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Development of a quantitative chemiluminescent immunoassay for the hepatitis B antigen detection

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Ai miei genitori

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Introduction

1. The hepatitis B virus

1.1. Morphology and epidemiology

The hepatitis B virus (HBV) infection is an important public health problem worldwide; 4.5 million people get infected every year. Over 95% of acutely infected adults completely and spontaneously recover from the infection (1) while the 5-10% is not able to produce antibodies against the virus triggering the major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). On a worldwide basis, 350 million people are chronically infected by HBV and about 1 million of them die each year from the complications of chronic infection (2). Acute infection may result in classical acute hepatitis or fulminant hepatitis. In chronic infection, HBV replication persists throughout the course of the infection, and the host immune response plays a pivotal role in HBV-related liver damage and control of HBV replication.
The HBV infection is prevalent in Asia, Africa, Southern Europe and South America (figure 1) where the prevalence of HBV in the general population ranges from 2 to 20%.

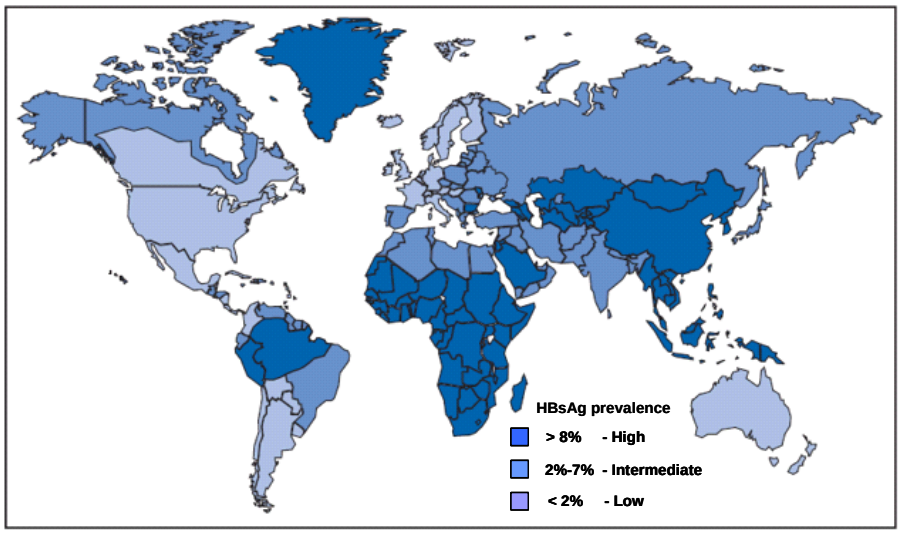


Figure 1. Geographic distribution of chronic hepatitis B virus (HBV) infection worldwide (3).

Hepatitis B virus is the smallest human DNA virus with a genome of 3200 base pairs which belongs to the *Hepadnaviridae* family (figure 2). The virus consists of a nucleocapsid and an outer envelope composed mainly of three surface antigens (HBsAgs). The nucleocapsid contains the hepatitis B core antigen (HBcAg), a DNA polymerase-reverse transcriptase, the viral genome and cellular proteins (4; 5).

Hepatitis B virus is a partially double-stranded circular DNA which replicates by a process that involves an RNA intermediate and reverse transcription. One of the two strands is incomplete and is associated with a DNA polymerase which is able to complete the strand in the presence of deoxynucleoside triphosphates. Analysis of the coding potential of genome reveals four overlapping open reading frames (ORFs). The C-ORF encodes for the core antigen (HBcAg), the major structural component of the capsid, and for a pre-core protein which is co-translationally processed and secreted as hepatitis B “e” antigen (HBeAg), which is a serological marker for high viral replication. The S-ORF encodes for surface antigens (HBsAgs) called preS1/S2 and S proteins; the surface antigen is a transmembrane glycoprotein usually shed in large amounts in the serum of infected people and plays a central role in the diagnosis of HBV infection (6); detection in the serum of the “Australian antigen”, namely hepatitis B surface antigen, was the Nobel prize discovery that identified hepatitis B virus about 40 years ago; to this day HBsAg remains the hallmark of HBV infection (7). The P-ORF encodes for the viral polymerase-reverse transcriptase. Finally the fourth X-ORF encodes for the “x” antigen (HBxAg): it has been shown to be a potent transactivator of cellular and viral genes. A variety of interactions with cellular proteins has been proposed but the precise function of HBxAg remains to be established (4; 5; 8).

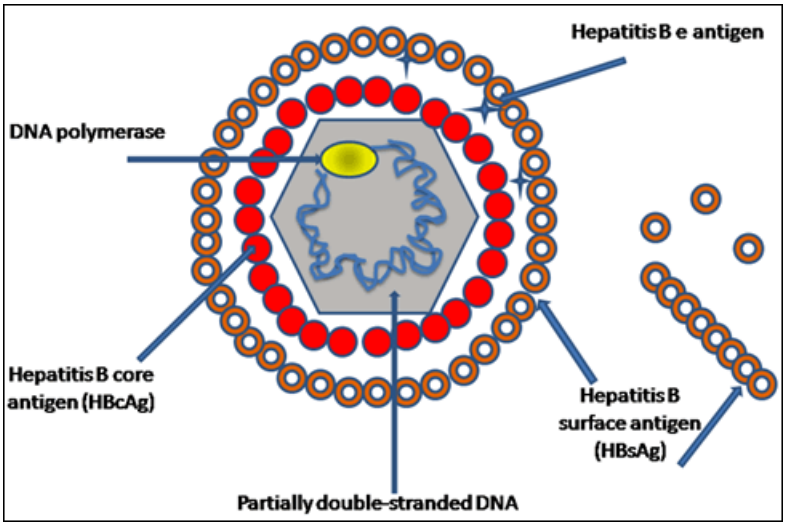


Figure 2. HBV structure (9).

Up to eight genotypes have been identified (A-H) due to a sequence difference greater than 8% in the entire HBV genome or 4% in the S region (10). Each genotype is further separated into subgenotypes if the divergence in the whole nucleotide sequence is between 4% and 8% (11); both genotypes and subgenotypes have different geographical distributions (figure 3) and often present demographic characteristics (12). Genotypes A1, A3, A4, A5 are endemic in Africa whereas A2 is endemic in Europe (13; 14; 15; 16); B and C are predominant in Asia and pacific islands (17). Genotype D is endemic in the entire old world including Africa, northern and south eastern Asia, the Mediterranean area and most European countries (18; 19; 20; 21; 15) while the E is endemic in western and central Africa (15; 22). The genotypes F, G, H are endemic in America; more subgenotypes have been found in different world areas (23; 24; 25).

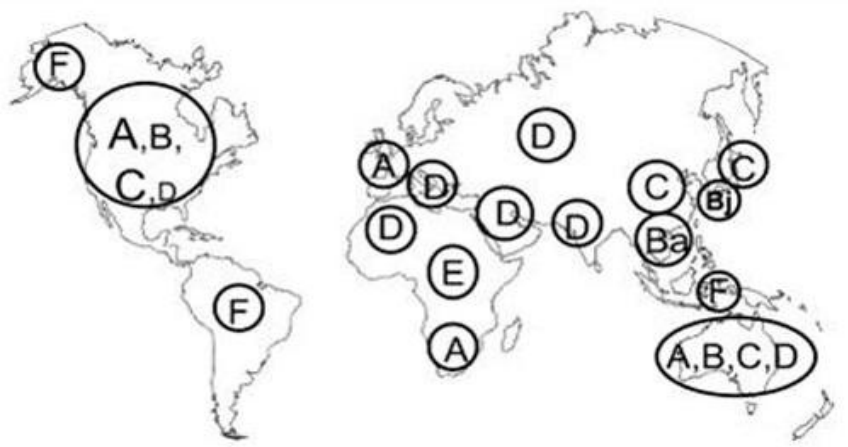


Figure 3. Geographic distribution of hepatitis B virus genotypes (26).

The genetic diversity of HBV and the geographical distribution of its subgenotypes (Table 1) provide clues to reconstruct the virus evolutionary history and understand the evolution and past migrations of man (12; 27).

1.2. The HBV envelope proteins

As mentioned before the envelope contains three related viral surface proteins expressed from one ORF containing three start sites for translation. The transcription starts at a promoter upstream of the ORF and at an internal promoter upstream the second translation initiation site (28). The translation of the larger mRNA yields the large envelope protein (L) consisting of 389 or 400 aminoacids depending on the genotype. The shorter mRNA gives rise to the middle size protein (M) 281 aa long and to the small (S) protein 226 aa long depending on which translation initiation site is used. The aa sequence present at the C termini of L and M is identical to the S protein and is referred to as S domain (29). The 55 aa long additional N-terminal domain of M, central in L protein, is called preS2 while the 108 or 119 aa long at the N-terminal domain unique to L is called preS1.

These envelope proteins are synthesized at the endoplasmic reticulum (ER) gaining a complex transmembrane topology. The S protein is expressed at the highest levels, predominates in both virions and subviral particles (7) and has a first N-terminal signal sequence (aa 8 to 22) for the insertion in the ER membrane and a

second one which directs the translocation of the peptide chain downstream this signal through the ER membrane into the ER lumen whereas the region upstream remains in the cytosol. The signal itself anchors the protein as a transmembrane domain in the lipid bilayer (30). The C-terminal hydrophobic 57 aa are believed to be embedded in the ER membrane oriented towards the ER lumen. This configuration leads the region between residues 23 and 79 to form a loop at the cytosolic side of ER membrane whereas the loop between aa 99 and 169 is on the lumen side. The luminal loop carries the major conformational epitope of the HBV surface protein antigen (HBsAg) and is in half of the S proteins N-glycosylated at asparagines 146.

Genomic group	Subtype	Area of prevalence
A	**adw2**	**Northwestern Europe**
	adyw1	**Central Africa**
B	**adw2**	**Indonesia, China**
	ayw1	**Vietnam**
C	**adw2**	**East Africa**
	adrq$^+$	**Korea, China, Japan**
	adrq$^-$	**Polynesia**
	ayr	**Vietnam**
D	**ayw2**	**Mediterranean area**
	ayw3	**India**
E	**ayw4**	**West Africa**
F	**adw4q$^-$**	**South America, Polynesia**

Table 1. Geographic distribution of HBV genomic groups and subtypes (31).

The M protein topology is identical to S and is regulated by the same promoter whereas the transcription of L is regulated by a specific but weaker promoter (32) and in the half of L chains the transmembrane topology changes after translation.
During budding at an intracellular membrane a short linear domain in the preS2 region interacts with binding sites on the capsid surface and the virions are secreted in the blood stream. After budding the HBsAg epitopes are located at the external surface of the viral particles (33; 34).

HBsAg exists in different forms in the serum: as competent virions of 42 nm (Dane particles) (7), as 20 nm diameter filaments of variable lengths and free floating 20-22 nm spherical defective particles corresponding to empty viral envelopes (figure 4) (35; 36).
The large surface protein L is an essential component of both virions and filaments and represents 10-20% of their envelope proteins. In contrast it represents only 2% of the 22 nm spherical particles (37; 38).
The S domain, but not the preS, contains multiple cystein residues; in the luminal loop they crosslink the envelope proteins with each other by multiple disulfide bridges. After synthesis disulfide-linked homo- and heterodimers between S, M and L proteins can be found (39).

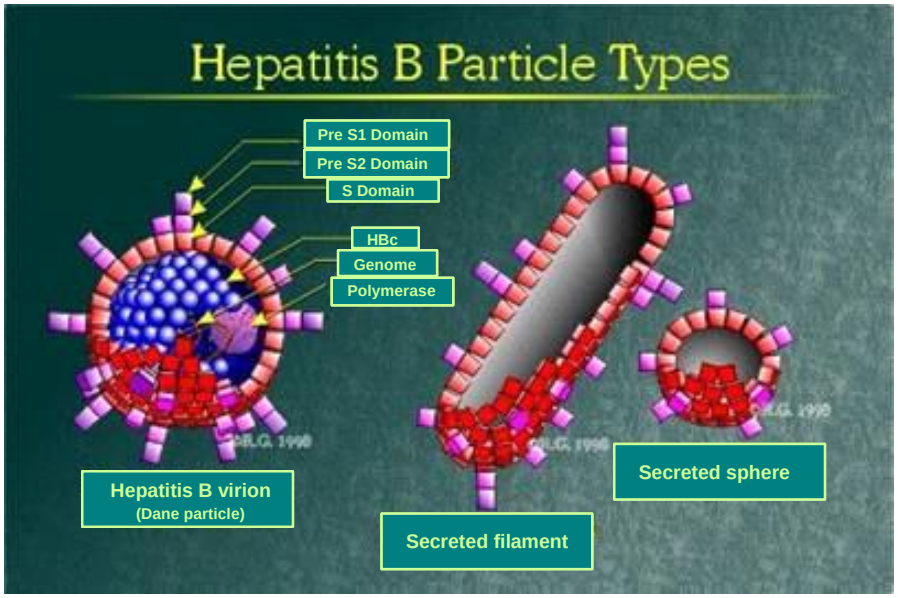


Figure 4. Different forms of HBV (40).

1.2.1. Subviral particles

The HBV surface proteins are not only incorporated into virion envelopes. They can bud efficiently from intracellular post-ER membranes without envelopment of capsids: they appear as subviral quasi-spherical or filamentous lipoprotein particles in the lumen of the compartment and are released from cell by secretion. The quasi-spherical particles have a diameter of 22 nm with an octahedral symmetry (41), the filaments have variable lengths and are composed of about 100 HBsAg monomers each (42; 39). The subviral particles

are highly overexpressed relative to virions and are 10000-fold higher concentrated in serum. Subviral particles and virions carry identical surface antigens (HBsAg) although the protein composition is not identical: spherical subviral particles contain low amounts of L protein whereas it is higher in filaments and especially in the virion envelope.
The S protein subunits interact tightly with each other during 22 nm particle formation (38; 43). Recombinant expression of S protein yields highly immunogenic intracellular 22 nm HBsAg particles which can be used as a vaccine against the hepatitis B.

