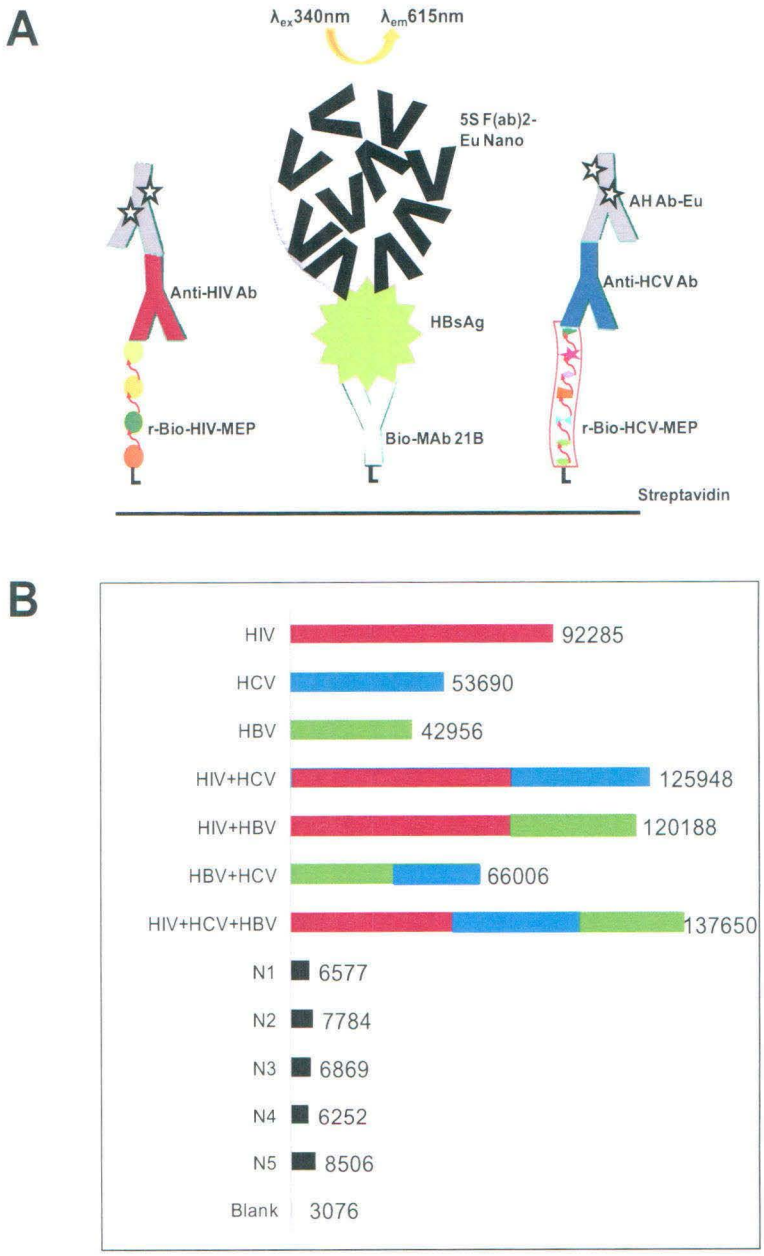


Figure 4.33. HIV, HCV and HBV multiplexed TRF immunoassay *(A) Diagrammatic representation of HIV, HCV and HBV multiplexed TRF immunoassay Format. Bio-MAb 21B, r-Bio-HIV-MEP and r-Bio-HCV-MEP immobilized on streptavidin coated plate. Step-1, addition of human serum containing one or more of the following analytes: HBsAg, anti-HIV antibody, anti-HCV antibody. Step-2, addition of 5S F(ab)$_2$ coated europium(III) nanoparticle and europium(III) chelate labeled anti-human antibody and detection by time-resolved fluorometry. (B) Histogram showing europium signals (CPS) obtained from different analytes in HIV, HCV and HBV multiplexed TRF Immunoassay. Different colours represent signals obtained from different analytes: red, anti-HIV antibody; blue, anti-HCV antibody; green, HBsAg.*

Table 4.10. Evaluation of HIV, HCV and HBV multiplexed TRF immunoassay using Viral Co-infection Performance Panel (PCA 201, Boston Biomedica Inc.)

PCA 201 Member ID#	Abbott EIA HBsAg[a]	Org. Tek. Anti HBc[a]	Abbott Anti-HTLV[a]	Genetic systems HTLV Blots	Result[b]	Abbott Anti-HIV1[a]	Dupont HIV Western Blots	Result[c]	Ortho anti-HCV[a]	HIV, HCV and HBV TRF multiplex immunoassay[d]
	2 MAbs + 1 PAb	1 Ag	Inactivated virus			2 Ag			3 Ag	This study 2Ag + 2 MAbs
01	53.4	9.0	2.1	68/61,55/53,42,36,29.24	P	0.2	Not done	-	6.3	**23.5** (+)
02	51.6	8.8	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.7	**38.7** (+)
03	49.3	8.7	2.1	68/61,55/53,42,36,29.24	P	0.1	Not done	-	6.3	**17.6** (+)
04	26.4	7.7	2.1	68/61,55/53,42,36,34,29,24,19	P	0.1	Not done	-	0.4	**ND**
05	0.4	7.4	2.1	68/61,55/53,46,36,34,29,24,19	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**12.8** (+)
06	0.5	0.5	0.1	N/A	-	0.1	Not done	-	0.5	**ND**
07	0.7	0.2	2.1	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**12.5** (+)
08	38.9	57.9	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	2.1	**39.9** (+)
09	1.0	0.6	2.1	68/61,55/53,42,36,29.24	P	13.5	15,24,31,41,53,55,64,120,160	P	2.2	**9.3** (+)
10	42.8	7.8	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.2	**44.7** (+)
11	48.6	8.5	2.1	68/61,55/53,42,36,29.24,19	P	0.1	Not done	-	6.3	**5.9** (+)
12	38.7	8.5	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**24.2** (+)
13	33.63	9.2	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.7	**30.9** (+)
14	51.8	8.2	2.1	68/61,55/53,42,36,29.24,19	P	0.32	Not done	-	0.5	**31.1** (+)
15	64.5	2.1	1.6	55/53,42,36,29.24	P	0.2	Not done	-	6.3	**23.5** (+)
16	0.5	5.4	2.1	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**13.1** (+)
17	42.3	8.1	0.1	N/A	-	13.5	15,24,31,41,53,64,120,160	P	1.1	**32.4** (+)
18	55.1	0.4	2.3	68/61,55/53,42,36,24	P	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**13.2** (+)
19	45.6	2.9	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.4	**31.2** (+)
20	12.2	6.2	0.1	N/A	-	1.1	15.24.vf31, vf41, vf53,vf55,f16	P	0.1	**0.3** (-) ?
21	42.2	2.7	0.2	N/A	-	13.5	15,24,31,41,53,64,120,160	P	0.9	**28.7** (+)
22	41.5	7.2	0.1	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	6.3	**29.8** (+)
23	46	8.1	2.1	68/61,55/53,42,32,24	P	0.2	Not done	-	6.3	**33.3** (+)
24	0.5	0.5	0.2	N/A	-	0.1	Not done	-	0.4	**0.7** (-)
25	50.1	8.5	0.2	N/A	-	13.5	15,24,31,41,53,55,64,120,160	P	0.9	**35.1** (+)

[a] Values indicate 'signal-to-cut-off'(S/Co) ratios, provided by the panel supplier (BBI) using the indicated commercial EIA kits. Org. Tek., Organon Teknika. S/Co values ≥ 1.0 are considered as positive.

[b] and [c] result of HTLV and HIV Western blot, respectively, provided by the panel supplier (BBI). 'P' and '-' indicate the presence and absence, respectively, of antigen bands in the blot assays.

[d] Values indicate 'signal-to-cut-off' ratios obtained using the in-house HIV, HCV and HBV multiplexed TRF immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values < 1.0 are designated as negative (-) and those with values ≥ 1.0 are designated as positive (+). 'ND' indicates 'not determined' due to lack of samples.

'?' indicates sample was not detected in in-house HIV, HCV and HBV multiplexed TRF immunoassay.

4.6.3. Conclusion

- A prototype multiplexed TRF immunoassay was developed for HIV, HCV and HBV for the simultaneous detection of one or more of the following analytes in infected human serum samples: anti-HIV antibody, anti-HCV antibody and HBsAg.
- In the multiplexed assay r-Bio-HIV-MEP, r-Bio-HCV-MEP and Bio-MAb 21B were used as capture on streptavidin coated plate and europium chelate labeled anti-human antibody and 5S F(ab)2 coated onto europium nanoparticles were used as tracer.
- The assay was evaluated with BBI viral co-infection performance panel. No false positive or false negative results were obtained except for PCA201#20, which was characterized as borderline sample for HIV and positive for HBsAg by commercial assays. This sample was also undetected with individual r-HIV-MEP based TRF immunoassay and HBsAg TRF immunoassay.
- All the samples that were positive by individual in-house immunoassays were detected by the multiplexed assay also. The results suggested that the objective of multiplexing was achieved successfully with excellent degrees of sensitivity and specificity.

5. Discussion

5. Discussion

In the present study, individual Time-resolved Fluorometric (TRF) immunoassays were developed for the detection of HIV, HCV, and HBV infections in human sera, and then they were combined to develop a multiplexed TRF immunoassay for the simultaneous detection of all three infections. The assays were optimized to high degrees of sensitivity and specificity. Our strategy was designed, based on the three rationales.

Firstly, as commercially available diagnostic EIAs have used multiple peptides and/or proteins as diagnostic intermediates, the development of single recombinant Multi Epitope Proteins (MEPs) for the detection of HIV and HCV, by linking the immunodominant epitopes of these viruses, has completely obviated the need to express multiple proteins.