1.3. Natural course of HBV infection and HBV serology

Hepatitis B infection induces liver injury via the host immune response. HBV enters the body in the bloodstream and targets hepatocytes, presumably since its receptor is found predominantly on these cells. There is a little cytopathic effect and the rate at which symptoms appear depends on the initial dose of virus. A cell-mediated response to an HBV infection results in cytotoxic T cells against both surface (HBsAg) and internal antigens (HBcAg and HBeAg). HBcAg epitope serves as the principal target of the cell-mediated immune response after being processed and presented by class I and II T-cell receptor: this expression triggers the immune response to affected hepatocytes.
In addition to intact virions, HBV-infected cells shed particles that are composed primarily or completely of aggregated HBs antigen. This combines with and blocks anti-HBs antibodies, thereby limiting the humoral response. The large amounts of antibody-HBsAg complexes cause type III hypersensitivity reactions and result in rash, arthralgia and damage to the kidneys.
The incubation period is 60-90 days (range 45-180 days), although virus replication starts a few days after infection. The first sign of infection is the characteristic appearance of HBsAg in infected cells. As with other hepatitis viruses, the symptoms are immune-mediated, resulting from inflammation and cell-mediated (cytotoxic T cell) responses to HBsAg on the surface of hepatocytes. In an acute HBV infection, symptoms last from 10 to 20 weeks after infection. Symptoms include jaundice, fatigue, abdominal pain, loss of appetite, nausea, vomiting and joint pain. If the cell-mediated immune response is weak, symptoms are mild but the infection does not resolve and chronic hepatitis ensues. This is frequently the case with younger patients who have lesser cell-mediated immunity. Chronic HBV infection can lead to chronic hepatitis. This leads to cirrhosis of the liver in up to a quarter of patients within five years. Of these patients, up to one quarter will develop hepatocellular carcinoma or

liver failure. Both of these are fatal in the absence of a liver transplant.

Four stages of hepatitis B infection have been described. During the stage one, viral replication occurs asymptomatically without liver inflammation with normal aminotransferase (ALT) levels. This immune-tolerance phase - typically observed in children or young adults - is associated with extremely high serum HBV DNA levels and positivity of HBeAg.

Acute or chronic liver injury derives from antiviral response mediated by cytotoxic T and natural killer cells during the stage two. With immune-mediated cytotoxic response towards infected hepatocytes, the liver cells suffer continuous or repeated bouts of damage as shown by elevated serum transaminase levels. After these immune attacks, HBV replication is temporarily suppressed and some patients may undergo HBeAg seroconversion with the loss of HBeAg and subsequent development of anti-HBe. If the host is not able to contain the viral replication the phase two continues resulting in a progressive liver damage (chronic hepatitis).

If the immune system eradicates all but a small number of infected cells, active viral replication decreases and the third stage begins: the loss of HBeAg, whose presence in serum is indicative of active viral replication, marks this change. HBeAg becomes undetectable and serum HBV DNA levels decrease to a level only detectable by sensitive polymerase chain reaction techniques (44).

A minority of patients relapse to stage two due to failure of the immune system to maintain viral suppression (subsequent elevation in viral DNA titers and HBeAg); most patients however continue to suppress viral replication reducing the infected hepatocytes via cell-mediated immunity or through the natural hepatocyte turnover. Many become negative for HBsAg over months to years and enter the stage four: anti-HBs antibodies neutralize the remaining viral particles (45).

The acute hepatitis B involves the passage from stage 1 to 4 over a period of several weeks to months. The chronic state indicates the persistence of the first stage where the viral replication continues in the absence of a significant immune response; patients with this stage disease rarely progress to cirrhosis, however one fourth of them will eventually enter stage two with a relapsing course and about half of them progress to cirrhosis within five years. The probability of developing a chronic stage or infection after an initial exposure, depends on the status of the immune system at the time of infection and on the age. The 90% of neonates exposed at birth will develop immune tolerance to HBV resulting in chronic infection while only 25% to 50% of children exposed at ages 1 to 5 years and 5% to

10% of older children or adults develop chronic hepatitis (44; 46). Moreover, an important mechanism for the development of viral persistence in adults may be the development of viral escape mutations.
Despite the clearance of HBsAg and development of anti-HBs in the stage 4 the viral DNA may be therefore detected in the serum as immune complexes many months after the clearance. Low level replication in the liver and peripheral blood cells after HBsAg seroconversion probably is responsible of cases of HBV reactivation reported in immunosuppressed and may contribute to hepatic carcinogenesis reported in HBsAg-negative patients (47; 48; 49; 50; 51; 52).

1.4. HBV immunopathogenesis

1.4.1. Innate immune response

As viruses infect target cells the host rapidly triggers the early innate defense mechanism to contain the viral spread. These mechanisms comprise the induction of apoptosis, the production of antiviral cytokines such as INF-$\alpha\beta$ by the infected cells and the activation of effectors of the innate immune system as NK and NKT cells (1; 53; 54; 55).
There is no currently evidence that HBV triggers apoptosis and liver injury: in fact in infected chimpanzees during the early phase there is neither histological nor biochemical evidence of hepatocellular injury (56). The evidence that HBV induces the production of INF-$\alpha\beta$ by the infected cells is also lacking. Although activation of NK, NKT cells and Toll-like receptors have been shown to inhibit viral replication in HBV transgenic mice (57), there is no evidence that these cells or any pathway of innate immune system play a central role in pathogenesis or viral clearance during the early phase of HBV infection before the entry of adaptive immune response (1; 58). These results indicate that early innate response may not significantly contribute to the pathogenesis of liver injury or to viral clearance and the HBV remains quite undetected till the adaptive response enters the liver (1).

1.4.2. Adaptive immune response

Virus specific $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) participate in tissue damage and viral clearance by killing infected cells or by producing soluble factors as cytokines and chemokines contributing to inflammatory process and inhibiting the viral replication (59). Cytokines and chemokines induce the antiviral

antibodies formation with neutralizing activity; their appearance is thought to occur late after HBV exposure thus they do not contribute to the eary phase of viral clearance during acute infection.
$CD4^+$ T helper cells do not have a pathogenetic role in the infection but they may contribute to the infection control by facilitating the induction and maintenance of virus specific CTLs.
The recognition of antigen by naive $CD8^+$ CTLs precursors circulating through secondary lymphoid organs start the development of effectors which clonally expand and leave the lymph nodes (60). Effectors $CD8^+$ CTLs play a fundamental role in the pathogenesis of liver disease and viral clearance. Studies on chimpanzees depleted of their $CD4^+$ or $CD8^+$ cells have been carried out: depletion of $CD4^+$ cells did not significantly affect the course of the infection; in contrast, depletion of $CD8^+$ cells greatly prolonged the infection and delayed the viral clearance until $CD8^+$ cells reappeared in the circulation and entered the liver (61).
The beginning of the liver injury corresponds with the influx of virus specific CTLs into the infected liver and the association between the magnitude of virus specific CTLs responses, liver disease severity and viral clearance has been reported in many studies. Patients with acute viral hepatitis who clear HBV, count relatively multispecific polyclonal CTL response to several HBV antigens that is associated with a severe degree of hepatocyte damage; in contrast liver injury is limited in chronically infected patients in whom the CTL response is weaker (62).
In addition to cause viral hepatitis, virus specific T cells as well as NK and NKT cells can abolish HBV expression and replication without killing the hepatocytes; this antiviral non-cytolytic activity is mediated by the local production by these cells of IFN-γ involving early after antigen recognition (63) and TNFα. HBV replication is inhibited early and non-cytopathically in a CD8 dependent and probably IFN-γ associated manner (61).
A large number of T cell-derived IFN-γ regulated genes are induced in the liver during viral clearance (64) reflecting the impact of the adaptive response that inhibits viral replication and kills the infected cells.
As CTLs reach the hepatocytes, the first step in the disease process is the antigen recognition by these cells which rapidly induces hepatocellular apoptosis. The initial apoptotic process involves a small number of hepatocytes; as time goes on, many host-derived, antigen non specific inflammatory cells are recruited into the liver.
Based on these facts it is apparent that HBV replicates noncytopathically in the hepatocyte and most of liver damage reflects the immune response. It is evident that the innate response does not

contribute to the pathogenesis of liver disease or viral clearance while the adaptive response contributes to both (1).

1.4.3. Antiviral therapy

Treatment of chronic hepatitis remains an important clinical objective. It is responsible 500000 to 1.2 million deaths every year from liver cirrhosis and hepatocellular carcinoma (HCC) (65). There are two main strategies for the prevention of the infection and for the treatment of those already chronically infected; these options consist of immunomodulatory and viral suppressant drugs (66). The chronic hepatitis therapy requires inhibiting the virus replication or eliminating the virus from cells.

With interferon therapy HBsAg loss occurs in 3%-10% of patients within one year of start of therapy and increases in sustained responders to 11%-32% (67). Treatment with interferon-α or PEG-interferon-α is of limited duration and response is often durable off-treatment. However side effects could appear leading to a poor tolerability and only a minority responds. Genotype proved to be an important predictor for the response to interferon-α or PEG-interferon-α therapy: for example genotypes A and B show higher end of treatment responses than genotypes C and D (67; 68; 69).

The use of reverse trancriptase inhibitors is an attractive target. Nucleoside analogues are chemically synthesized drugs which mimic natural nucleosides (70). Nucleoside analogues are well tolerated and most patients are responsive but the therapy is impaired by the selection of drug resistant mutants leading to loss of efficacy and to the relapse after treatment. Four oral nucleoside analogues, lamivudine, adefovir, entecavir and telbivudine are currently marketed and approved as a first line of therapy (66). HBsAg loss is rare in patients treated with nucleoside analogues (less than 2% after one year of therapy); however in a little amount of patients 14% HBsAg loss was observed (71; 72); although all of them are potent viral suppressors, none is able to permanently eradicate HBV (73).

Treatment of non-responders to previous treatment is little studied and most of the results are of difficult interpretation. Nucleoside analogues appear to be effective in interferon failures, although efficacy may be different and data are limited (74).

The major problem with current treatments is the emergence of drug resistant variants. Several new anti-HBV nucleoside analogues are in different stages of clinical trials and over time there should be an increase in the compounds designed to target specific molecules (70).

1.5. Diagnosis and clinical application of HBV markers

Serum hepatitis is usually first diagnosed from the clinical symptoms. Liver enzymes are also detected in the bloodstream during the symptomatic phase. Much further information can be obtained from serology and the presence of HBV antigens. Tests that detect HBsAg and HBcAg and antibodies against HBcAg, HBsAg and HBeAg (the hepatitis B panel) are used in diagnosis. There is a characteristic sequence in the appearance of HBV markers following infection. HBsAg is the hallmark of HBV infection and is the first serological marker to appear in acute hepatitis B. Persistence of HBsAg for more than 6 months refers to chronic HBV infection (CHB). Anti-HBs is a neutralizing antibody and its presence suggests the recovery from hepatitis B and confers a long-term protective immunity against HBV infection. In addition, it is the only detectable serological marker in those who successfully respond to hepatitis B immunization. Because of the large amounts of HBsAg that are not associated with infectious virus, the presence of HBeAg is the best marker for infectious virus. Hepatitis B e antigen usually indicates active HBV replication and risk of transmission of infection. Seroconversion from HBeAg to anti-HBe is usually associated with serum HBV DNA undetectable by hybridization technology and remission of liver disease. Nevertheless, a certain proportion of anti-HBe-positive patients continue to have HBV replication and active liver disease. These patients usually harbor precore/core promoter mutations in the HBV genome that prevent or decrease the production of HBeAg. The prevalence and clinical significance of HBeAg-negative chronic hepatitis B have been increasingly recognized in many Asian countries.

In acute HBV infection, HBeAg declines before HBsAg, then is replaced by anti-HBe. As anti-HBe increases, HBsAg starts to decline. Anti-HBs appears some months after the disappearance of HBsAg; detectable anti-HBsAg antibodies do not rise until about eight months after infection. The failure to detect anti-HBsAg early in infection is not because of a lack of the antibodies; instead, they are undetectable because they are complexed with the large amount of the antigen that is shed from infected cells. The period from about six to eight months when neither free HBsAg nor its antibody can be detected is known as the “HBsAg window”. This phenomenon also applies to HBeAg which is shed from infected cells, though to a much lesser extent; thus, the best tool for diagnosis of an acute HBV infection during the window is the presence of anti-HBc IgM. Anti-HBc appears early and all three antibodies remain in the blood for many years (figure 5) (31; 75; 6).

Chronic persistent HBs antigenemia may take two different courses. In both HBsAg remains in the blood for years but in chronic infection with late seroconversion, anti-HBe is eventually detectable, preceded by a decline in HBeAg. In chronic HBV infection without seroconversion, anti-HBe is not detectable, and the level of HBeAg remains high. In both types of chronic illness anti-HBc IgM is produced early followed by anti-HBc IgG (31).
Recent developments in the treatment of chronic hepatitis and several studies have led to interest in biomarkers for the prediction of virological response to antiviral therapy. Quantitative serology for HBsAg and HBeAg are two candidates for clinical utility (76).

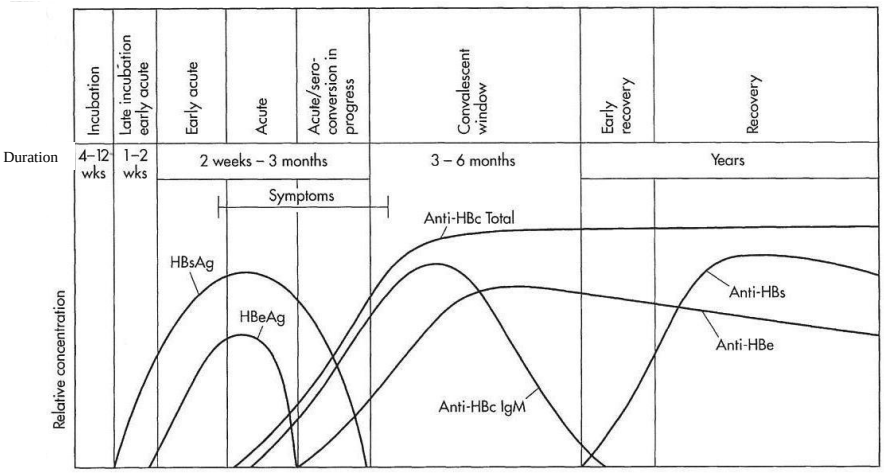


Figure 5. Hepatitis B diagnostic profile; serological profile in 75-85% of patients with acute hepatitis B (31).