Secondly, as passive coating of capture antigen on the solid surface exhibits several disadvantages [Schetters, 1999], the sensitivity and specificity of the assay were enhanced by immobilization of biotinylated MEPs and antibody on streptavidin coated plates. The high affinity binding surface of streptavidin coated plates have high functional capacity and stability [Huhtinen et al., 2004; Välimaa and Laurikainen, 2006]. Moreover, the coating on streptavidin coated surfaces maintains the nature of antigenic determinants of biotinylated recombinant proteins [Schetters, 1999]. The interaction of biotin to avidin, or streptavidin, has been used widely in biochemistry and molecular biology. Key features of the interaction between biotin and avidin/ streptavidin are their extraordinarily high affinity for each other and their extremely slow dissociation rate [Choi-Rhee et al., 2004]. Routinely, the proteins have been *in vitro* biotinylated, whereby lysine residues of target proteins have been modified by chemical agents. By *in vitro* biotinylation of proteins, the degree of biotin labeling cannot be effectively controlled. Moreover the biotinylation may involve amino acids which are part of an important epitope, which may modify the nature of the epitope, thus reducing its detectability and decreasing the assay sensitivity [Smith et al., 1998].

In the present study, the recombinant MEPs have been *in vivo* biotinylated, using cloning strategies in *E. coli*. A versatile plasmid expression system was designed, where biotinylation takes place with the help of BirA (biotin holoenzyme

synthetase), which covalently attaches biotin to the specific lysine residue of the BAP (biotin acceptor peptide), which is the minimal substrate for biotinylation by BirA [Smith et al., 1998; Tsao et al., 1996]. The genes encoding MEPs were cloned into this expression system and biotinylated MEPs and BirA were co expressed. This *in vivo* biotinylation strategy assured high efficiency of biotinylation of recombinant MEP at a single unique lysine residue in the BAP, which is a fusion partner of the biotinylated MEP but not part of any of the epitopes involved in antibody recognition. Hence, it proved to be an efficient, simple and a cost effective way over *in vitro* biotinylation strategies. This approach further increased sensitivity of the immunoassay, as enzymatic biotinylation at a unique position in every recombinant fusion protein ensured uniform biotinylation of every protein molecule, such that they will be oriented in the same fashion during immobilization on a streptavidin coated plate. A 6x-His tag was also incorporated in the design of expression vector, so that the biotinylated MEP can be purified by Ni-NTA affinity chromatography in a single step. The recombinant biotinylated MEP was expressed in *E. coli,* considering the cost perspective and ease of handling.

Thirdly, the immunoassays for HIV and HCV were developed in an indirect format, where the biotinylated MEPs, immobilized on streptavidin coated plates, were used to capture the specific antibodies in the infected sera, and europium(III) chelate labeled anti-human antibody was used to monitor captured antibodies, through measurement of time-resolved fluorescence. An immunoassay, for the detection of HBsAg in human serum samples was developed in a sandwich format, where a biotinylated monoclonal antibody (MAb), immobilized on streptavidin coated plate was used to capture HBsAg in human sera, and $F(ab)_2$ fragments of another MAb, coated onto europium(III) chelate doped polystyrene nanoparticles were used to monitor captured HBsAg through time-resolved fluorescence. 107 nm polystyrene europium(III) nanoparticle, used as a label in this study, is doped with ~30 000 europium(III) chelates, which enhances the signals tremendously, thus enabling high sensitivity of the assay [Härmä et al., 2001; Soukka et al., 2001; Valanne et al., 2005]. The use of fluorescent europium(III) chelate and europium(III) nanoparticles enabled easy measurement of fluorescence, directly from the dry wells of microtiter plate, without addition of any substrate or stopping the reaction, contributing to the

simplicity and rapidity of the procedure. Well characterized serum panels were used to evaluate the optimized immunoassays.

Our earlier work has established the utility of recombinant MEPs in the detection of infections by different pathogens (Dengue and HCV) [AnandaRao et al., 2005; AnandaRao et al., 2006; Chugh et al., 2006]. The study on HIV diagnostics was based on the premise that the use of a single diagnostic intermediate, designed to have HIV-specific immunodominant epitopes from all known genotypes and expressed to high levels in an *E. coli* expression system, could effectively address the issues of cost and specificity associated with the currently available multiple antigen-based anti-HIV diagnostic assays. To develop this single recombinant antigen, we focused on four proteins expressed by HIV, namely, p24 of HIV-1, gp41 of HIV-1 group M, gp41 of HIV-1 group O and gp36 of HIV-2 [Dorn et al., 2000; Gupta and Chaudhary, 2003; Janvier et al., 1996]. The ability of these proteins to elicit humoral immune response has been well documented and their antigenic determinants have been identified using a variety of different approaches [Cano et al., 2004; Lottersberger et al., 2004]. Using the available information, epitopes were selected from these antigens, for designing a synthetic MEP antigen as a single diagnostic intermediate for HIV. The choice of these epitopes was based on four criteria, namely, they had to be (i) immunodominant, (ii) specific to anti-HIV antibodies (iii) linear, and, (iv) phylogenetically conserved in most genotypes of HIV circulating in different parts of the world. These criteria were based on a number of considerations.

Firstly, in order for a synthetic MEP to be capable of efficiently recognizing HIV specific antibodies, it is necessary that its constituent epitopes exhibit significant reactivity to HIV-infected patient sera worldwide. Secondly, since we wanted to use *E. coli*-based over-expression, from the cost perspective, it was necessary to work with linear epitopes, so that when incorporated into the synthetic protein, they would presumably retain their immunoreactivity to multiple HIV types and groups. On the other hand, if conformational epitopes were to be used, it is highly unlikely that they would retain their conformational integrity upon expression in *E. coli*, compromising their immunoreactivity in the synthetic antigen [Chugh et al., 2006; Horal et al., 1991; Janvier et al., 1996]. The selected epitopes ranged in length from 28-51 amino acids residues. The four selected epitopes were linked by tetra glycyl flexible linkers, which

are considered to be the preferred linkers while designing flexible chimeric proteins [Robinson and Sauer, 1998]. Analysis of the structure of the synthetic MEP by computer modeling showed that all the chosen epitopes appeared to be accessible for interaction with antibodies. This suggested that the four chosen epitopes would collectively contribute to the overall specificity and sensitivity of the single recombinant molecule, towards anti-HIV antibodies.

The r-MEP was *in vivo* biotinylated and over-expressed in *E. coli*. The biotinylated (r-Bio-HIV-MEP) and non-biotinylated (r-HIV-MEP) antigens were purified under denaturing conditions as they were insoluble, despite their fusion to thioredoxin. Further, the efficacy of r-HIV-MEP, as a diagnostic intermediate, was evaluated in an in-house TRF immunoassay. In this assay, r-HIV-MEP, passively coated on microtiter plate, was used to capture anti-HIV antibodies and commercially available HIV serum panels and normal human sera were used as test samples. Anti-human antibody, labeled with europium(III) chelate, was then used as a tracer and the captured anti-HIV antibody was monitored through TRF. The results showed that our synthetic diagnostic intermediate could indeed recognize and bind to anti-HIV antibodies, elicited by both HIV-1 as well as HIV-2. However, it is to be noted, that the design of r-HIV-MEP precludes differentiation of HIV-1 from HIV-2. As HIV exhibits a distinct geographical distribution, a Worldwide HIV Performance Panel (WWRB 302) was used to evaluate if r-HIV-MEP could recognize the HIV infected sera from different parts of the world. Panel members included specimens characterized as HIV-1 Group M (subtypes A to G), HIV-1 Group O and HIV-2. Our results demonstrated that r-HIV-MEP was able to recognize antibodies to a diverse set of HIV infections from India and from other countries such as Argentina, China, Ghana, Ivory Coast, Mozambique, Spain, Thailand, Uganda, USA and Zimbabwe.

The efficacy of r-Bio-HIV-MEP, as a diagnostic intermediate, was also evaluated in an in-house TRF immunoassay. In this assay, r-Bio-HIV-MEP immobilized on a streptavidin coated plate was used to capture anti-HIV antibodies, and a commercially available HIV serum panel, and normal human sera were used as test samples. Anti-human antibody, labeled with europium(III) chelate was then used as a tracer and the captured anti-HIV antibody was monitored through TRF. The sensitivity of the r-HIV-MEP and r-Bio-HIV-MEP in the immunoassays was

evaluated by using HIV-1 Seroconversion Panel (PRB 931), and the results were found to be in complete agreement with the best performing Abbott HIV 1/2 EIA kit, in the early diagnosis of anti-HIV antibodies in human sera. Our immunoassays were able to detect panel member #6 of HIV Seroconversion Panel (PRB 931), which showed immuno-reactivity with Abbott HIV 1/2 EIA kit only, and not with other EIA kits. r-Bio-HIV-MEP based TRF immunoassay showed slightly higher signal-to-cut-off ratios than r-HIV-MEP based TRF immunoassay for the detection of members of seroconversion panel. The specificity of r-HIV-MEP in the TRF immunoassay, was evaluated by using Viral Co-infection Performance Panel (PCA 201). This single diagnostic intermediate performed as well as the other commercial anti-HIV kits from Abbott, Genetic Systems and Organon Teknika except for panel member # 20 of the Viral Co-infection Performance Panel, which was a borderline sample. The information regarding the subtype of this member was unavailable. Overall, the data attest to the utility of our designer antigen, in detecting HIV infection from diverse geographical locations, with high specificity and sensitivity. It was also useful in monitoring seroconversion. In conclusion, the high density of HIV-specific, phylogenetically conserved and immunodominant epitopes, selected for designing the r-HIV-MEP, contributed to high degrees of sensitivity and specificity. The high level of expression of r-HIV-MEP in *E. coli*, and its single step affinity purification design, make this approach highly cost effective for anti-HIV screening in blood banks, in most developing countries. The yield of purified r-HIV-MEP from one liter of induced culture is sufficient for ~40,000 assays [Talha et al., 2010].

In a previous study from our laboratory, a single recombinant antigen, HCV multiepitope protein (HCV-F-MEP V1) was designed for the detection of HCV infections and was evaluated as a diagnostic intermediate. Five conserved and immunodominant epitopes from the structural and non-structural proteins of HCV polyprotein, namely, Core (1b), Core (3g), NS3 (1b), NS4A (1b), NS 4B (1b) and NS5A (1b) were selected and linked with flexible linkers to form HCV-F-MEP V1 [Chugh et al., 2006]. To enhance the sensitivity of the immunoassay, HCV-F-MEP V1 was modified to develop HCV-MEP V2. For the design of HCV-MEP V2, additional epitopes from non-structural proteins of HCV, namely, NS4A (3a) and NS4A (2b) were incorporated. A longer region of NS3 (1b) of 266 amino acids (longer than the previous sequence of 48 amino acids in HCV-F-MEP V1), slightly

longer region of NS4B (1b) and NS5A (1a) (HCV-F-MEP V1 contained NS5A of subtype 1b] were also incorporated. Incorporation of these epitopes exhibiting higher sensitivity was based on published literature [Duo et al., 2002; Park et al., 1995]. Core 1b and 3g epitopes of HCV-F-MEP V1 had 89 % similarity with each other, thus core 3g epitope was omitted in the design of HCV-MEP V2. Another reason for the omission of core 3g epitope was to reduce the size of the antigen because expression of large protein in *E. coli* resulted into low level of expression [Samuel et al., 1988]. Finally, for the design of recombinant HCV-MEP V2 (r-HCV-MEP) seven conserved and immunodominant epitopes from the non-structural [(NS3 (1b), NS4A (3a, 2b, 1b), NS4B (1b) and NS5A (1a)] and structural [Core (1b)] proteins were selected and linked with flexible tetra-glycyl linkers. The ability of these structural and non-structural proteins to elicit humoral immune response has been well documented and their antigenic determinants have been identified using a variety of different approaches [Claeya et al., 1995; Goeser et al., 1994]. The choice of these epitopes was based on four criteria, namely, they had to be (i) immunodominant, (ii) specific to anti-HCV antibodies (iii) linear, and (iv) phylogenetically conserved in most genotypes of HCV circulating in different parts of the world [Khudyakova et al., 1995; Sailberg et al., 1992]. These criteria were based on a number of rationales.

Firstly, in order for synthetic multiepitope protein to be capable of efficiently recognizing HCV specific antibodies, it was necessary that its constituent epitopes exhibit significant reactivity to HCV infected patient sera worldwide. Secondly, it was necessary to consider linear and conserved epitopes, so that when expressed in *E. coli*, they would presumably retain their immunoreactivity to multiple HCV serotypes. Analysis of the structure of the synthetic MEP by computer modeling showed that all the chosen epitopes appeared to be accessible for interaction with antibodies. This suggested that the seven chosen epitopes would collectively contribute to the overall specificity and sensitivity of the single recombinant molecule towards anti-HCV antibodies.

The recombinant antigen was *in vivo* biotinylated and over-expressed in *E. coli*. The biotinylated antigen (r-Bio-HCV-MEP) was purified under denaturing condition as it was insoluble, despite its fusion to thioredoxin. Further, the efficacy of r-Bio-HCV-MEP, as a diagnostic intermediate, was evaluated in an in-house TRF

immunoassay. In this assay, r-Bio-HCV-MEP, immobilized on a streptavidin coated plate, was used to capture anti-HCV antibodies and commercially available HCV serum panels and normal human sera were used as test samples. Anti-human antibody, labeled with europium(III) chelate, was then used as a tracer and captured anti-HCV antibody was monitored through TRF. The results showed that our synthetic diagnostic intermediate could indeed recognize and bind to anti-HCV antibodies. This protein did not exhibit any reactivity with normal human sera. We proceeded to evaluate its potential utility as an anti-HCV diagnostic intermediate, with several well-characterized HCV panels from Boston Biomedica, USA. As HCV exhibits a distinct geographical distribution, a Worldwide HCV Performance Panel was used to evaluate if r-Bio-HCV-MEP could recognize the HCV infected sera from different parts of the world. The Boston Biomedica panel WWHV 301 was used for this purpose. Panel members included specimens characterized as HCV types 1, 2, 3, and 4 and also samples that were un-typable as well as non-consensus specimens. Our results demonstrated that r-Bio-HCV-MEP was able to recognize antibodies to a diverse set of HCV from several countries such as Argentina, Uganda, Ghana, China, Egypt and USA.

The sensitivity of r-Bio-HCV-MEP in the TRF immunoassay was evaluated by using HCV Seroconversion Panel (PHV 901), and the results were found to be in complete agreement with both the commercial EIA kits from Abbott and Ortho, in the early diagnosis of anti-HCV antibodies in human sera. The earliest time point at which seroconversion was detected in this panel was represented by panel member #3. It was found that both the commercial kits tested, Abbott HCV 2.0 EIA and Ortho HCV 2.0 EIA, and our in-house assay, picked this member from PHV 901, but the signal-to-cut-off ratios obtained with the in-house assay were higher than the commercially available kits tested. On the other hand, the specificity of r-Bio-HCV-MEP in the TRF immunoassay was evaluated by using Viral Co-infection Performance Panel (PCA 201). Significantly, regardless of the presence or absence of antibodies to HBV, HIV or HTLV, the results of the in-house immunoassay for HCV antibodies closely matched the results obtained with the commercial assays. Overall, the data showed that the performance of our single r-Bio-HCV-MEP-based TRF immunoassay was in total agreement with the commercially available multi antigen based anti-HCV EIA kits, namely, Abbott HCV 2.0, Abbott HCV 3.0 and Ortho HCV

3.0. In conclusion, the high density of HCV specific, phylogenetically conserved and immunodominant epitopes, selected for designing the r-Bio-HCV-MEP, contributed to high degrees of sensitivity and specificity. The high level of expression and *in vivo* biotinylation of r-Bio-HCV-MEP protein in *E. coli*, and its single step affinity purification design, makes this approach highly cost effective for anti-HCV screening in blood banks in most developing countries. The yield of purified r-Bio-HCV-MEP from one liter of induced culture is sufficient for ~300,000 assays.

A TRF immunoassay, for the detection of HBsAg in human serum samples was developed utilizing the hybridoma clones available in our laboratory, producing monoclonal antibodies (MAbs) 21B and 5S specific for HBsAg. In this assay, biotinylated MAb 21B, immobilized on streptavidin coated plate, was used to capture HBsAg, and in-house and commercially available serum panels were used as test samples. Further, 5S $F(ab)_2$ coated on europium(III) nanoparticles was used as tracer to detect the bound HBsAg on MAb 21B and europium fluorescence was measured by time-resolved fluorometry directly from dry wells of microtiter plate. It has been shown, that removal of the Fc region of the mouse IgG and using only the $F(ab)_2$ fragment, resulted in lower background in TRF immunoassay [Väisänen et al., 2006]. We proceeded to evaluate the potential utility of HBsAg TRF immunoassay with in-house serum panel and well-characterized Viral Co-infection Performance Panel from BBI. The in-house assay did not show any false positive results which indicated that the assay was highly specific. Performance of the in-house assay was further evaluated by analyzing a dilution series of r-HBsAg and it was found that sensitivity of the in-house assay for the detection of r-HBsAg was 0.02 ng/ml, which was several folds higher as compared to the commercially available HBsAg EIA kits [Kuhns and Busch, 2006]. The signal-to-cut-off ratios obtained with the in-house assay were several folds higher than the commercially available kits tested for the detection of HBsAg positive samples. There was some discrepancy in the detection of HBsAg in a few samples, in the in-house assay as compared to commercially available HBsAg EIA kits. The most probable reason for this can be the inadequate recognition of the monoclonal antibodies used in this assay, to all HBsAg subtypes. This may be resolved by the use of one or more additional monoclonal antibodies as capture and/or tracer or the use of polyclonal antibodies, specific for HBsAg, as tracer.

The saturating amount of both r-Bio-HIV-MEP and r-Bio-HCV-MEP antigens immobilized on a streptavidin coated plate was 100 ng/well, when low binding capacity streptavidin plates were used; while the saturating amount of both r-HIV-MEP and r-HCV-MEP antigens in passively coated maxisorp plate was 500 ng/well. The data suggested that the use of streptavidin coated plate also reduced the amount of recombinant antigens required, apart from other advantages [Nakanishi et al., 2008; Schetters, 1999; Välimaa et al., 2003]. When high binding capacity, Nunc immobilizer streptavidin plate, was used, for the immobilization of biotinylated antigen, the best signal-to-background ratios were obtained with 25 ng/well of biotinylated antigen, whereas the non-biotinylated antigen required in passive coating was 250 ng/well to achieve the same signal-to-background ratios (unpublished data from our laboratory).

In-house individual TRF immunoassays, for the detection of HIV, HCV and HBV infections, were combined to develop a multiplexed TRF immunoassay, for the simultaneous detection of one or more of the following analytes in infected human serum samples: anti-HIV antibody, anti-HCV antibody and HBsAg. In the multiplexed assay r-Bio-HIV-MEP, r-Bio-HCV-MEP and Bio-MAb 21B were used as capture on streptavidin coated plate, and europium(III) chelate labeled anti-human antibody and 5S $F(ab)_2$ coated onto europium(III) nanoparticles, were used as tracer. The assay was evaluated with BBI Viral Co-infection Performance Panel. All the samples that were positive by individual in-house immunoassays were detected by the multiplexed assay as well; this suggests that the objective of multiplexing was achieved successfully with high degrees of sensitivity and specificity. This is a prototype multiplexed TRF immunoassay, and needs to be corroborated with larger number of HIV, HCV and/or HBV infected serum samples, as well as the serum samples infected with auto-immune diseases, other co-infections, and pregnancy samples. The TRF multiplexed immunoassay allows the simultaneous detection of three analytes from a single human serum sample, saving time and resources in a blood bank setting, where blood is screened for several transfusion-transmissible diseases. The current assay has the potential for multiplexing the detection of other infectious diseases e.g. syphilis, malaria and dengue. Though, currently, the assay is time consuming but it can be adapted to a rapid test format.