Quantification of HBsAg was introduced more than 20 years ago but only recently it has been significantly improved by new automated quantitative assays (77). HBsAg appears useful to identify non responders; its secretion is highly dynamic and varies along chronic HBV infection both quantitatively and qualitatively. The variations of total serum HBsAg in the different phases of HBV infection proposes quantitative HBsAg as a new diagnostic tool for the characterization of HBV carriers and be very useful in clinical practice to define their specific condition (7).
HBsAg seroconversion is the ultimate laboratory marker of successful therapy for patients with chronic disease. Data from clinical trials suggest a role for quantification of HBsAg at baseline and/or on treatment in identifying patients most likely to achieve HBsAg seroconversion (78; 79).

HBeAg seroconversion is an established therapeutic endpoint for the management of HBeAg-positive CHB and is associated with reduced morbidity and mortality. HBeAg and HBsAg titers have both been proposed as surrogates for infected liver cell mass (61; 80).
Hepatitis B core antigen is an intracellular antigen that is not detectable in serum. Its antibody, anti-HBc, indicates a prior exposure to HBV, irrespective of the current HBsAg status. IgM anti-HBc is the first antibody detectable in acute HBV infection, which is usually detected within one month after HBsAg appearance. Thus, an acute infection can be distinguished from a chronic infection by the presence of antibodies (IgM) against HBcAg. The presence of IgM anti-HBc with high index value usually indicates a recent HBV infection and this antibody usually disappears within 6 months. However, the titration of IgM anti-HBc may increase to levels usually detectable in acute infection in 10-20% of chronic hepatitis B patients with acute exacerbation or hepatitis flare that often leads to a misdiagnosis of acute hepatitis B. IgG anti-HBc is not a neutralizing antibody and remains detectable throughout the patient's life. It can be found in patients recovered from acute hepatitis B and in those with chronic HBV infection. Of particular note is that isolated IgG anti-HBc can be seen in the window period of acute HBV infection, many years after recovery from hepatitis B or chronic HBV infection. This unique serological profile occurs in 1% of blood donors in low-prevalence areas and in 20% of those in endemic areas. Recent studies also indicated that isolated IgG anti-HBc may suggest an occult or silent HBV infection. Occult HBV infection is currently defined as the absence of circulating HBsAg in individuals positive for serum or tissue HBV DNA irrespective of other HBV serological markers (81; 82).
There are several clinical application of those markers:

- Screening to identify those at risk of spreading the disease (blood donors, pregnant women, health care workers, transplant donors and recipients, donors of semen, drug abusers);
- Differentiation of hepatitis B from other causes of hepatitis;
- Monitoring disease progression;
- Management of treatment of chronic hepatitis patients (31).

1.6. Antigenicity and escape mutants

Naturally occurring mutations have been identified in the structural and non structural genes. Although HBV is a DNA virus, it contains a polymerase without the proofreading activity. The mutation rate is 1.4 to 3.2 x 10^{-5} substitutions per site per year in chronic infections and

100-fold higher in the liver transplantation setting (83); because of the polymerase error rate (one error per 10^7 bases), in active infection 10^7 base-pairing errors can be generated per day over the 3200 base-pairs genome (42). Due to the low fidelity of the polymerase, the high replication rate and the overlapping reading frames, mutations occur throughout the genome including the immunodominant *"a"* determinant of the envelope (84).

The best characterized mutants are the pre-core stop codon mutations resulting in a loss of the "e" antigen resulting in an enhanced viral replication and mutations in the polymerase-reverse transcriptase genes conferring resistance to antivirals. Several mutations in the surface gene have been identified altering the antigenicity of the viral surface protein and the structure of the viral envelope (85).

Amino acids substitutions within the S gene involving the major antigenic *"a"* determinant of HBsAg, common among all subtypes, have been detected in cases of failure of immunization against the virus (8).

The 226 amino acids long HBs antigen is a membrane protein with four transmembrane domains; it consists of two pairs of type-specific determinants, "d/y" and "w/r" antigenic determinants and HBV is classified into subtypes or serotypes (*dw*, *yw*, *dr* e *yr*). All of them share a common "alpha" *"a"* determinant (86; 87), a surface conformational epitope located on the extra-membranous loop spanning 101-159 amino acids: this region contains eight cysteine residues which form disulphide bridges to maintain the correct conformation of the "a" loop and consists in a highly conformational epitope cluster (88). Some authors, using a combination of conformational peptides and phage display experiments, constructed a model of the *"a"* determinant: the key feature of this model includes a laminar loop stabilized by bonding between cysteine residues 108-138 with a finger projection stabilized by disulphide-bonded cysteine residues 121-124 (figure 6) (42).

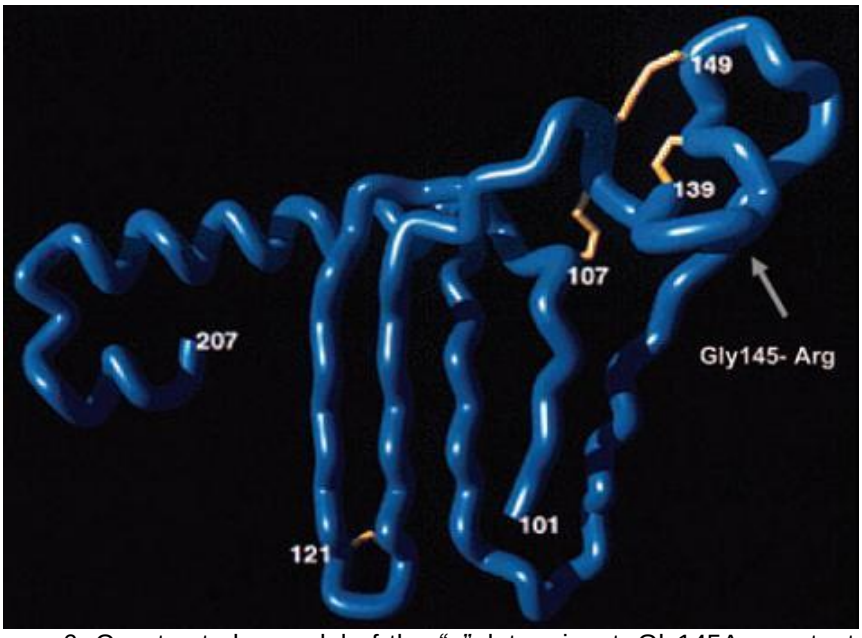


Figure 6. Constructed a model of the *"a"* determinant. Gly145Arg mutant is the projecting amino acid 139-147 antigenic loop of the *"a"* determinant (89).

Most of the antibodies that appear after natural infection and found in patients sera are specific for the epitopes localized between 124-147 amino acids (90; 91) even if there are several epitopes located on different regions of HBsAg particle (90). It is well documented that mutations onset in this region (amino acid substitutions, insertions or deletions) result in antigenic and immunogenic changes (87; 92; 93; 94; 95) and may enable the virus to escape the neutralizing antibodies through a conformational alteration which impairs the antibody-antigen interaction. Furthermore site-directed mutagenesis and amino acids replacement investigations (96) have identified important HBsAg residues. As a consequence there could be a decrease in the vaccine efficacy and the infection develops even in presence of anti-HBs antibodies ("vaccine escape mutant") (4). The hepatitis B virus vaccine consists of a yeast derived recombinant surface antigen (HBsAg) protein producing protection against 95% of the recipients. Antibodies induced by the vaccine are predominantly directed towards the *"a"* determinant (97; 98).
A large number of amino acid substitutions are found within the region of aa 124 to 147 : the most frequent substitutions are the sG145R, which is known to change the antigenicity and immunogenicity of HBsAg (87; 99) being the major vaccine and

immunological escape variant (94), and the sD144A, but many other substitutions have been described within the *"a"* determinant, in the HBs antigen outside of the *"a"* determinant sequence and in the pre-S region. As well, some published works suggest the amino acid residues at positions 120 to 123 may be also crucial for the antibodies recognition (100): the cysteine at 121 is important in maintaining the correct conformation through the formation of intramolecular disulphide bonds. The presence of Arg/Lys at position 122 is essential for HBsAg antigenicity since that position is known as an HBsAg subtype determinant (*ay/ad*) and substitutions in this position (e.g., K122I) were identified in some chronically infected patients (101; 102); similarly the substitution T123N has a great impact on antibodies recognition (88). However the natural isolated mutants have multiple mutations that may contribute synergistically to the HBsAg antigenicity.
Selection of HBs antigen mutants has been observed principally in two clinical settings, including vaccinated newborns of HBV infected mothers, and liver transplant recipients receiving human monoclonal anti-HBs antibodies. The HBV detection in pregnant women would reduce the virus transmission from mother to fetus, and the detection in blood donors would avoid the infection through the blood transfusions. Mutations of HBV S gene have been reported in infants born to carrier mothers despite an adequate anti-HBs response after vaccination (87; 103; 104) or in individuals after liver transplant or blood transfusions despite the presence in each case of anti HBs antibodies; in addition to these 'vaccine and immune escape mutants', 'diagnostic escape mutants' have been described since most of the HBsAg detection assays are based on anti-HBs antibodies (4; 8; 105), thus monoclonal antibodies against the wild-type "a" determinant used in conventional HBs antigen diagnostic methods fail to find occurring HBV infection (4; 91). Since many HBsAg immunoassays use monoclonal antibodies with epitopes directed against the major hydrophilic region, in particular against the *"a"* determinant, amino-acid mutation in this region may account for immunoassay false negative results (106).

1.7. Serological assays

1.7.1. Immunoassay overview

Immunoassays use reagents to generate a signal from a sample. They are very sensitive analytical tests based on the three important properties of the antibodies:

- Their ability to bind an extremely wide range of natural and chemical man-made molecules.
- The huge specificity and selectivity for the substances to which the antibody binds even in the presence of many other molecules with high concentration in a sample.
- The strength of the binding with their target.

In an immunoassay the antibodies are usually immobilized onto a solid surface, the so called solid phase. In the simplest type of immunoassay the antibody immobilized on the solid phase captures the analyte from the sample, named antigen, and a different antibody specific for another epitope of the antigen is used as a signal generation system. This antibody is labeled with a probe, which could be an enzyme (EIA), a chemiluminescent (CLIA) compound or a radioisotope, and called tracer (RIA).

After an incubation which allows the antibody to bind with the analyte, unbound labeled antibody is removed through a separation or washing step. Since the antibodies form a sandwich around the analyte, immunometric assays are also known as sandwich assays.

The HBsAg common immunometric assay is a direct solid-phase immunoassay (figure 7). Generally anti-HBs antibodies are attached to the surface of a solid support (microparticles, test tubes, microtiter wells); the solid phase antibodies are then utilize for HBsAg determination in serum or other fluids. If the HBsAg is present, it is bound to the solid phase via the immobilized antibodies after an incubation step followed by a washing step; in a second step a highly purified labeled probe (a second antibody) will bind to the free antigenic sites of each captured antigen. The measurement of the amount of the probed antibody gives a quantitative indication of the HBs antigen present in the serum specimen. This direct solid phase technique is generally applicable to the macromolecules and complex structure having two or more antibody binding sites. Similar assay have been constructed for HBcAg and HBeAg determination (31).

1.7.2. Molecular vs immunochemical assays

The development of serological assays to detect HBsAg has played a central role in the diagnosis of hepatitis B virus infection. The majority of acute as well as chronic infections are asymptomatic and simple and inexpensive methods that may be useful to discriminate acute from chronic hepatitis are needed. A diagnosis of acute or chronic HBV infection, past infection or successful vaccination can be determined. The quantification of HBV DNA provides to the monitoring of the effectiveness of antiviral therapy and detecting development of drug resistance (107). Molecular diagnostics are also

being applied to infected liver tissue; the use of molecular techniques to quantify intrahepatic HBV DNA and other replicative intermediates may provide additional informations for monitoring and predicting treatment efficacy. Recent improvement in molecular technology enabled the detection of as few as 10 copies/ml of HBV DNA in serum leading to redefinitions of chronic infection as well as the thresholds for antiviral therapies.
As the sensitivity of these molecular techniques continues to improve, the challenge will be to standardize these assays and define clinically the significant levels of HBV replication. However, the need to assess viral dynamics several times during the management of treatment makes the cost a challenge. Many studies with the aim to investigate if there is a correlation between quantitative HBsAg immunoassays (simple, sensitive, reproducible and inexpensive methods) and

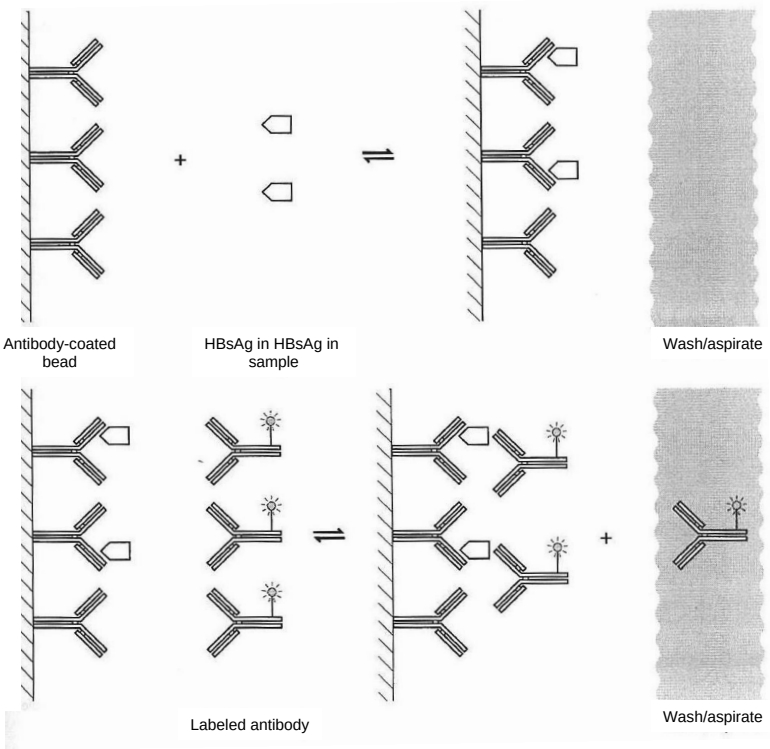


Figure 7. Immunometric assay for HBsAg (31).