6. Summary

6. Summary

- A novel recombinant multiepitope diagnostic intermediate, r-HIV-MEP was designed by linking four virus specific, immuno-dominant, linear and phylogenetically conserved epitopes from core and envelope proteins of HIV.
- The synthetic gene encoding r-HIV-MEP was *in vivo* biotinylated using cloning strategies in *E. coli.* Non-biotinylated (r-HIV-MEP) as well as *in vivo* biotinylated (r-Bio-HIV-MEP) antigens were purified to homogeneity using Ni-NTA affinity chromatography.
- In-house TRF immunoassays were developed based on r-HIV-MEP and r-Bio-HIV-MEP as single diagnostic intermediates for the detection of anti-HIV antibodies in infected human serum samples. The assays were evaluated using commercially available and well-characterized serum panels from Boston Biomedica Inc (BBI).
- r-HIV-MEP based TRF immunoassay detected HIV infection from diverse geographical locations, with high specificity and sensitivity. It was also useful in monitoring seroconversion. The use of fluorescent europium(III) chelate labeled antibody as tracer further simplified the immunoassay by easy measurement of time-resolved fluorescence directly from the dry wells of microtiter plate without addition of substrate and stopping the reaction.
- A novel recombinant multiepitope diagnostic intermediate HCV-MEP V2 (r-HCV-MEP), was designed by modifying previous construct HCV-F-MEP V1, by linking additional virus specific, immuno-dominant, linear and phylogenetically conserved epitopes from the non-structural and structural proteins of HCV.
- The synthetic gene encoding r-HCV-MEP was *in vivo* biotinylated using cloning strategies in *E. coli.* The *in vivo* biotinylated (r-Bio-HCV-MEP) antigen was purified to homogeneity using Ni-NTA affinity chromatography.
- An in-house TRF immunoassay was developed based on r-Bio-HCV-MEP as single diagnostic intermediate for the detection of anti-HCV antibodies in infected human serum samples. The assay was evaluated using commercially available and well-characterized serum panels from BBI.
- r-Bio-HCV-MEP based TRF immunoassay detected HCV infection from diverse geographical locations, with high specificity and sensitivity. It was also useful in monitoring seroconversion. The use of fluorescent europium(III) chelate labeled

antibody as tracer further simplified the immunoassay by easy measurement of time-resolved fluorescence directly from the dry wells of microtiter plate.

- Monoclonal antibodies (MAbs) 21B and 5S (from hybridoma clones available in our laboratory) specific for HBsAg were purified to homogeneity. An in-house HBsAg TRF immunoassay was developed using the two purified MAbs. Purified chemically biotinylated-MAb 21B immobilized on streptavidin-coated wells was used to capture HBsAg in human serum samples. Bound HBsAg were detected using 5S $F(ab)_2$ fragments coated on europium nanoparticles. Time-resolved fluorescence of europium was measured directly from dry wells of microtiter plate.
- The HBsAg TRF immunoassay was evaluated by an in-house Finnish human serum panel and a commercially available and well-characterized serum panel from BBI. The immunoassay detected HBV infection with high specificity. With the in-house assay, out of the total 44 HBsAg-seropositive serum samples tested, one sample was found to be on the borderline and 4 samples remained undetected.
- Individual TRF immunoassays developed, based on r-Bio-HIV-MEP and r-Bio-HCV-MEP and HBsAg TRF immunoassays were combined to develop an HIV, HCV and HBV multiplexed TRF immunoassay for the simultaneous detection of one or more of the following analytes in human serum samples from the same well – anti-HIV antibody, anti-HCV antibody and HBsAg. The multiplexed assay was evaluated with commercially available and well-characterized viral co-infection performance panel from BBI.
- The multiplexed TRF immunoassay detected co-infections from diverse geographical locations with high specificity and sensitivity except one serum sample (PCA 201 #20) which was characterized as a borderline case for HIV and positive for HBsAg by commercial assays. The sample also remained undetected with individual in-house TRF immunoassays developed for anti-HIV antibodies and HBsAg.
- The objective of multiplexing was successful as all the samples that were positive by individual in-house TRF immunoassays were also detected by the TRF multiplexed assay.
- The 'know-how' of the design and production of the novel r-HIV-MEP has been transferred to a leading diagnostics manufacturing company in India.

7. Future perspectives

7. Future perspectives

In the present study, a prototype multiplexed TRF immunoassay for the detection of HIV, HCV and HBV infections was developed. Analyte specific biotinylated reagents were mixed and immobilized randomly on streptavidin coated microtitration wells. The presence of one, or more than one, analytes can be detected, but the origin of the response cannot be identified. In future, a rapid test can be developed in a lateral or vertical flow format, in which the analyte specific reagents can be immobilized as separated test lines on a nitrocellulose strip, followed by, step-1, addition of human serum and, step-2, addition of 5S $F(ab)_2$ coated europium(III) nanoparticle and europium(III) chelate labeled anti-human antibody and detection by time-resolved fluorometry. The analytes can be identified, based on the presence of bands at their respective capture zones (Figure 7.1). Another approach can be to immobilize analyte specific reagents on certain localized areas of microtitration well bottom (spotted wells) followed by, step-1 and step-2, as mentioned above. From the measured signal pattern, the analyte can be identified, based on the location of signal. This will require multiple readouts (scanning) from each well (Figure 7.2).

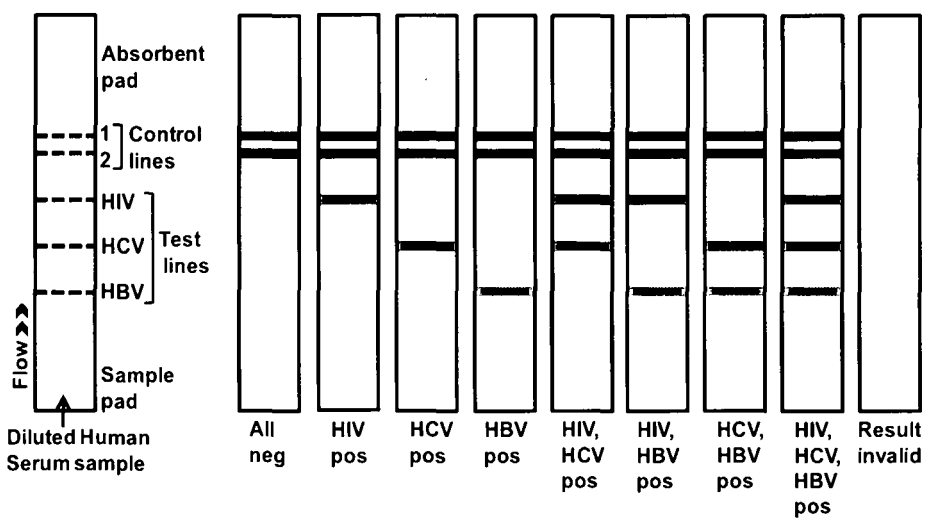


Figure 7.1. Hypothetical representation of HIV, HCV and HBV multiplexed TRF lateral flow immunoassay format. *In the lateral flow immunoassay, different capture reagents, r-HIV-MEP, r-HCV-MEP and MAb 21B are immobilized on nitrocellulose strip as sharp lines and are shown in figure as HIV, HCV and HBV test lines, respectively. Control line-1 represents a positive control for HBV and control line-2 represents a positive control for HIV and HCV. Red lines represent anti-HIV antibody, blue lines represent anti-HCV antibody and green lines represent HBsAg, in human sera.*

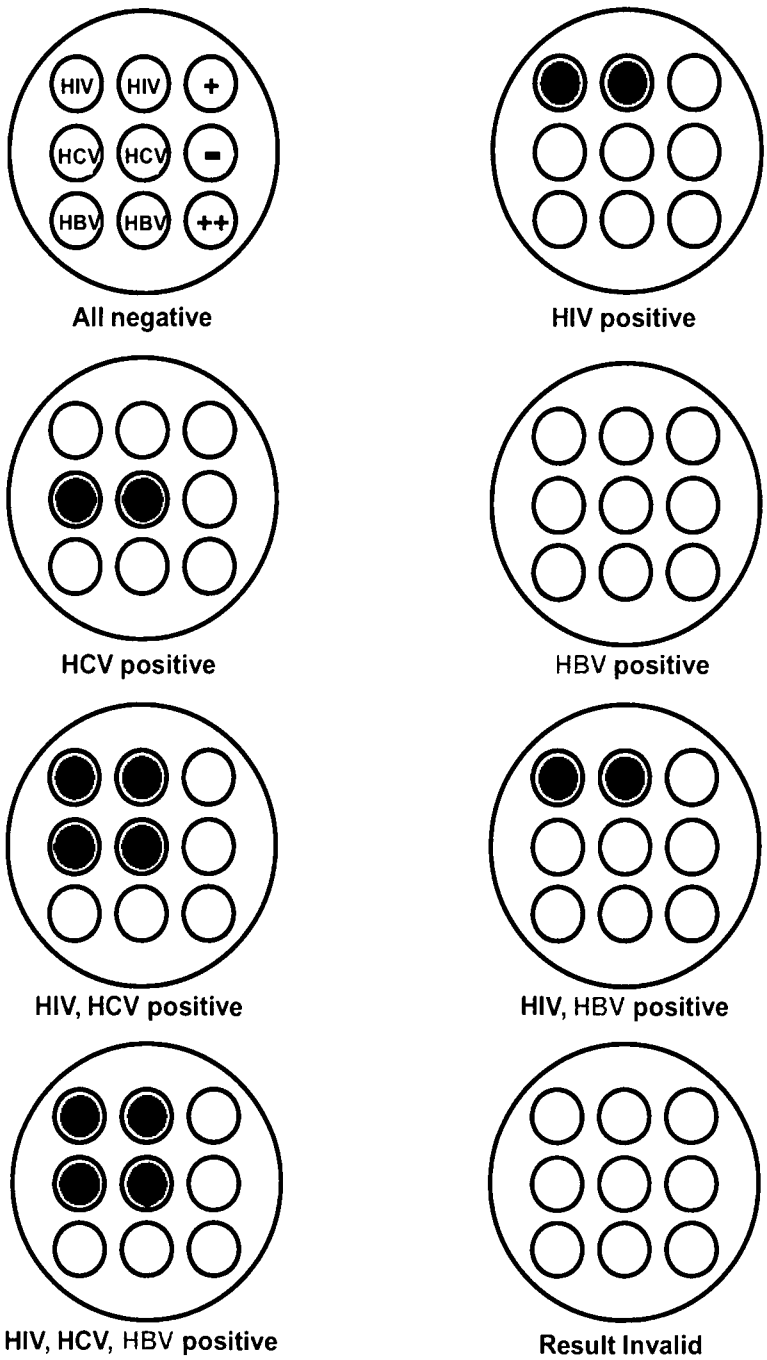


Figure 7.2. Hypothetical representation of HIV, HCV and HBV multiplexed TRF spot immunoassay format. *In the spot immunoassay, different capture reagents, r-Bio-HIV-MEP, r-Bio-HCV-MEP and Bio-MAb 21B are immobilized on streptavidin coated spots and are shown in figure as HIV, HCV and HBV, respectively. '+' represents a positive controls for HIV and HCV, '++' represents a positive control for HBV and '−' represents a negative control. Red spots represent anti-HIV antibody, blue spots represent anti-HCV antibody and green spots represent HBsAg, in human sera.*

8. References

8. References

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9. Publications

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1. **Talha SM**, Salminen T, Chugh DA, Swaminathan S, Soukka T, Pettersson K, and Khanna N. (2010) Inexpensive designer antigen for anti-HIV antibody detection with high sensitivity and specificity. Clin. Vaccine Immunol. **73**:335-341.

2. Batra G, **Talha SM**, Nemani SK, Dhar N, Swaminathan S, Khanna N. (2010) Expression, purification and characterization of *in vivo* biotinylated dengue virus envelope domain III based tetravalent antigen. Protein Expr. Purif. (doi:10.1016/j.pep.2010.04.017).

3. **Talha SM***, Myyryläinen T* *et al.* Dual-label time-resolved immunofluorometric assay for simultaneous detection of HIV and HBV infections. (Manuscript communicated).

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4. **Talha SM** *et al.* Highly Sensitive Time-Resolved Fluorometric Immunoassay for the Detection of Antibodies to HIV-1 and HIV-2 with Europium(III) Nanoparticle Label Technology. (Manuscript communicated).

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Inexpensive Designer Antigen for Anti-HIV Antibody Detection with High Sensitivity and Specificity▽

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A novel recombinant multiepitope protein (MEP) has been designed that consists of four linear, immunodominant, and phylogenetically conserved epitopes, taken from human immunodeficiency virus (HIV)-encoded antigens that are used in many third-generation immunoassay kits. This HIV-MEP has been evaluated for its diagnostic potential in the detection of anti-HIV antibodies in human sera. A synthetic MEP gene encoding these epitopes, joined by flexible peptide linkers in a single open reading frame, was designed and overexpressed in *Escherichia coli*. The recombinant HIV-MEP was purified using a single affinity step, yielding >20 mg pure protein/liter culture, and used as the coating antigen in an in-house immunoassay. Bound anti-HIV antibodies were detected by highly sensitive time-resolved fluorometry, using europium(III) chelate-labeled anti-human antibody. The sensitivity and specificity of the HIV-MEP were evaluated using Boston Biomedica worldwide HIV performance, HIV seroconversion, and viral coinfection panels and were found to be comparable with those of commercially available anti-HIV enzyme immunoassay (EIA) kits. The careful choice of epitopes, high epitope density, and an *E. coli*-based expression system, coupled with a simple purification protocol and the use of europium(III) chelate-labeled tracer, provide the capability for the development of an inexpensive diagnostic test with high degrees of sensitivity and specificity.

Human immunodeficiency virus (HIV) is a lentivirus of the family *Retroviridae*, whose members characteristically have an RNA genome within a capsid and a lipid envelope. HIV infection induces a profound immune dysfunction, with abnormalities in every arm of the immune system, resulting in AIDS (5). In 2007, there were 2.7 million new HIV infections and 2 million HIV-related deaths. Globally, there were an estimated 33 million people living with HIV in 2007. India is one of the largest and most populated countries in the world, with a population of over 1 billion. Of this number, it is estimated that around 2.4 million Indians were living with HIV in 2007 (26). The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins. These proteins are divided into three classes: the major structural proteins (Gag, Pol, and Env), the regulatory proteins (Tat and Rev), and the accessory proteins (Vpu, Vpr, Vif, and Nef) (11). The *gag* gene of HIV type 1 (HIV-1) encodes a polyprotein precursor, p55, which is cleaved by the virus-encoded protease into three proteins, p24, p17, and p15. Linear B-cell epitopes have already been identified within p24 (14). The antigen p24 is of special significance because of its ability to be expressed first in body fluids after HIV-1 infection. The linear immunodominant epitope of p24 serves as an important diagnostic intermediate to detect antibodies to HIV-1 in human sera (23). The envelope glycoproteins (gp), gp41 of HIV-1 and gp36 of the closely related HIV-2, are highly immunogenic and are important diagnostic intermediates for the detection of antibodies to these viruses in human sera (17, 24). HIV-1 comprises three lineages, denoted M, N, and O (22). HIV-2 and divergent forms have been detected in West African or West Africa-related patients with AIDS (7–9). Several enzyme immunoassay (EIA)-based diagnostic kits are available on the market for the detection of antibodies to HIV in human sera. These anti-HIV EIA kits use synthetic peptides and/or recombinant proteins mainly from the envelope gp of HIV-1 group M, HIV-1 group O, and HIV-2. The fourth-generation kits also have antibodies to p24 antigen. The requirement of multiple peptides and/or multiple recombinant proteins for reliable diagnosis of HIV infections adds to the cost of these EIA kits. The high cost of anti-HIV EIA kits becomes prohibitive for routine use in many developing countries, precluding early detection and prevention of new infections (18, 25, 27). We have designed a single recombinant multiepitope protein (MEP) antigen, consisting of several immunodominant, linear, and conserved virus-specific epitopes from structural proteins of HIV-1 and HIV-2. DNAs encoding these epitopes have been assembled in tandem in a single open reading frame, with intervening sequences encoding flexible linkers, and expressed in *Escherichia coli*. A polyhistidine tag has also been included which allows for facile purification of recombinant MEP by Ni-NTA chromatography. The purified protein has been used as the coating antigen for developing an anti-HIV indirect immunoassay. We have evaluated the performance of this assay with that of other multiple-antigen-based immunoassay kits currently available on the market, using well-characterized commercially available serum panels.

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MATERIALS AND METHODS

Materials. *E. coli* host strains DH5α and BL21(DE3) were purchased from Invitrogen Life Technologies, Carlsbad, CA. Plasmid vector pET-32a(+) was obtained from Novagen, Madison, WI. The synthetic gene, codon optimized for *E. coli* expression, encoding the recombinant HIV-MEP (r-HIV-MEP) was custom synthesized by Geneart, Regensburg, Germany. Restriction endonucleases, calf intestine alkaline phosphatase, and T4 DNA ligase used in all routine cloning and transformation experiments were procured from MBI Fermentas, Burlington, Canada. *Taq* polymerase for PCR screening was an in-house preparation. Ni-NTA super flow resin was purchased from Qiagen, Maryland. Goat anti-human IgG was purchased from Pierce, Rockford, IL. Isopropyl-ß-D-thiogalactopyranoside (IPTG) was procured from Calbiochem-EMD Biosciences, La Jolla, CA. Well-characterized international serum panels were purchased from Boston Biomedica Inc. (BBI), now SeraCare Life Sciences Inc., Milford, MA. The BBI panels were the worldwide HIV performance panel (WWRB 302-01 to WWRB 302-30), HIV seroconversion panel (PRB 931-01 to PRB 931-09), and viral coinfection panel (PCA 201-01 to PCA 201-25). The europium(III) chelate, {2,2′,2″,2‴-{[2-(4-isothiocyanatophenyl) ethylimino] bis (methylene)bis {4-{[4-(α-galactopyranoxy)phenyl] ethynyl}pyridine-6,2-diyl}bis (methylene-nitrilo)} tetrakis(acetato)} europium(III), was synthesized in the Department of Biotechnology, Turku University, Turku, Finland. This is referred to in this paper as Eu^{3+}-9d-chelate. The computer modeling of r-HIV-MEP was done using online software available at http://www.sbg.bio.ic.ac.uk/~3dpssm.