HBV DNA levels have been performed to overcome this problem (108). A lot of data revealed and demonstrated a significant correlation of HBsAg serum concentration, detected through different automated chemiluminescent immunoassays, with HBV DNA levels. HBsAg quantitative measurement can be a surrogate of viral load during the management of chronic infection (109; 110). In contrast, the direct sequencing of HBV often does not enable to detect mutant HBV when it is found coexisting with the wild-type in a certain proportion. Usually, it is assumed that direct sequencing does not detect a second population if it represents <20% of the sample viral load. In this case the HBsAg immunoassay, due to its better sensitivity, could give a positive result by detecting this minor wild-type population (111).

The serological diagnosis of HBV has relied for many years on a combination of qualitative assay results. In chronic hepatitis quantitative testing of markers represents an attractive alternative to HBV-DNA determinations which are quantitative but a little less specific, fairly imprecise, difficult to standardize and too expensive. The possible role of quantitative determination of HBsAg has been prospected initially by Froesner in 1982 (112) who studied the concentration of HBsAg over time in patients with acute and chronic infections and concluded that in acute hepatitis B a reduction of at least 50% of levels was present in patients who recovered spontaneously. When the first automated quantitative assays were available other authors demonstrated the role of this marker also in the monitoring of chronic disease and in particular its predictive value on the outcome of interferon therapy (113).

Since few years ago HBsAg was used typically as a qualitative serological marker to diagnose an ongoing HBV infection; more recently, quantitative automated immunoassays have been developed and a wide range of concentrations has become available (110). Over the past years, assays for HBsAg detection have been improved to detect 0,1 ng HBsAg/ml of serum which means about 2 x 10^7 HBsAg particles/ml of serum (109). Usually the quantitation by automated instruments is based on a calibration curve standardized using the World Health Organization (WHO) standard and expressed in IU/ml (International Units) or the Paul-Ehrlich-Institut (PEI) standard.

The inability to detect HBsAg mutants using commercial assays has been identified as a critical problem and the ability to detect them has a critical role in the diagnosis of the disease.

Using recombinant mutation panels, some substitutions were not detected by some commercial kits and these assays failed also to detect the most frequent HBsAg mutation, G145R. Most of the new

generation assays (launched after 2000), using combination of monoclonal and polyclonal antibodies, can detect some of the common HBsAg mutations and someone also any rare mutation but there is the potential risk for a decreased analytical sensitivity for wild-type detection. However most of them, even using mixtures of monoclonal antibodies that recognize mutant as well as wild-type HBsAg can fail to detect multiple mutations (106). There are three categories of HBsAg mutant detection of assay performance: 1) mutants detected as well as wild-type, 2) mutants that cannot be detected by the assays and 3) intermediate cases in which recognition is significantly decreased than to the wild-type HBsAg (114). Immunoassays using combination of monoclonal and polyclonal antibodies fall in the first and third category.
The choice of the assay in the routine diagnosis should not be guided only by the ability to detect HBsAg mutants but also consider the sensitivity. Very low level of antigenemia are found under circumstances like: 1) window period of the primary infection, 2) low-level HBV carrier, 3) co-infection with HCV and/or HDV that may interfere with virus replication and/or HBsAg expression (115). An ideal HBsAg test should have high sensitivity for the early primary infection, for all HBV genotypes and for mutants recognition; therefore the analytical sensitivity is considered the major criteria for the assay selection for routine diagnosis.

1.7.2.1. Confirmatory HBsAg assay

Generally, the immunoassay protocols foresee that samples deemed reactive for HBsAg must be repeated (116). False-positive HBsAg are not frequently found when performing solid phase immunoassay. HBsAg presence is well established by confirmatory neutralization tests in which the percentage of signal reduction is measured (117) utilizing high-titered human anti-HBs for this purpose. The HBsAg screening is repeated in presence and in absence in parallel of these antibodies. There are two basic confirmation procedures, a first step neutralization and a second step neutralization. The sample is treated with two buffers, with and without anti-HBs: if the sample results positive again with both treatment this means it is a false-positive. If the sample is too much positive a second treatment follows with sample dilution.
Weakly reactive samples which do not result negative at the confirmatory tests should be considered as negative: to be sure of this, these samples should be investigated in other ways as HBV viral load and other serological markers to understand the infectious status and to provide a clearer picture of the patient's health otherwise they

could be false-negative samples. Laboratories performing HBsAg tests should determine the significance of the weak reactive assay results looking first of all at the whole profile of the HBV tests.

2. Chemiluminescence principles and immunoassays

Luminescence spectrometry has become a firmly established and widely employed branch of analytical chemistry applied to qualitative and quantitative analytical detections. Bioluminescence and chemiluminescence (CL) present many advantages including low limits of detection (for highly luminescent molecules) and applicability to complex samples without using hazardous labels (e.g., radioisotopes) (118).

2.1. Enzyme labels

Enzymes are now used more widely than any other type of label. They are measurable at very low concentrations by utilizing their catalytic properties to generate colored, fluorescent or luminescent compounds. The main disadvantage of enzyme immunoassays (EIAs) is that they have susceptibility to interferences and assay conditions. The signal generation step must be controlled, optimized and kept free from interferences; the enzyme-substrate incubation is sensitive to time, temperature, pH and inhibitory substances (31).
Well designed fluorimetric EIAs are generally more sensitive than the colorimetric ones but their sensitivity could be limited by interference from background fluorescence and quenching effects.
Chemiluminescent immunoassays depend on the use of luminescent compound which emits light during the course of a chemical reaction (31). Unlike fluorescence there is no incidence light and the signal emanates only from the luminescent molecules. Unlike the radioactive disintegration all the emitted photons can be triggered in very short times. Luminescent immunoassays have achieved sensitivity levels several orders of magnitude better than radioisotopic and fluorimetric assays.

2.2. Chemiluminescence

Most enzyme mediated luminescent reactions have low quantum yields producing weak light emission that rapidly decays. The addition of another chemical can enhance the light output. For example the horseradish peroxidase in the presence of hydrogen peroxide causes the oxidation of luminol with emission of light (31).
Chemiluminescent reactions generally yield a product in an electronically excited state which reverts to the ground state with the emission of photons. These reactions are oxidative, requiring

oxidants such as oxygen or hydrogen peroxide, and are biphasic: there is a rapid initial phase in which the excited state is populated and the rate dependent on reagent concentration. This is followed by a decay phase characteristic of the chemiluminescent derivative (118).

Chemiluminescent emission intensities are sensitive to some environmental factors as temperature, pH, ionic strength and other species present in the system. For many CL systems there is a low background level even in absence of labeled molecule: the absence of a high background signal, as in absorption spectroscopy for example, improves the detection limit.

There exists a wide variety of chemiluminescent labels and reactions used for analytical and clinical purposes in immunoassays as luminol which in presence of a catalyst reacts with hydrogen peroxide in alkaline solutions to emit light of about 425-435 nm; this molecule and its derivatives such isoluminol and N-(4-aminobutyl)-N-ethylisoluminol (ABEI) are applied to label compounds with carboxylic acids or amines as amino acids as well as bis-acridinium salts (119).

Some constraints arise from coupling the chemiluminescent molecules to analytes or antibodies in a way that the label properties are not affected. Usually the conjugated derivatives have less than 1% of luminescence of the native molecule. In particular the conjugation of luminol with proteins can drastically affect the quantum yield of the reaction while with isoluminol derivatives as ABEI, which can be coupled to molecules with low molecular weight, there is few loss in quantum yield. Due to this, ABEI and related molecules have been used as labels for different immunoassays including steroids immunoassays with a degree of performance comparable to the radioimmunoassay (RIA) (120; 121).

A universal problem of these methods remains the signal quenching when the label is coupled with proteins. The unique advantage of radioactive labels is that the quantitative determination is unaffected by their environment: luminescent molecules are susceptible to quenching by biological fluids as serum. This problem has been in part avoided by using the solid phase antibody system which enables the separation of the formed immunocomplex from the unbound material through some washing steps eliminating the interfering compounds before the signal detection.

Immunoassays are become centrally important in the analysis of drugs, hormones, pesticides and proteins; immunoanalytical methods are based upon the reaction between a labeled analyte/antibody highly specific for antibodies/analytes. Analysis is done by measuring chemical or physical properties associated with the label thus enabling the construction of a standard curve which gives back a

measured signal as a function of the labeled species concentration. Unknown ligand concentrations are extrapolated from this calibration curve (122).
The essential components required for a chemiluminescence reaction are the chemiluminescent substrate, oxidants, inorganic ions and a catalyst. To be used as a chemiluminescent label a molecule must satisfy some requirements: to be attachable to antigens or antibodies, to form stable conjugates until the reaction is triggered, to not significantly alter the physical-chemical properties of the attached molecule in particular its immunological activity.
The luminescence could be glow or flash of light: in the first case the emission persists for hours whereas the second type foresees the emission to take place in few seconds. Generally the glow chemiluminescence is produced by enzymes, e.g., horseradish peroxidase or alkaline phosphatase; the enzyme can directly act on the chemiluminescent molecule which behaves as substrate, providing the breakage between a phosphate group and the molecule activating it in its excited state.
In the luminescent reaction enzymes can indirectly act on the target molecule catalyzing a reaction between it and another chemical as oxygen or hydrogen peroxide as it occurs in the flash chemiluminescence. The most used molecules in the flash chemiluminescence are acridinium esters and isoluminol.
The luminol does not present an high chemiluminescent efficiency; nevertheless, it has been observed that the employment of aromatic compounds in the mixture with the enzyme enhanced the light emission. In the luminol-HRP system this enhanced chemiluminescence provides a light emission 1000 fold higher in intensity than in absence of enhancers and a background reduction up to 90%.
The chemiluminescence based immunoassays are a good alternative to radioimmunoassay without less performance, especially in the field of clinical chemistry: its feasibility has been demonstrated for a range of both high and low molecular weight analytes. Moreover the development of immunochemiluminometric assay based on labeled antibodies has the potential to increase and improve the assay sensitivity in the detection and monitoring of infective agents as well as tumor markers (123; 118).

2.2.1. The LIAISON® system

The Liaison® system is a fully automated instrument designed to perform immunometric analyses of biological fluid samples (such as serum or plasma) employing the chemiluminescence phenomenon

(figure 8). Up to 15 different tests can be performed at once on up to 144 samples in a sequential or random access mode. The output of the analysis is generated through the formation of an immune complex, followed by a chemiluminescent reaction that produces an emission of light.

Figure 8. The Liaison® system.

The instrument is composed of two modules, designed to be both allocated on a single workbench (figure 9); the first module is a personal computer with touch screen, that hosts the user interface software and all the system data (assay protocols, reagent cartridges database, output of analyses, calibration history, network controls etc.). The second module is the actual analyzer, that performs the analysis from sample loading all the way to the final output for the user. Key components of the analyzer include:

- ✓ Cuvette loader and stacker: two conveyor belts allow continuous loading of the reaction modules that are stored on a multilevel rack (7 levels).
- ✓ Sample rack slots: in the left-hand part of the instrument, a storage area can hold up to 12 sample racks, each carrying up to 12 samples. A barcode reader avoids error in cataloguing of samples.
- ✓ Reagent slots: in the right-hand part of the instrument, another storage area can hold up to 15 different reagent cartridge simultaneously. This area is kept at a constant temperature of 15°C for optimal conservation of the reagents, while a stirring device keeps always the paramagnetic beads used as solid

phase in homogenous suspension. Barcode reader for cartridge identification is also present.

- ✓ Robot dispensation arms: two robotic arms each carrying a dispensing needle are present. The left arm is usually dedicated to sample dispensation while the right one to the reagents. Each one has a separate washing well to clean the needle after each dispensation.
- ✓ Incubator: this area hosts the reaction cuvettes at a constant temperature of 37°C during the incubation steps.
- ✓ Washing station: through the application of a magnetic field, this part allows the retention of the paramagnetic beads and the removal of the "supernatant". Different number of washing steps can be set.
- ✓ Signal measuring area: it contains the injection devices for the trigger reagents and the chemiluminescence detection system with a photomultiplier tube.

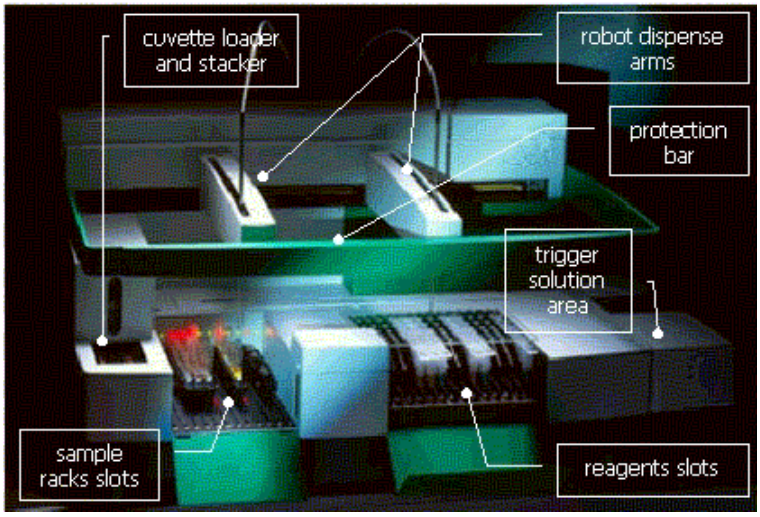


Figure 9. The Liaison® analyzer components.