Cloning of synthetic r-HIV-MEP gene. A synthetic gene (0.54 kb) encoding the r-HIV-MEP antigen, codon optimized for expression in *E. coli* (21), was custom synthesized as a BamHI/HindIII fragment in the Geneart vector pPCRscript. Regions of very high (>80%) or very low (<30%) GC content, internal TATA boxes, chi-site stretches, internal ribosomal entry sites, AT-rich or GC-rich sequence stretches, repeat sequences, and RNA secondary structures were avoided where possible. The lengths of individual epitopes varied from 28 to 51 amino acid (aa) residues, and the adjacent epitopes were joined together by flexible tetraglycyl (Gly-Gly-Gly-Gly) linkers (20). The *r-HIV-MEP* gene was inserted into the expression vector pET-32a(+), in frame with the vector-encoded thioredoxin gene and six-His tag-encoding sequence, under the control of the tightly regulated T7 promoter. This expression vector was transformed into *E. coli* strain BL21(DE3).

Expression and purification of r-HIV-MEP. Transformants harboring the r-HIV-MEP plasmid were expression screened to choose a clone that expressed r-HIV-MEP maximally. As the r-HIV-MEP antigen is expressed as a thioredoxin fusion, the predicted size of the induced protein is ~41 kDa (data not shown). A localization experiment performed with this clone showed that the r-HIV-MEP antigen was localized predominantly with the insoluble fraction of the lysate. For purification of the recombinant antigen, a 1-liter culture was induced at log phase with IPTG for 4 h. Bacteria from the induced culture were centrifuged in a Sorvall SLA3000 rotor at 8,000 rpm for 10 min at 4°C, suspended in lysis buffer (6 M guanidine HCl, 10 mM Tris-HCl, 100 mM sodium phosphate, 300 mM NaCl, 1% Tween 20, pH 8) and sonicated, at 4°C, in a Sonics Vibracell sonicator (amplitude setting of 60), using 20 pulses of 10 s each, with 30 s off time between the pulses. The lysate was clarified by centrifugation at 16,000 rpm at 4°C in an SS34 rotor for 45 min. The recombinant protein from this material was purified by affinity chromatography on a 5-ml Ni-NTA superflow resin column essentially as described earlier (6).

Eu^{3+}-9d-chelate and its conjugation to anti-human antibody. The synthesis and full description of fluorescent properties of the Eu^{3+}-9d-chelate have been published previously (15, 19). Goat anti-human IgG was labeled using a 40-fold molar excess of the Eu^{3+}-9d-chelate. The labeling and removal of excess free label were performed essentially as described before (19). The protein concentration of the labeled antibody preparation was determined by Bradford assay (3). The level of label incorporation was determined to be 2.6 Eu^{3+}-9d-chelate per antibody. Bovine serum albumin (BSA) and sodium azide were added to final concentrations of 0.1% and 0.05%, respectively. The solution was filtered through a 0.22-μm membrane and stored at 4°C until used further.

In-house indirect HIV immunoassay. Time-resolved fluorometry (TRF) measurements of Eu^{3+}-9d-chelate-labeled anti-human antibody in indirect immunoassays with r-HIV-MEP as the capture antigen were made using a Victor^{3}V 1420 Multilabel counter (Perkin Elmer, Singapore), which allows the measurement directly from a solid phase. Briefly, 5 μg/ml of r-HIV-MEP was prepared in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) and 100 μl of this was added into each well of a 96-well plate and incubated overnight at 37°C. The wells were aspirated and blocked with 300 μl of blocking buffer (37.5 mM Tris-HCl, pH 7.75, 25% goat serum, 115 mM NaCl, 0.05% NaN_3, 0.038% Tween 40, 15 μM EDTA, 1.38% BSA) and incubated for 2 h at room temperature with shaking. The wells were washed two times using COLUMBUS Plus-BASIC (TECAN, Grödig, Austria) with wash buffer (10 mM KH_2PO_4, 40 mM K_2HPO_4, pH 7.2, 150 mM NaCl, 0.1% Tween 20, 0.5 M KCl). After washing, 2 μl of each serum sample in 50 μl assay buffer (37.5 mM Tris-HCl, pH 7.75, 25% goat serum, 115 mM NaCl, 0.5 M KCl, 0.05% NaN_3, 0.038% Tween 40, 0.1% Triton X-100, 15 μM EDTA, 0.38% BSA) was incubated in each well for 30 min at room temperature with shaking. The wells were washed four times with wash buffer. One microgram per milliliter of Eu^{3+}-9d-chelate labeled anti-human antibody was made in assay buffer, and 50 μl of this was added into each well and incubated for 30 min at room temperature with shaking. The wells were washed seven times with wash buffer, and TRF for Eu^{3+}-9d-chelate was measured from dry wells using the following parameters: excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 400 μs; window time, 400 μs; cycling time, 1 ms; measurement time, 1s (i.e., counts resulting from 1,000 sequential excitations were integrated for each measurement). To designate sera as either positive or negative, we used a cutoff value of 6,500 counts/s (obtained by adding three times the standard deviation to the mean of HIV-negative sera). Sera with a "signal-to-cutoff" (S/Co) ratio of <1.0 were designated negative, while those with S/Co ≥ 1.0 were designated positive. To ensure the validity of the cutoff value, we included positive- and negative-control samples in each run. All measurements were made with a single lot of reagents using the same instrument.

TABLE 1. List of HIV-specific immunodominant epitopes selected from the literature in designing the r-HIV-MEP antigen

Viral protein	Position of epitopes in HIV proteins[a]	% positivity with patient sera	Reference
HIV-1 p24	aa 272–322 of p55 (505)	ND[b]	14
HIV-1 group O gp41	aa 580–616 of gp160 (863)	84	10
HIV-2 gp36	aa 587–614 of gp160 (858)	100[c]	12
HIV-1 group M gp41	aa 580–625 of gp160 (853)	100	10

[a] Numbers in parentheses indicate the total numbers of amino acid residues of the corresponding full-length proteins.
[b] ND, not done.
[c] In combination with HIV-1 gp41 (31 aa) and p24 (146 aa).

RESULTS

Design of r-HIV-MEP antigen. To design a MEP that could be of diagnostic utility, linear and conserved immunodominant epitopes, known to elicit anti-HIV antibodies, were selected based on published literature, summarized in Table 1 (10, 12, 14). These epitopes were from HIV-1 p24 and the Env antigens of HIV-1 and HIV-2. The r-HIV-MEP was designed by linking these epitopes in tandem using $(Gly)_4$ peptides. The DNA and predicted amino acid sequence and computer modeling analysis of the three-dimensional structure of the r-HIV-MEP are shown in Fig. 1. Computer modeling analysis suggests that the design of the MEP permits easy accessibility of all its constituent epitopes. All these epitopes would therefore be freely available for interaction with their cognate antibodies and would contribute to the overall sensitivity and specificity of the MEP in terms of reactivity with patient sera. Multiple sequence alignment of the epitopes of the r-HIV-MEP antigen with the corresponding epitopes of different HIV types and groups is shown in Fig. 2. It is apparent that the epitopes selected for inclusion in r-HIV-MEP manifest a considerable level of conservation among the specific types and groups of HIV, suggesting that this synthetic antigen may exhibit immunoreactivity to antibodies specific to different types and groups of HIV.

Expression and purification of r-HIV-MEP. We expressed a synthetic gene encoding the r-HIV-MEP protein in *E. coli* using

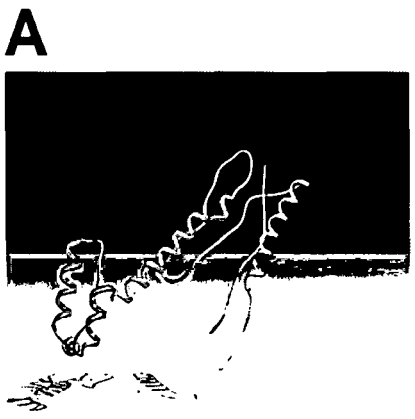

FIG. 1. The r-HIV-MEP antigen designed for this study. (A) Computer-generated graphic visualization (http://www.sbg.bio.ic.ac.uk/~3dpssm) of r-HIV-MEP. (B) Complete nucleotide (lowercase letters) and predicted amino acid (capital letters) sequences of the r-HIV-MEP gene showing four epitopes (aa 1 to 51, p24 of HIV-1; aa 56 to 92, gp41 of HIV-1 group O; aa 97 to 124, gp36 of HIV-2; and aa 129 to 174, gp41 of HIV-1 group M) linked together with flexible tetraglycyl linkers (underlined). The asterisk indicates the engineered stop codon.

IPTG induction from a 1-liter shake-flask culture. As localization experiments showed that the r-HIV-MEP was associated exclusively with the insoluble pellet fraction of lysates, we solubilized it using guanidinium and purified it under denaturing conditions by Ni-NTA affinity chromatography (data not shown). We obtained ~21 mg of >95% purified protein from a liter of induced culture.

Evaluation of the r-HIV-MEP with Boston Biomedica serum panels. Next, we sought to establish an in-house anti-HIV

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HIV-1 p24     NKIVRMYSPT SILDIRQGPK EPFRDYVDRF YKTLRAEQAS QEYKNWMTET L
HIV-1 grM_A   .........V .......... .......... F........T ..V.......  .
HIV-1 grM_B   .......... .......... .......... .......... ..V.......  .
HIV-1 grM_C   .........V .....K.... .......... F........T .DV.....D.  .
HIV-1 grM_D   .........V .......... .......... .......... .DV.......  .
HIV-1 grM_F   .........V .....K.... .......... F.V....... .DV.G...D.  .
HIV-1 grM_G   .........V .......... .......... F.C....... .DV.G...D.  .
HIV-1 grM_H   .........V .....K.... .......... F........T .DV.......  .
HIV-1 grM_J   .........V .......... .......... F.A......T .DV.....D.  .
HIV-1 grN     .R.......V ...E.K.... .......... .........T .DV.......  .
HIV-1 grO     ..M.K....V .....K.... .......... .........T ..V.......  .
HIV-2_A       Q.C....N.. N....K.... ...QS..... ..S.....TD PAV.....Q.  .
HIV-2_B       Q.C....N.. N....K.... ...QS..... ..S.....TD PAV.....Q.  .