To analyze samples, the Liaison® platform is based on two key features: the use of paramagnetic micro particles bound to immunoreactive species as solid phase (figure 10) and the generation of signal by means of a chemiluminescent probe. The adoption of micro beads as solid phase instead of the classic immunoassay supports, as the ELISA microwells, gives a clear edge in terms of available reaction surface, which in turn increases the kinetic rates of the antigen-antibody complex formation. Moreover, diffusion of both

the analyte and the solid phase in the reaction volume is allowed, while in ELISA system only the analyte can diffuse, decreasing the possibility of the immune complex formation. The micro beads adopted in the Liaison® based immunoassays are colloidal particles composed of a ferric oxide core covered by a polystyrene layer formed by spontaneous coalescence of polystyrene linear chains. This structure is in turn coated with another layer composed of polyurethane activated with tosyl- groups. The tosyl- group (4-toluenesulfonyl chloride) can undergo nucleophilic attack, allowing the beads to covalently bind proteins through their available aminic groups (e.g., ε-amino groups of lysines, N-terminal end) (figure 10). The paramagnetic properties of these micro particles allow easy manipulation through the application of a magnetic field. The particles respond to a magnet but they are not magnetic by their own and do not retain any residual magnetism after removal of the magnet.
The tracer molecule is an antigen or antibody conjugated to a signal generating compound. Chemiluminescent tracers are formed by conjugating the antibody (or antigen) to a molecule that can generate a photon emission upon addition of trigger reagents. The entity of this photon emission is measured with a luminometer, usually equipped with a photomultiplier tube.

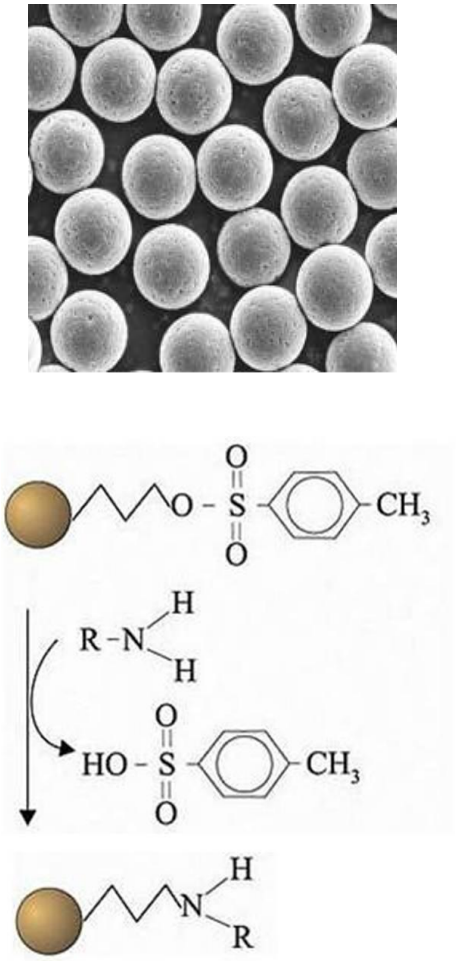


Figure 10. Paramagnetic micro beads are functionalized with a tosyl-group, to allow binding on amino-groups of proteins.

The chemiluminescent molecule used in the Liaison® assays is the luminol derivative ABEI (N-(4-Amino-Butyl)-N-Ethyl-Isoluminol), which is converted to its activated ester to allow conjugation with the antibody or antigen (figure 11). The amino-butyl group acts as spacer allowing the binding reaction with the protein to be probed; moreover the spacer, increasing the distance from the protein, enables the molecule to be more available to the emission reaction. Without ethyl and amino-butyl groups the generated luminescence would be much lower.

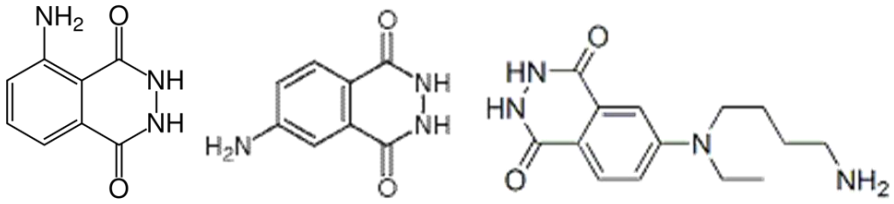


Figure 11. Luminol, isoluminol and ABEI.

In a basic environment and in presence of a microperoxidase (deuteroferriheme), the ABEI molecule reacts with H_20_2 reaching an excited state; this reaction causes the breakage between two N atoms delivering the bond energy as light signal detectable at 420-425 nm (figure 12). The light signal is measured and integrated in just 3 seconds time ("flash" chemiluminescence) (figure 13) by a photomultiplier as relative light units (RLU) and will be related to the tracer amount bound to the immunological complex formed during the reaction.

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

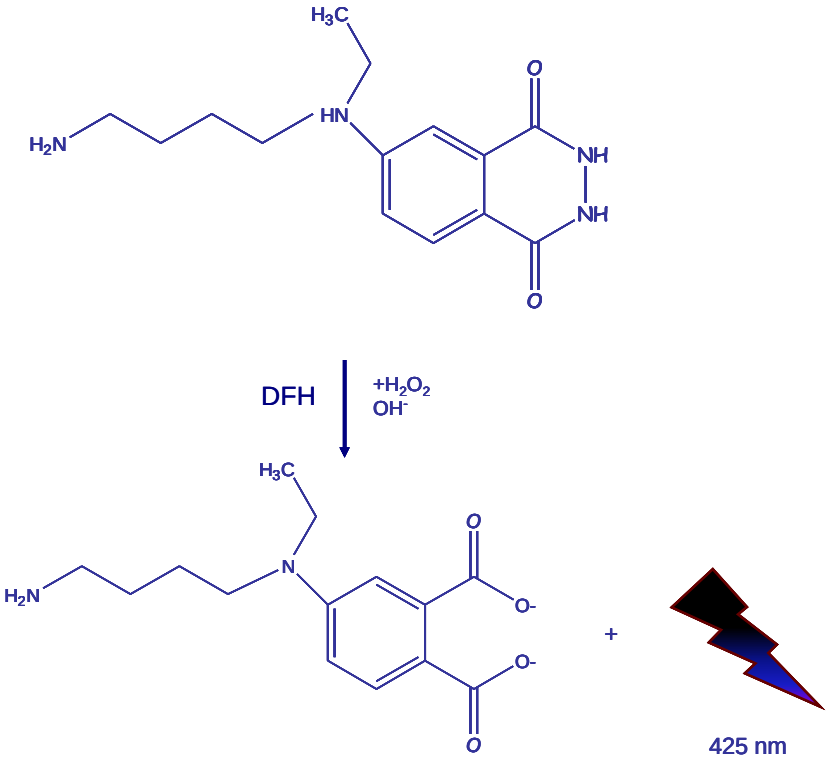


Figure 12. Chemiluminescence reaction of ABEI.

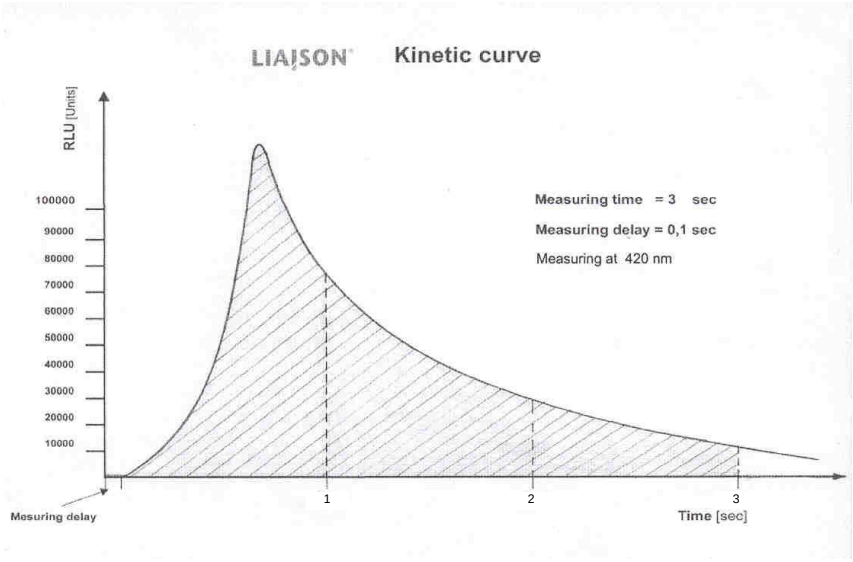


Figure 13. Flash chemiluminescence signal integration in the Liaison® system.

Aim of the work

As mentioned in detail in the introduction, the hepatitis B virus infection is largely diffused worldwide and the HBV detection at the early stage of the infection would enable 1) a promptly treatment of the disease but most importantly 2) would reduce the infection transmission in pregnant women and 3) the detection in blood donors would avoid the infection through the blood transfusions. This could be done by continuously improving the specificity and the sensitivity of the already marketed diagnostic kits and particularly by developing their ability to detect also the mutant species of the hepatitis B virus. These mutants are not responsive to the vaccine so the disease develops even in presence of anti-HBsAg antibodies and it becomes so clear the necessity to diagnose also these escape species. Up to now, as most of the available diagnostic kits foresees the use of monoclonal or polyclonal antibodies directed against the most common and antigenic as well as the mutation prone loop of the "a" determinant, some of the commonly found single or multiple mutations are not detected by a lot of commercial immunoassays.

For these purposes, in the present project some innovative bio-reagents have been investigated to obtain, always for the Liaison® platform, a novel quantitative prototype kit improved than the already marketed semi-quantitative DiaSorin HBsAg Liaison® kit (DiaSorin S.p.A., Saluggia, Italy) in collaboration with Advanced Life Science Institute Inc. (Saitama, Japan), the chemistry laboratory of the Department of Biotechnologies and Biosciences at Milano-Bicocca University and the immunology laboratory of the Department of Medical Sciences at Novara University.

In particular in this work, 1) some monoclonal antibodies have been selected in order to recognize as well the wild-type as the mutant species of HBV, 2) the production of a panel of the most common HBsAg recombinant mutant species (from literature collection and competitors analyses) has been carried out, 3) a novel ABEI-derivative chemiluminescent molecule has been evaluated to provide a better performance and 4) the comparison of the prototype with the marketed DiaSorin HBsAg Liaison® kit and other competitors kits has been carried out.

To allow this prototype to be launched, validation studies have been performed in accordance to the WHO and/or PEI HBsAg Ad and Ay serotypes sensitivity panel as reference.

Materials and methods

3.1. ALSI monoclonal antibodies and ALSI buffer

Seven mouse monoclonal antibodies were produced from ALSI institute (Saitama, Japan) according to their patented manufacturer procedures (91).
Peptides representing epitopes of the surface antigen, containing at least an epitope of amino acid sequence corresponding to the positions 26 to 80 in HBs antigen in the inside of a lipid bilayer, have been obtained by recombinant gene technology and used as immunizing agents for BALB/c mice. When the titer of antibodies produced by the host is increased, a final immunization is carried out and then the spleen excised. The spleen cells are fused with mouse myeloma cells to give hybridomas. Antibodies produced are specifically selected against the antigen and cloned. The screening of the antibodies to be cloned is made in presence of a denaturant buffer containing a strong surfactant able to destroy the lipid bilayer of the HBV particles (the ALSI buffer). Monoclonal antibodies resistant to the denaturation action of the buffer can be obtained.
Antibodies for the solid phase have been digested to Fab molecules and biotinylated while the other for the tracer are maintained as whole IgG.
The ALSI buffer, used to select the monoclonal antibodies and capable to destroy the lipid bilayer of viral particles or the protein aggregates, is a 100 mM Tris-HCl protein denaturant due to the combination of denaturating agents, surfactants and detergents at pH 7.

3.1.1. Solid phase preparation

Dynabeads® M-280 tosylactivated paramagnetic microbeads (Invitrogen, Oslo, Norway) are coated with 150-200 µg/ml biotin-BSA in 10 mM PBS buffer pH 7.4 at 1% beads concentration for 18-24 hours at 37°C; the following step foresees their incubation with 50-100 µg/ml streptavidin in 10 mM PBS buffer pH 7.4 always at 1% beads concentration for 3 hours at 37°C. The streptavidinated beads are now ready for the biotin-Fab coatings according to manufacturer procedures.
Single biotin-Fab coating occurs at 20 µg/ml in 10 mM PBS buffer pH 7.4 at 1% streptavidinated beads concentration for 18-24 hours at 37°C; the post-coating (or blocking) step follows with 0.1% biotin-BSA

in 10 mM PBS buffer pH 7.4 for 5 hours at 37°C. After that the beads are washed, resuspended and finally diluted to 0.25%, the usual working concentration, in the storage buffer (10 mM PBS buffer pH 7.4).
The final mix of the three single coated Fabs occurs by adding the three separated coatings at 0.25% in a ratio 1:1:1 (each Fabs is also diluted 1:3 with the storage buffer and tested as control).
The co-coating of the pre-mix Fabs solution occurs as the single Fab procedure but using concentrations of 20 and 40 µg/ml of the mixture at 1-2% streptavidinated beads. Different final coated beads dilutions have been tested from 0.25% to 0.125%.

3.1.2. Tracer preparation

The antibodies conjugation with dextran-BSA-ABEI or simply with ABEI or ABEI derivative to give the tracer is done according to ALSI and DiaSorin manufacturer procedures.
The ABEI molecule is purchased from Sigma-Aldrich while the ABEI derivative is synthesized by the chemistry laboratory of Milano-Bicocca University and the procedure will be patented soon.
Different molar ratio and incorporation factors have been tried: for the Japanese conjugates the ratio dextran: BSA refers to moles BSA per moles of dextran and BSA: Fab equal to 1:1 refers to their weights. The ABEI 10-20-30x refers to 10-20-30 ABEI molecules per moles of BSA.
The different incorporation factors of simple ABEI and ABEI derivative, varying from 30x to 10x, refer to ABEI or ABEI derivative molecules per moles of antibodies.
After the ABEI-ABEI derivative conjugation the tracer is then passed through a semi-preparative HPLC column (HiLoad Superdex 200 prep grade xk 16/60, GE Healthcare), collected and diluted to 20 ug/mL with dilution buffer (10 mM PBS, BSA 0.1%, NaN_3 0.1%). This sample is then ready to dilute at the desired concentration in the conjugate buffer (whose final formulation is 10 mM PBS, 15% BSA, 0.03% PVP, 0.01% Tween, 200 µg/ml mouse IgG, 10% human serum, 5% bovine serum, 5% sheep serum and other preservatives and antifoam reagents at pH 7.4).