HIV-1 grO gp41       WGIRQLRARL LALETLLQNQ QLLSLWGCKG KLVCYTS
HIV-1 grO ANT70      .......... .......... .......... .......
HIV-1 grO MVP-5180   .......... Q.....I... .R.N...... ..I....
HIV-1 grO 99CMU4122  .......... Q.....I... ...N...... R.I....
HIV-1 grO 99USTWLA   .......... Q.....M... ...N...... .SI....
HIV-1 grO VAU        .......... .....FI... ..HN.....N R.I....

HIV-2 gp36 QDQARLNSWG SAFRQVCHTT VPWVNDSL
HIV-2_A    .......... C......... ........
HIV-2_B    K...Q..... C......... ...P.ET.
HIV-2_A/B  K...Q..... C......... ........
HIV-2_U    K...S..A.. C......... ...I..T.
HIV-2_G    K......... C......... ...DALGA

HIV-1 grM gp41  WGIKQLQARI LAVERYLKDQ QLLGIWGCSG KLICTTAVPW NASWSN
HIV-1 grM_A     .........V .......Q.. R...M..... .H....F... .S....
HIV-1 grM_B     .........V .......R.. .......... ......T... .T....
HIV-1 grM_C     .......T.V ..I..H.R.. .......... .......... .S....
HIV-1 grM_D     .......... .......... .......... .H....N... .S....
HIV-1 grM_F     .........V .......... .......... ......N... .S....
HIV-1 grM_G     .......S.V ..I....... .......... ......N... .T....
HIV-1 grM_H     .........V .......R.. .......... ......N... .S....
HIV-1 grM_J     .........V .......... .......... ......N... ......
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FIG. 2. Multiple sequence alignment of the four r-HIV-MEP epitopes with the corresponding epitopes of different HIV types, groups, and subtypes (http://bioinfo.genotoul.fr/multalin/multalin.html). The black dots indicate identical residues. Variants are indicated by the standard single-letter amino acid code. Letters in the virus names indicate subtypes. In the case of HIV-1 group O gp41, the alignment has been done with different isolates within the group.

TABLE 2. Evaluation of r-HIV-MEP-based indirect immunoassay using HIV seroconversion panel (PRB 931; Boston Biomedica Inc.)

Member ID	Bleed date[a]	Days since first bleed	S/Co value					
			Abbott[b]		Gen. Sys.[b]		OT HIV[b]	MEP[c]
			HIV-1	HIV-1/2	HIV-1	HIV-1/2		
01	14	0	0.2	0.1	0.2	0.1	0.3	0.1 (−)
02	16	2	0.2	0.1	0.1	0.1	0.3	0.2 (−)
03	21	7	0.2	0.1	0.2	0.1	0.4	0.3 (−)
04	23	9	0.2	0.1	0.2	0.1	0.3	0.3 (−)
05	29	15	0.2	0.1	0.2	0.1	0.3	0.3 (−)
06	11	28	0.9	6	0.3	0.4	0.6	1.2 (+)
07	16	33	3.9	>18.7	0.8	1.1	2.3	5.6 (+)
08	18	35	5.7	>18.7	1.3	1.9	3.1	9.2 (+)
09	25	42	10.5	>18.7	2.9	4	4.6	>10 (+)

[a] Bleed dates for member IDs 01 to 05 were in August 1995; those for IDs 06 to 09 were in September 1995.

[b] Values indicate signal-to-cutoff ratios provided by the panel supplier (Gen. Sys., Genetic Systems; OT, Organon Teknika) using the indicated commercial kits. S/Co values ≥1.0 are considered positive.

[c] Values indicate signal-to-cutoff ratios obtained using the in-house indirect immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values <1.0 are designated negative (−), and those with values ≥1.0 are designated positive (+).

indirect immunoassay using the purified r-HIV-MEP. In this assay, the purified r-HIV-MEP antigen was used to capture anti-HIV antibodies in sera. Bound anti-HIV antibodies were detected by TRF using anti-human antibody labeled with Eu^{3+}-9d-chelate. To establish the specificity of r-HIV-MEP protein as an intermediate for anti-HIV immunoassay, more than 50 HIV-negative human serum samples were evaluated. The results demonstrated that r-HIV-MEP did not exhibit any false positivity with normal human serum samples. These results unequivocally established the high degree of specificity of r-HIV-MEP protein for the detection of anti-HIV antibodies.

Next, 57 serum samples from various well-characterized BBI panels were used to evaluate our in-house r-HIV-MEP-based anti-HIV immunoassay. Table 2 compares the ability of our in-house assay to detect early seroconversion with those of other commercial kits using a set of nine sera constituting the HIV seroconversion panel (PRB 931). The earliest time point at which seroconversion is detected in this panel is at 28 days, using the in-house assay, represented by panel member 6. In addition to our in-house assay, only one kit, namely, the Abbott HIV-1/2 kit, out of the five commercial kits tested could pick up this member. We assessed the sensitivity of the r-HIV-MEP antigen to detect anti-HIV antibodies further by testing it against BBI's worldwide HIV performance panel (WWRB 302) consisting of 25 sera. Of these sera, 21 sera were HIV-1 positive, representing genotypes A, B, C, D, E, F, G, and O, from diverse geographical locations such as the United States, Spain, and several countries in Asia and Africa. Of the remaining four sera in this panel, two were HIV-2 positive and two were HIV negative (Table 3). Interestingly, the in-house immunoassay using r-HIV-MEP identified all 21 HIV-1 samples and the 2 HIV-2 samples. Further, the two sera that were HIV seronegative using five different commercial kits were also seronegative in the in-house assay. To examine specificity in the background of other infections, we evaluated the in-house immunoassay using BBI's viral coinfection panel consisting of 25 sera (PCA 201). Of these, 9 were HIV seronegative while the rest (n = 16) were HIV seropositive, based on commercial assays. We tested 7 of the HIV-seronegative and all 16 of the HIV-seropositive samples using the r-HIV-MEP-based immunoassay. Many of these samples were also seropositive for hepatitis B virus (HBV), hepatitis C virus (HCV), and/or human T-cell leukemia virus (HTLV). The results are summarized in Table 4. Significantly, regardless of the presence or absence of antibodies to HBV, HTLV, or HCV, the results of the in-house immunoassay for HIV antibodies closely matched the results obtained with the commercial assays. The lone exception was provided by panel member 20. This serum, which scored as HIV positive with the commercial kits, was assigned as HIV negative using the r-HIV-MEP-based assay. A closer examination reveals that this discrepancy is attributable to this sample being a borderline specimen. The S/Co ratios, which must be ≥1.0 to designate a sample as seropositive, were 1.1 and 1.0 for the two commercial kits and 0.9 for the in-house assay.

Overall, the data show that the performance of our single r-HIV-MEP-based immunoassay is in near total agreement with the commercially available multiantigen-based anti-HIV EIA kits, namely, Abbott HIV-1, Abbott HIV-1/2, Genetic Systems HIV-1, Genetic Systems HIV-1/2, and Organon Teknika HIV-1.

DISCUSSION

Our earlier work has established the utility of recombinant MEPs in the detection of infection by different pathogens (1, 2, 6). The present study is based on the premise that the use of a single diagnostic intermediate designed to have HIV-specific immunodominant epitopes from all known genotypes and expressed to high levels in an *E. coli* expression system could effectively address the issues of cost and specificity associated with the currently available multiple-antigen-based anti-HIV diagnostic assays. To develop this recombinant antigen, we focused on the HIV-1 and HIV-2 antigens shown in Table 1. The ability of these proteins to elicit humoral immune response has been well documented, and their antigenic determinants have been identified using a variety of different approaches (4, 13, 16). We selected immunodominant, linear, and phylogenetically conserved epitopes from these antigens for incorporation into the synthetic antigen r-HIV-MEP. In order for a synthetic MEP to be capable of efficiently recognizing HIV-specific antibodies, it is necessary that its constit-

TABLE 3. Evaluation of r-HIV-MEP-based indirect immunoassay using worldwide HIV performance panel (WWRB 302; Boston Biomedica Inc.)

Member ID	Origin	Gtyp[a]	S/Co value					
			Abbott[b]		Gen. Sys.[b]		OT HIV-1[b]	MEP[c]
			HIV-1	HIV-1/2	HIV-1	HIV-1/2		
01	Spain	O	1.1	1.8	0.8	5.6	1.3	>10 (+)
02	Ghana	A	>11.5	>16.1	6.9	8.7	7.0	>10 (+)
03	Ghana	G	>11.5	>16.1	7.1	8.8	7.2	>10 (+)
04	Ghana	G	>11.5	>16.1	7.1	8.8	6.5	>10 (+)
05	Ghana	A	>11.5	>16.1	7.1	8.7	7.0	1.8 (+)
06	Ghana	G	>11.5	>16.1	6.9	8.8	7.1	9.2 (+)
08	Ivory Coast	G	>11.5	>16.1	6.9	8.7	6.7	>10 (+)
09	Ivory Coast	A	>11.5	>16.1	6.9	8.6	6.5	>10 (+)
10	Ivory Coast	Neg[d]	0.4	0.2	0.1	0.4	0.4	0.5 (−)
11	Mozambique	HIV-2	1.2	14.6	0.6	9.7	3.0	1.8 (+)
12	Mozambique	C	>11.5	>16.1	7.1	8.9	6.9	>10 (+)
14	Uganda	D	>11.5	>16.1	4.5	8.5	6.2	6.4 (+)
15	Uganda	D	>11.5	>16.1	6.3	8.1	7.2	3.5 (+)
16	Uganda	D	>11.5	>16.1	7.0	8.8	6.9	8.8 (+)
17	Uganda	D	>11.5	>16.1	6.8	9.8	7.0	2.7 (+)
19	Zimbabwe	C	>11.5	>16.1	6.0	9.9	7.0	>10 (+)
21	China	B	>11.5	>16.1	6.7	8.8	7.0	>10 (+)
22	Thailand	E	>11.5	>16.1	7.3	9.8	7.0	>10 (+)
24	Thailand	E	>11.5	>16.1	7.4	9.8	6.9	>10 (+)
25	India	HIV-2	0.4	15.4	3.8	10	2.1	>10 (+)
26	USA	D	>11.5	>16.1	7.4	9.8	7.1	>10 (+)
27	USA	B/D	>11.5	>16.1	7.0	9.8	7.2	>10 (+)
28	Argentina	F	>11.5	>16.1	7.0	8.9	6.8	>10 (+)
29	Argentina	B	>11.5	>16.1	6.9	8.5	6.6	>10 (+)
30	Argentina	Neg	0.3	0.2	0.2	0.2	0.4	0.3 (−)