3.2. Samples

HBsAg negative sera from blood donors are obtained from the hospital of Lille (France) while the routine panel from the hospital of Chivasso (Italy).

The positive sera and the standard curves derive from DiaSorin QC department in Saluggia (Italy).
The sensitivity panel PHA-08 and the sample NIBS 00/588 are from Boston Biomedica Inc. (Boston, USA).
The mutant samples of DiaSorin panel are from the immunology laboratory of Novara University; the native and other mutant panels are from DiaSorin QC department in Saluggia (Italy).

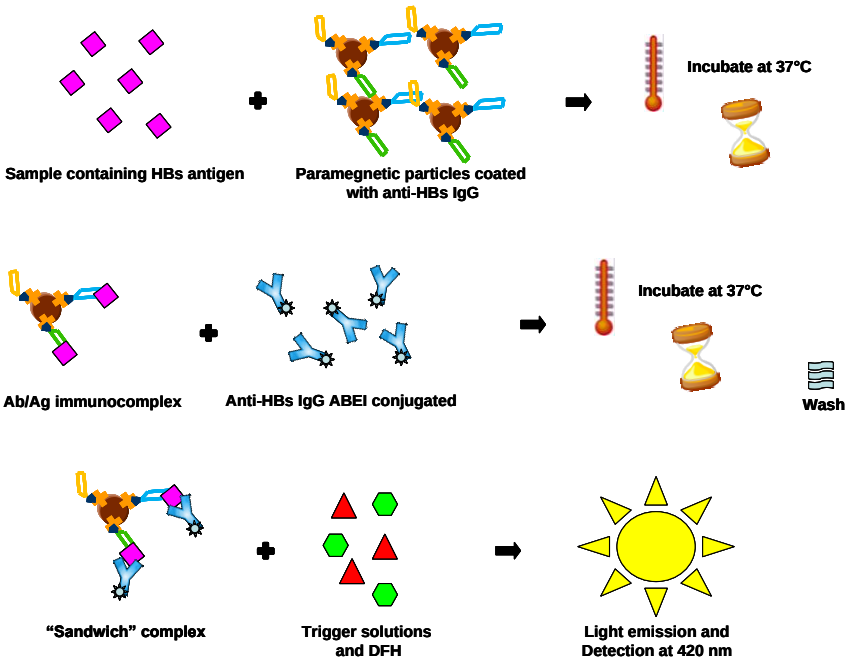
3.2.1. Mutant panel

Surface antigen gene sequences containing defined mutations were cloned in the pcDNA3.1(+) vector designed for expression in mammalian cells. Inserted genes were verified by sequencing and the expression vector used to transiently transfect human HeLa/293T cells according to Novara university procedures. The presence of recombinant protein was evaluated on both fresh culture supernatant and following lyophilization and analyzed with the ETI-MAK 4 ELISA assay (DiaSorin,Saluggia), a number of commercial assays and the novel prototype assay developed in-house.

3.3. Liaison® immunoassay

The assay format will involve the quantitative HBs antigen determination through the fully automated Liaison® platform (DiaSorin, Saluggia, Italy) which employs the chemiluminescence phenomenon. The light signal is measured by a photomultiplier as relative light units (RLU) and will be related to the tracer amount bound to the immunological complex formed during the reaction.
The marketed DiaSorin kit has been employed as reference and its sequences of the immunochemical reactions and their parameters (the protocol assay or method file) were used at the beginning as shown in scheme 1. The commercial 310100 HBsAg kit (DiaSorin, Saluggia, Italy) foresees a direct, sequential one step assay format.
During the first incubation (5 TIC), HBsAg present in samples (150µl) binds to the solid phase (0.25%, 20µl). During the second incubation (10 TIC), the antibodies for conjugate (200µl of 800 ng/ml sheep anti-HBsAg ABEI-IgG 35x) react with HBsAg already bound to the solid phase. After each incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents (hydrogen peroxide and 800ng/ml deuteroferriheme in 1M NaOH) are added and a flash chemiluminescence reaction is thus induced. The light signal, and hence the amount of conjugate, is measured by a photomultiplier as relative light units (RLU) and is directly proportional to HBs concentration present in calibrators, samples and controls.

To achieve a quantitative antigen determination, a two steps protocols was then used by adding another wash after the first incubation to the protocol in the scheme 1 and adjusting the incubation times. In the first step the sample (150µl) is allowed to react with the solid phase coated with a mixture of three monoclonal antibodies against the HBs antigen. In the reaction mixture the ALSI buffer (30µl) is added to dissolve the lipid bilayer constituent the envelope allowing the transmembrane region to react with the antibodies. At the end of incubation a wash cycle removes the unbound material. A second incubation in which the tracer with or without ALSI buffer is added follows. The tracer reacts with the antigen present in the previous formed immuno complex. A second wash cycle is needed before measuring the light signal in a way that it could be directly related to the total amount of antigen present in the sample.



Scheme 1. The sequential one step protocol of the 310100 HBsAg DiaSorin kit.

Results

HBsAg quantification has been demonstrated to be helpful to evaluate the response to treatment and for the early detection of drug-resistant strains, in addition to HBV DNA quantification.
The Liaison HBsAg assay to be product will be a chemiluminescent immunoassay (CLIA) for the quantitative detection of HBsAg in human serum and plasma. The production of a more specific and sensitive assay able to detect mutants species is at the basis of this work.

4. Comparison between DiaSorin Commercial HBsAg Kit and first prototypes: "different raw materials"

In a first set of experiments all the reagents purchased or made in collaboration with ALSI were tested to provide a starting point of the new prototype development. The marketed DiaSorin kit has been employed as reference and its sequences of the immunochemical reactions and their parameters (the protocol assay or method file) were used.
The commercial kit foresees a direct, sequential one step assay format as shown in materials and method paragraph 3.3. scheme 1.

4.1. Solid phase

The first differences consist in the employment of different bio-reagents starting from the antibodies immobilized onto the solid phase: in the commercial kit a mixture of three mouse monoclonal antibodies, having balanced reactivity for ad and ay subtypes, are immobilized but only one of them has been characterized with an epitope mapping. It is the 4B5 monoclonal antibody which is directed against the linear sequence between residues 141-146. The other two antibodies are called 3A3 and H2 and are supposed to map a region of the "a" loop and in any case an external accessible region of the antigen.
The antibodies utilized for the prototype solid phase are produced by ALSI institute and are the following (figure 14):

IgG ID	*Use*	*a.a. epitope*	*HBs region*
5C3	Solid Phase	124-147 conformational	"a loop"
121	Solid Phase	151-170 linear	Transmembrane
163	Solid Phase	31-50 linear	Inner hydrophilic

Figure 14. ALSI monoclonal antibodies used for the solid phase with epitope and HBs region recognized.

The first one is directed against a conformational sequence inside the "a" loop region, the second one against a transmembrane region and the last one against a linear epitope positioned in the inner hydrophilic portion of the viral particle. First of all these antibodies should be tested to verify if they can replace the current ones.
In order to achieve an optimal performance of the solid phase the antibodies to be coated are treated with pepsin to obtain Fab(2) fragments; these are divided through a chromatographic separation and finally biotinylated. The necessity to develop such a complex process araised from
the fact that the direct coating of the whole IgG determined a sub-optimal performance. The actual current kit foresees biotinylated Fabs as well.
The coating of each single biotinylated Fab (indicated as Fab1 Fab2 and Fab3 for simplicity) at 20 μg/ml, employing streptavidinated beads with 50 μg/ml of streptavidin, has been carried out and tested alone or in presence of the other two Fabs; also the contemporary coating (co-coating) of a mixture of the three Fabs together has been tested. The beads concentration during the coating is 1% and the final working concentration is 0.25%. The commercial kit has been always tested as reference for the performance comparison.
As shown in figure 15 the Fab 1 alone has the reactivity comparable to the coating of the commercial kit and it is enough to reach the same performance; Fab 2 and Fab 3 in these conditions show low reactivity. The best performance, in which an additive effect of each Fab is observed, is reached by coating the three pre-mixed Fabs together in a stoichiometric way (20 μg/ml referred to the final mixture coated).
To explain the lack of reactivity of Fabs 2 and 3 we should consider

that they are selected against hide regions of HBs to enable the detection of mutants. In normal conditions, such as for the commercial assay, they are not able to interact with their own epitopes because these found inside the lipid bilayers of HBV particles or are masked by the protein aggregation among HBs antigens in small spherical and tubular particles (91).
ALSI institute patented a particular buffer for the sample dilution to dissolve the lipid bilayer constituent the envelope (figure 16) allowing the transmembrane region to react with the antibodies and the aggregated protein antigens to be disrupted. ALSI sample diluent has been formulated for the new prototype kit; this assay buffer is optimized in order to expose internal epitope of HBs antigen.

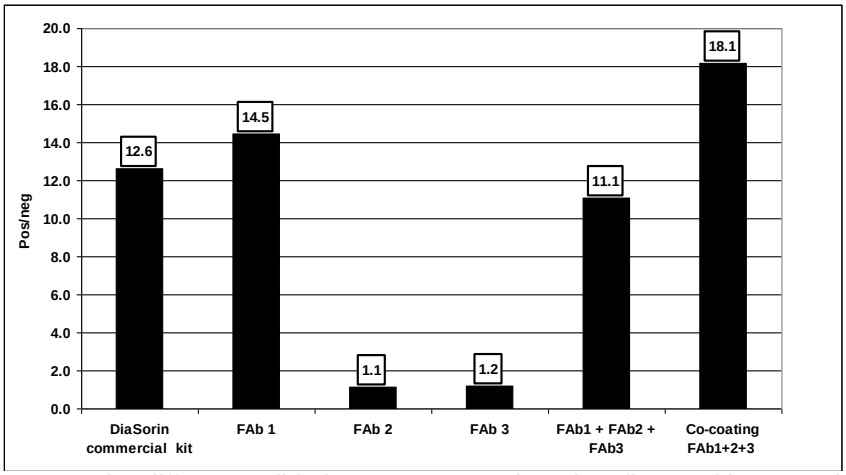


Figure 15. The different solid phases are tested against five positive samples with ~1 IU/ml concentration of HBsAg and five negative samples. Positive to negative ratio has been calculated from a mean of two replicates for each sample. The single coating concentrations are 20µg/ml for each Fab and 20µg/ml for the total pre-mixed Fabs solution.

Among the first preliminary experiments the effect of this new buffer was tested and compared with the commercial one. The results show (table 2) that it increases the sensitivity even of the commercial kit because displaying the entire denaturated antigen the anti-HBs immobilized on the solid phases and present in the tracer can bind to other sites previously masked by the viral envelope increasing the amount of detection.

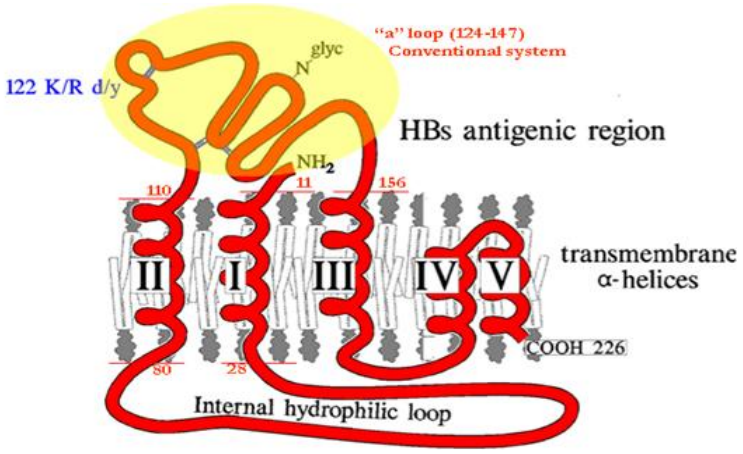


Figure 16. Mouse monoclonal antibodies are able to detect I/V inner region if combined with the use of a complex detergent mixture. The use of detergents in the assay buffer renders the approach manifest.

4.1.1. MP Stability

The solid phase, as the other reagents, must respond to some stability requirements to be employed in a kit; for the preliminary screening of the prototype reagents, accelerated heat stress stabilities are generally performed.
The PMPs reactivity has been tested after their storage at 37° and 45° C for three and five days and signals have been compared with the beads stored at 4°C. The average of positive samples at 1 IU/ml with the standard deviation after five days of storage at the three temperatures has been reported (figure 17) with the loss of signal referred to the signal at 4°C and expressed in percentage. Generally the acceptance limits are a loss of reactivity up to 20% at 37° and up to 30% at 45°C. In this particular case the solid phase shows to be highly stable; a longer stability will be carried out during the optimization and validation phases when the final prototype is developed.
This is the starting point to find out a new prototype which will be better than the already marketed HBsAg kit. The following experiments will maintain the best solid phase found up to now, the co-coating of a mixture of three Fabs with a 1:1:1 ratio, and the ALSI buffer as sample diluent. The variations in the reagents formulation, volume and incubation time will be varied to improve the

immunochemical reactions once the suitable prototypes will be obtained.

	Commercial buffer	ALSI buffer
pos / neg	18.1	24.1

Table 2. Improving effect of the ALSI sample diluent testing the same positive and negative samples. The mean of positive and negative values are calculated and the positive to negative ratio has been reported for both buffers.

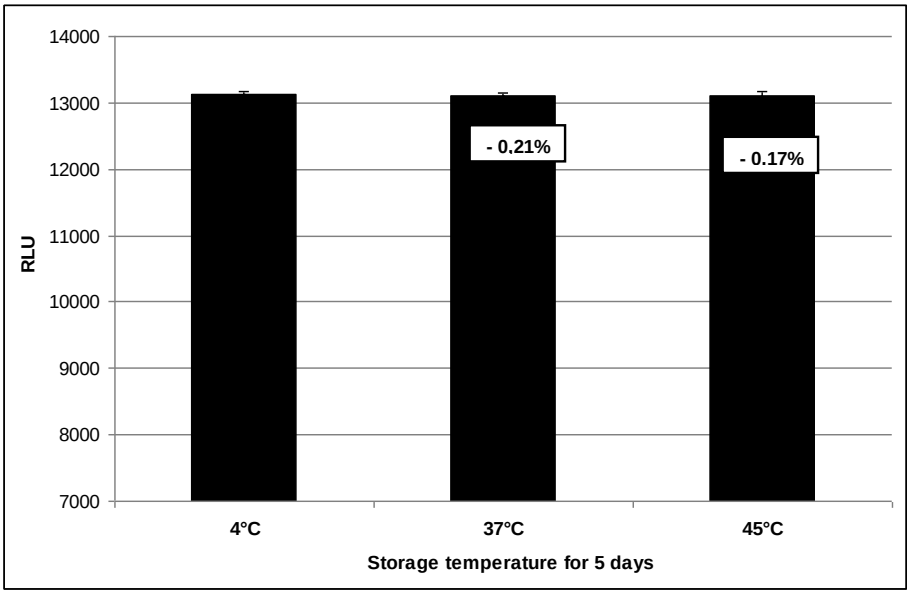


Figure 17. Magnetic particles stability measured over the five positive samples with ~1IU/ml concentration of HBsAg. The mean of three replicates of each sample with the relative standard deviation has been reported.

4.2. Tracer

The commercial kit is using sheep polyclonal antibodies to HBsAg having balanced reactivity for ad and ay subtypes and directly conjugated to ABEI molecules as tracer. The working concentration is 800 ng/ml with 35x ABEI ratio (35 ABEI molecules per antibody mol).
For the new prototype ALSI provided four mouse monoclonal antibodies to employ as tracer as shown in figure 18 below:

IgG ID	*Use*	*a.a. epitope*	*HBs region*
124	Conjugate	124-147 conformational	"a loop"
320	Conjugate	124-147 conformational	"a loop"
128	Conjugate	121-130 linear	Surface (Mainly conserved)
111	Conjugate	61-69 linear	Inner hydrophilic

Figure 18. ALSI monoclonal antibodies used as conjugated tracer with epitope and HBs region recognized.

The first two antibodies are directed against the same conformational epitope present in the "a" loop region recognized also by the first Fab of the solid phase to gain an additive detection; the third one is against the mainly conserved surface region and the last one against a linear epitope positioned in the inner hydrophilic portion of the viral particle.
Due to the difficulties of the current available DiaSorin about the conjugation technology for HbsAg conjugate, which is the weak technological point of current kit (too much polyclonal consumption with too much ABEI ratio), the need to investigate new conjugation technologies for the new prototype arised. In order to increase the performances different strategies were evaluated:

a) The use of complex matrix as carriers of ABEI and IgGs / FAbs. With these matrixes ABEI incorporation should be easily increased without the direct ABEI conjugation on monoclonals. This technology is part of ALSI know how. The ALSI approach in synthesizing the tracer is different from Diasorin manufacturing procedure; some different lots of dextran-BSA-Fabs ABEI tracers differing for dextran molecular weight, BSA and Fabs ratios and ABEI incorporation factors have been produced and tested.

b) The use of ABEI derivatives that can improve conjugate solubility and reduce aggregation. The use of purer and more

soluble ABEI derivates can reflect lower incorporation factor increasing conjugate solubility.

c) The use of new chemiluminescent derivates of isoluminol.

At first the dextran matrix has been employed as scaffold to link BSA, antibodies and ABEI (figure 19). The four antibodies have been attached as Fabs to reduce a further steric hindrance. The first conjugates are made by fixing the molar ratios dextran-BSA 1: 5 (5 moles of BSA per mole of dextran) and BSA-Fabs 1:1 weight referred to BSA. The only variable studied was the ABEI incorporation factor 10x, 20x and 30x referred to BSA moles.
All available tracers have been run with three different concentrations (1000, 500, 250 ng/mL). In figure 20 only the most acceptable results for further investigations have been shown. 30X ABEI conjugate reaches higher RLU response for positive samples, but the RLU of negative samples are higher than 1000 RLU and hazardous intra-assay cv% has been observed. 20X ABEI conjugate shows lower RLU response for positive samples (70-80%) and normal sera (50-80%) with a higher ratio between positive and negative samples. Considering also cv% response this tracer seems the current best option, with working concentration equal to 500ng/mL. 10X ABEI conjugate seems able to reach the same reactivity of 20x ABEI conjugate but with higher (double) working concentration.

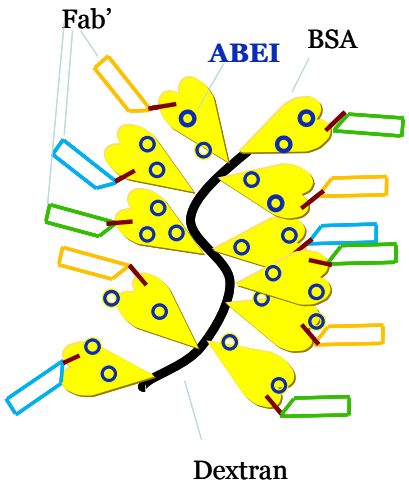


Figure 19. Dextran-BSA-ABEI-Fabs (DBAF) complex conjugate.

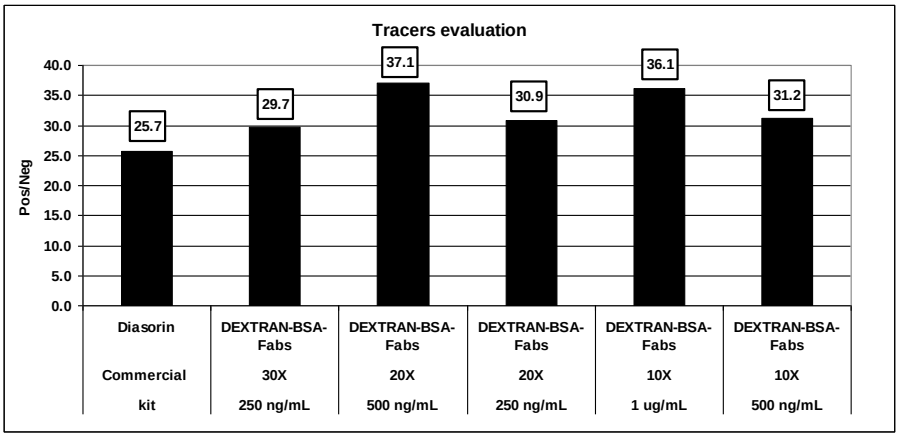


Figure 20. First screening of conjugates tested over positive samples at 1 IU/ml (n=5) and negative samples (n=5). The mean of positive and negative values are calculated and the positive to negative ratio has been reported.

This first approach has been done by using a basal buffer composition and a preliminary accelerated heat stress stability with the 20x ABEI conjugate has been performed after 3 days at 37° and 45° C with a huge loss in reactivity (-60% and -77% respectively).
Several conjugates have been made with dextran higher molecular weight, different dextran-BSA ratios or different coupling chemistries but, although the reduction in loss of signal, none of them has found to be stable according to the design goal requirements after the heat stress stability. Even different conjugate buffer compositions have been evaluated by adding some detergents in order to reduce any aggregation event that could occur.
To be sure that ABEI activity is not affected by heat treatment the comparison of the total light emission of both heat stressed and not stressed conjugates has been carried out (figure 21). The light emission should be the same (+/-10%) between treated and untreated conjugates otherwise a decrease in the potential ABEI emission can be supposed. The light emission of two conjugates is comparable so the lower signal of heat stressed conjugates depends on the conjugate structure.
In order to discard the possibility that the decrease of signal depends on IgG low stability to heating conditions an ELISA assay has been done in which heat treated IgG-HRP conjugates have been used (not shown). All available IgG for Dextran-BSA conjugates have been assessed; their immuno-reactivity is not affected by heat treatment.

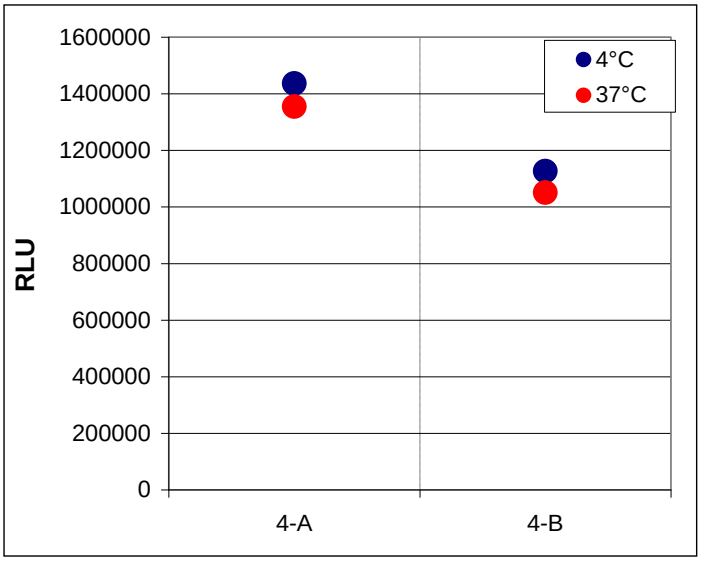


Figure 21. Comparison of the light emission of ABEI of a conjugate with (A) and without (B) heat stress treatment for 3 days at 37°.

As suggested by DiaSorin department and by the literature, the aging at 45°C of sugar structures (dextran in this case) gives the Maillard – polymerization reaction (124) with primary amino groups of the proteins. By carrying out a HPLC analysis, to highlight if aggregation states occur during heat treatment procedures on an available Dextran-BSA-ABEI-FAb conjugate, no aggregations or molecular weight differences before and after thermal treatment have been observed (data not available). These results could suggest that if the aggregation occurs, it involves an arrangement at the tertiary structure by forming oligomeric states rather than the polymerization of oligomers. If the stability depends on re-arrangement of tertiary oligomeric structure of dextran it is difficult to obtain acceptable stability results with the assessment of dextran-BSA-ABEI structure.
Other efforts have been made to improve sensitivity and stability testing a new synthesis of dextran-BSA-Fab ABEI which foresees the addition, before ABEI conjugation, of the EMCS and sulfo-EMCS cross linkers. Different ratios dextran : EMCS : ABEI have been produced and the best solution appeared to be 1 : 30 :40 which is more sensitive than the previous DBAF with higher positive RLU and comparable negative RLU (not shown). The stability study has been performed by changing the conjugate buffer compositions and pH (below pH 7) to enhance the EMCS stability. Surprisingly pH 5.0

remarkably stabilized the conjugate with no effect on the signal (table 3).

	pH5.0		pH5.5		pH6.0		pH6.5	
	4°C	45°C	4°C	45°C	4°C	45°C	4°C	45°C
mean pos	8707	7194	9000	7095	8995	6458	8441	5210
mean neg	632	636	505	508	637	496	515	478
pos/neg	13.8	11.3	17.8	14.0	14.1	13.0	16.4	10.9
DELTA RLU pos	-	-17%	-	-21%	-	-28%	-	-38%

Table 3. Stability evaluation changing the pH of conjugate diluent tested over positive samples at 0.25 IU/ml (n=5) and negative samples (n=5). The mean of positive and negative values are calculated and the positive/negative ratio has been reported for each condition.

New structures without BSA have been tried to reduce undesired sugars-proteins oligomerization by coupling the Fabs or IgG to the dextran always as scaffold for ABEI or directly to the chemiluminescent molecule (ABEI 20x IgG 1:1:1): an improvement on the stability after heat treatment has been observed (not shown) but these conjugates have a too low positive to negative ratio due to high negative values and a low specificity (table 4) and assessments and optimization of the conjugate buffer are needed.

	Diasorin Commercial Kit	Prototype A (MP biot-Fabs / DBAF)	Prototype B (MP biot-Fabs / IgG-ABEI)
Mean pos	11.816	18723	6668
Mean neg	416	487	634
Pos / neg	28.4	38.4	10.5

Table 4. The prototype B has a very low ratio value when compared to the A and the commercial kit. The mean of positive (n=5, 1 IU/ml) and negative (n=5) values are calculated and the positive to negative ratio has been reported.

A preliminary optimization of the conjugate buffer has been tried by adding casein or BSA to the conjugate buffer in order to reduce the not specific binding and to increase if possible the positive/negative ratio. Casein at a concentration of 0.3% has been found to decrease the background without affecting the positive signal.
There are two final prototypes, which are the most stable up to now, and are the two candidates for a further investigation for the

specificity and sensitivity requirements and for further optimization: the prototype A with the DBAF conjugate obtained with the use of EMCS during the coupling reaction and the prototype B (Benchmark) with ABEI directly coupled to the IgG antibodies.

4.2.1. Preliminary sensitivity and specificity assessment

The marketed DiaSorin kit has an adequate sensitivity to be maintained with the new prototype (0.020-0.024 PEI Unit/ml – 0.05 IU/ml – 0.11 ng/ml), a specificity corresponding to 94-98% to be improved (greater than 99.5%) as well as the conjugate working concentration which is currently too high. The target cut-off value in RLU to be reached with the new prototype should be higher or equivalent to four times the average of the negative population, very difficult to obtain, or the average added to ten times the relative standard deviation should be acceptable.
With the two prototypes built up, two panels of negative samples have been tested. The first series of samples is from Italian routine panel found to be negative for HBsAg; the second series is from French blood donors samples. The two series have been assessed with both prototypes. Together with sera panels, low positive DiaSorin QC samples with values around 0.1 – 0.03 PEI units were assessed to set the target assay performance requirements. These DiaSorin QC samples Ad-G and Ad-H had previously been calibrated against the BBI Sensitivity panel #808 using DiaSorin ETI-MAK 4 ELISA kit and during the assessment the following data have been obtained (table 5 A and B).