[a] Gtyp, genotype.
[b] Values indicate signal-to-cutoff (S/Co) ratios, provided by the panel supplier (Gen. Sys., Genetic Systems; OT, Organon Teknika) using the indicated commercial kits. S/Co values ≥1.0 are considered positive.
[c] Values indicate signal-to-cutoff ratios obtained using the in-house indirect immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values <1.0 are designated negative (−), and those with values ≥1.0 are designated positive (+).
[d] Neg, negative.

uent epitopes exhibit significant reactivity to HIV-infected patient sera worldwide. Since we wanted to use *E. coli*-based overexpression from the cost perspective, it was necessary to work with linear epitopes, so that when incorporated into the synthetic protein, they would presumably retain their immunoreactivity toward anti-HIV antibodies. Finally, the phylogenetically conserved epitopes would facilitate the recognition of multiple HIV types and groups. The selected epitopes, which ranged in length from 28 to 51 amino acid (aa) residues, were fused in frame using flexible tetraglycyl linkers between adjacent epitopes. These linkers are preferred in designing flexible chimeric proteins (20). Computer modeling analysis of the MEP antigen which showed all the chosen epitopes to be accessible suggested that they would collectively contribute to the overall specificity and sensitivity of the molecule.

We overexpressed the recombinant antigen in *E. coli* and purified it under denaturing conditions, as it was insoluble despite its fusion to thioredoxin. We then evaluated the efficacy of r-HIV-MEP as a diagnostic intermediate in an in-house Eu^{3+}-9d-chelate-based indirect immunoassay. In this assay we used the recombinant protein as the capture antigen and panels of HIV-infected (commercially available) and normal human sera as test samples. We then used anti-human antibody labeled with Eu^{3+}-9d-chelate as a tracer and monitored captured anti-HIV antibody through TRF. The results showed that our synthetic diagnostic intermediate could indeed recognize and bind to anti-HIV antibodies, elicited by both HIV-1 and HIV-2. However, it is to be noted that the design of r-HIV-MEP precludes differentiation of HIV-1 from HIV-2. As HIV exhibits a distinct geographical distribution, a worldwide HIV performance panel (WWRB 302) was used to evaluate if r-HIV-MEP could recognize the HIV-infected sera from different parts of the world. Panel members included specimens characterized as HIV-1 group M (subtypes A to G), HIV-1 group O, and HIV-2. Our results demonstrated that r-HIV-MEP was able to recognize antibodies to a diverse set of HIV infections from India and from other countries such as Argentina, China, Ghana, Ivory Coast, Mozambique, Spain, Thailand, Uganda, the United States, and Zimbabwe. The HIV seroconversion panel (PRB 931) was utilized to evaluate the sensitivity of the r-HIV-MEP in the immunoassay, and the results were found to be in complete agreement with those of the best-performing Abbott HIV-1/2 EIA kit in the early diagnosis of anti-HIV antibodies in human sera. Our immunoassay was able to pick up panel member 6 of the HIV seroconversion panel (PRB 931), which showed immunoreactivity with the Abbott HIV-1/2 EIA kit only and not with other EIA kits. The viral coinfection panel (PCA 201), on the other hand, was used for the evaluation of specificity of the r-HIV-MEP in the immunoassay. This single diagnostic intermediate performed as well as the other commercial anti-HIV kits from Abbott, Genetic Systems, and Organon Teknika except for

TABLE 4. Evaluation of r-HIV-MEP-based indirect immunoassay using viral coinfection performance panel (PCA 201; Boston Biomedica Inc.)

Panel ID	S/Co value										
	Hepatitis B virus[a]			HTLV[a]			HIV-1[a]			HCV[a]	MEP[b]
	a	b	c	d	e	f	g	h	i		
1	53.4	68.9	9.0	2.1	2.6	P	0.2	0.3	–	6.3	0.3 (–)
2	51.6	68.9	8.8	0.1	0.2	–	13.5	15.2	P	0.7	>10 (+)
3	49.3	68.9	8.7	2.1	2.6	P	0.1	0.2	–	6.3	0.2 (–)
4	26.4	68.9	7.7	2.1	2.6	P	0.1	0.2	–	0.4	ND
5	0.4	0.4	7.4	2.1	2.6	P	13.5	15.5	P	6.3	>10 (+)
6	0.5	0.1	0.5	0.1	0.1	–	0.1	0.3	–	0.5	ND
7	0.7	0.2	0.2	2.1	2.6	P	13.5	15.5	P	6.3	>10 (+)
8	38.9	68.9	57.9	0.1	0.2	–	13.5	15.5	P	2.1	>10 (+)
9	1.0	1.3	0.6	2.1	2.6	P	13.5	15.5	P	2.2	>10 (+)
10	42.8	68.9	7.8	0.1	0.2	–	13.5	15.5	P	0.2	>10 (+)
11	48.6	68.9	8.5	2.1	2.6	P	0.1	0.2	–	6.3	0.5 (–)
12	38.7	68.9	8.5	0.2	0.2	–	13.5	15.5	P	6.3	>10 (+)
13	33.6	38.6	9.2	0.1	0.3	–	13.5	15.5	P	0.7	>10 (+)
14	51.8	68.9	8.2	2.1	2.6	P	0.32	0.2	–	0.5	0.1 (–)
15	64.5	68.9	2.1	1.6	2.2	P	0.2	0.3	–	6.3	0.7 (–)
16	0.5	0.3	5.4	2.1	2.6	P	13.5	15.5	P	6.3	>10 (+)
17	42.3	68.9	8.1	0.1	0.4	–	13.5	15.5	P	1.1	>10 (+)
18	5.1	1.0	0.4	2.3	2.6	P	13.5	15.5	P	6.3	>10 (+)
19	45.6	50.8	2.9	0.2	0.2	–	13.5	15.5	P	0.4	>10 (+)
20	12.2	11.9	6.2	0.1	0.2	–	1.1	1.0	P	0.1	0.9 (–)*
21	42.2	57	2.7	0.2	0.2	–	13.5	15.5	P	0.9	>10 (+)
22	41.5	68.9	7.2	0.1	0.2	–	13.5	15.5	P	6.3	>10 (+)
23	46	68.9	8.1	2.1	2.6	P	0.2	0.3	–	6.3	0.6 (–)
24	0.5	0.2	0.5	0.2	0.2	–	0.1	0.2	–	0.4	0.4 (–)
25	50.1	68.9	8.5	0.2	0.3	–	13.5	15.5	P	0.9	9.7 (+)

[a] Assays performed using commercial kits. HBs (columns a and b), HTLV (columns d and e), and HIV-1 (columns g and h) assays were done with kits from Abbott. In each of these instances, assays were done twice, independently by BBI (columns a, d, and g) and a reference lab (columns b, e, and h). Other kits used were from Organon Teknika (for HBc, column c) and Ortho (for HCV). Values indicate signal-to-cutoff (S/Co) ratios provided by the panel supplier using the indicated commercial kits. S/Co values ≥1.0 are considered positive: HTLV (column f) and HIV-1 (column i) assays were also done in blot format using kits from Genetic Systems and Dupont, respectively. P and – indicate the presence and absence, respectively, of antigen bands in the blot assays (columns f and i).

[b] HIV detection using the r-HIV-MEP-based in-house assay. Values indicate signal-to-cutoff ratios obtained using the in-house indirect immunoassay. Samples with S/Co values <1.0 are designated negative (–), and those with values ≥1.0 are designated positive (+). The results are indicated in parentheses. The asterisk indicates a borderline result in the in-house assay. ND, not determined due to lack of sample.

panel member 20 of the viral coinfection panel, which happened to be a borderline sample. The information regarding the subtype of this member is unavailable. Overall, the data attest to the utility of our designer antigen in detecting HIV infection, from diverse geographical locations, with high specificity and sensitivity. It is also useful in monitoring seroconversion. However, these are preliminary data and need to be corroborated with a larger number of serum samples. The use of fluorescent Eu^{3+}-9d-chelate enables simplified, rapid, and universal test protocols to be constructed for a wide range of analytical applications because of the ease of antibody labeling with Eu^{3+}-9d-chelate and easy measurement of fluorescence directly from the dry wells without adding any substrate or stopping the reaction. Though the current assay takes a few hours, it has the potential to be adapted to a rapid test format.

In conclusion, the high density of HIV-specific, phylogenetically conserved, and immunodominant epitopes selected for designing the r-HIV-MEP contributed to a high degree of sensitivity and specificity. Further, our strategy of using a single recombinant MEP completely obviates multiple peptide synthesis and multiple protein expressions, and our Eu^{3+}-9d-chelate-labeled antibody as a tracer further simplifies the immunoassay. These factors, together with the high level of expression of r-HIV-MEP in *E. coli* and its single-step affinity purification design, make this approach highly cost-effective for anti-HIV screening in blood banks in most developing countries. The yield of purified r-HIV-MEP from 1 liter of induced culture is sufficient for ~40,000 assays.

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