A

	Doses			DiaSorin
	ng/mL	IU/mL	PEI units	EIA S/CO
Ad-G	0,73	0,17	0,11	3,24
Ad-H	0,37	0,06	0,05	1,46

B

	RLU		
	LIAISON® HBsAg 310100	Prototype A	Prototype B (Benchmark)
Ad-G	1746	6872	1807
Ad-H	1108	3784	1306

Table 5. Ad-G and Ad-H QC samples doses expressed in ng/ml, IU/ml and PEI units obtained after calibration against the BBI Sensitivity panel (A) and the relative RLU obtained with the Liaison Commercial kit and the two prototypes (B).

As determined from the target assay performance requirements, the average RLU from negative population shall not exceed 850 RLU. Ideally, the average RLU from negative population should be comprised between 500 and 750 RLU. The Standard Deviation (SD) of the RLU from negative population shall not exceed 1/10 of the average RLU from negative population. In addition, the resulting specificity shall be not less than 99.5% at first result. RLU described below are used as rough evaluation of the cut off value for prototypes performance assessment at preliminary analysis.
After the routine panel assessment, samples with RLU higher than 2000 (n = 16) for prototype A and 1000 RLU (n = 12) for prototype B were retested.
After retest 3 samples out of 16 were judged to be outliers for prototype A, and 1 sample out of 12 was judged to be an outlier for prototype B; the other samples showed comparable RLU after re-test.
Considering routine panel assessment, the standard deviation is out from target assay performance requirement and average RLU added to 10 standard deviation does not permit to have requested sensitivity.
After the blood donors assessment, samples with RLU higher than 1500 (n=11) with prototype A and 1000 RLU (n=7) with prototype B were retested.

After retest 2 samples out of 11 were judged to be outliers for prototype A, and 5 samples out of 7 was judged to be outliers for prototype B while other samples showed comparable RLU after re-test.
Even in this case, the standard deviation is out from target assay performance requirement and average RLU added to 10 times the standard deviation does not permit to have requested sensitivity.
In order to roughly highlight if the bad normal sera assessment depends on the ABEI content, other two lots of tracer for prototype A with lower incorporation of ABEI (30x and 15x) have been tested.
With 15X ABEI conjugate all these samples can be correctly classified as negative (not shown).
From this first attempt to verify the performances of the prototypes for normal sera, data combined from the two sets of results indicate that they are out of target assay for standard deviation analysis, specificity and sensitivity. This seems to depend essentially on high ABEI content as verified with prototype A.
In order to reduce the background, especially for the prototype A, and so to improve the specificity, some changes in the conjugate buffers, as addition of surfactants and animal sera to reduce not specific binding and interactions and mouse IgG antibodies, able to interact with human anti-mouse IgG if present, have been tried. After some of these assessments the routine and blood donor panels have been continuously tested. 0.025 and 0.05 PEI units standard points have been assessed together with normal sera in order to have a rough idea of cut off value. Outliers for prototype A and benchmark have been found and no positive or equivocal results were found after retest (table 6). After retest prototype A seems to be able to reach target assay performance requirements (RLU corresponding to 0.025 PEI standard point is comparable to four times the average RLU) (see also figure 22) except for standard deviation, double than expected while benchmark is almost comparable to the commercial kit.
These assessments show a problem of outliers that could depend on sub-optimized conditions of the conjugate buffer or the assay buffer volume during reaction and could be considered a weak point of the assessment.

FIRST TEST

A

	LIAISON 310100	Benchmark	Prototype-A
average	502	514	688
std dev	98	248	390
	19%	48%	57%
average + 10 std dev	1479	2999	4593
pos sample	6	4	1
eqv	2	1	5
tot sample	281	281	328
specificity	97%	98%	98%

AFTER RETEST

B

	LIAISON 310100	Benchmark	Prototype-A
average	489	484	583
std dev	53	50	120
	11%	10%	21%
average + 10 std dev	1019	983	1781
average*4	1956	1938	2333
pos sample	0	0	0
eqv	0	0	0
tot sample	281	281	328
specificity	100%	100%	100%

Table 6. Specificity referred to the three test kits before (A) and after samples retest (B). For each the statistical parameters are reported. The Benchmark is almost similar to the current kit while prototype A is near to the target requirements except for the standard deviation still too high.

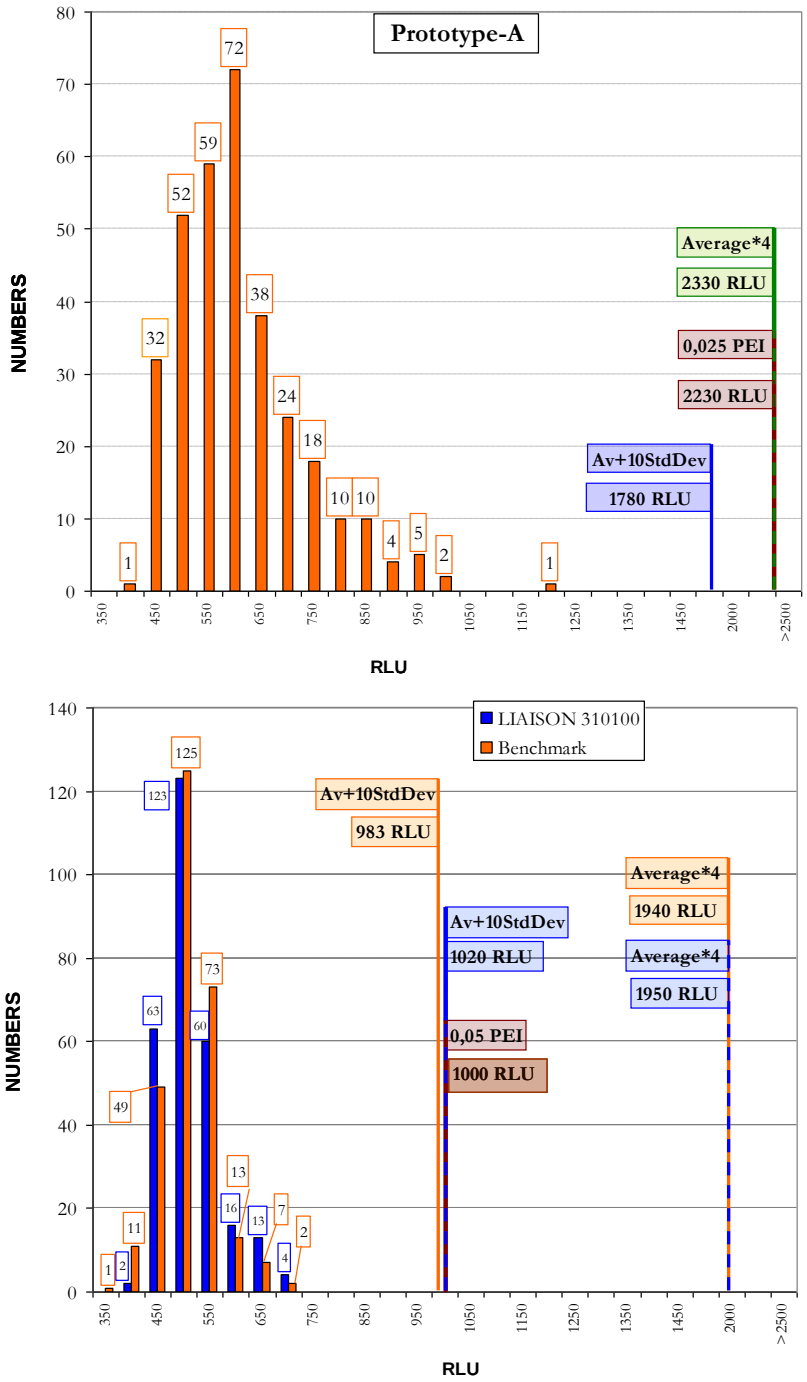


Figure 22. Frequency distribution obtained with prototype A over 381 normal samples and with Benchmark and the commercial kit over 281 normal samples.

Generally different assay panels are used to determine the sensitivity level of an immunoassay kit. The most used are the Paul Ehrlich Standard curve (which comprises both Ad and Ay subtypes) ranging from 0.0125 to 1.0 PEI units/ml and the Boston Biomedica Inc. HBsAg sensitivity panel PHA 808 (Ad and Ay subtypes) from 0.1 to 3.0 ng/ml.
The minimum target sensitivity of the new prototype assay shall be less than 0.03 PEI/ml and at least 0.1 ng/ml and 0.05 IU/ml. The RLU value at the nominal concentration of 0.03 PEI/ml should not be less than four times the average RLU of the negative population (4xAv.Neg RLU) as target requirement or the average RLU of the negative population with 10 times the relative standard deviation (Av.Neg+10SD) as acceptable requirement.
Paul Ehrlich Standard curve has been obtained from DiaSorin QC department and has been tested with commercial kit, prototype A and benchmark together with normal sera assessment (table 7).
For prototype A, and Benchmark in particular, the slope is not enough especially for low PEI values (figure 23). This could depend on matrix constituted by fetal calf serum and no conclusion can be done from PEI standard curve assessment. Other assessments should be done and may be other standard curves in different matrices.
For prototype A, the RLU value at 0.025 PEI units is similar to four times the average in RLU, of normal sera after retest of outliers. Considering the retest we can have cut-off sensitivity equal to four times the average of RLU able to reach target assay performance requirements, even if there is still the problem of outliers in a normal panel.

		Prototype A	Benchmark	LIAISON® 310100
	PEI units	RLU		RLU – Index gray zone = 0.9-1.1
STAU - C	1	31848	5832	21300 – 50 Index
STAU - D	0,5	11525	2579	8527 – 17 Index
STAU - E	0,2	7180	1791	3387 – 6,05 Index
STAU - F	0,1	4805	1321	2260 – 4 Index
STAU - G	0,05	3184	1185	1411 – 1,98 Index
STAU - H	**0,025**	2232	1149	993 – 1,16 Index
STAU - I	0,0125	2105	1046	727 – 0,54 Index

Table 7. Paul Ehrlich Standard curve (Ad + Ay) from DiaSorin QC department obtained by spiking HBsAg positive sera in fetal calf serum. The relative RLU and Index obtained with the 3 kits have been reported.

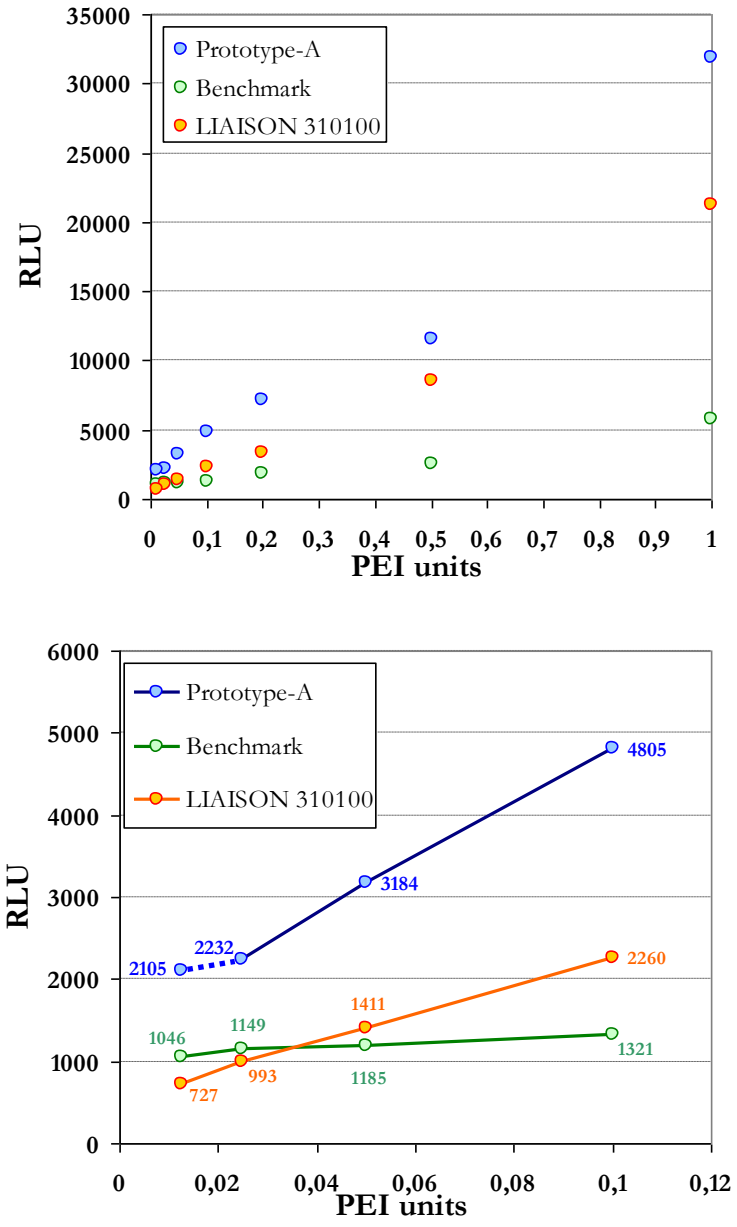


Figure 23. Correlation comparison between PEI units and RLU for two prototypes and the commercial kit. For Benchmark in particular, the slope is not enough steep.

The sensitivity panel from Boston Biomedica Inc. tested with both prototypes gave the following results shown in figure 24.
With PHA-08 panel, for the prototype A, a cut off $\cong$ 1300 RLU should be obtained in order to assure the sensitivity equal to 0.1 ng/ml.
Obtained RLU is lower than four times the average RLU and lower than the average RLU with 10 standard deviations, even if it is able to give 100% of specificity after retest.
Up to now the prototype A does not seem to be able to reach target assay performance sensitivity requirements with PHA-08.
Obtained RLU with the benchmark are obviously lower than four times the average RLU and lower than average RLU with 10 standard deviations. Again as the specificity study, RLU for BBI are comparable to HBsAg commercial kit for the cut off – low positive values (figure 25).
High doses of Ad panel seem to give lower RLU (-25%) when comparing benchmark to LIAISON HBsAg 310100 (while comparable data are shown for Ay Panel).

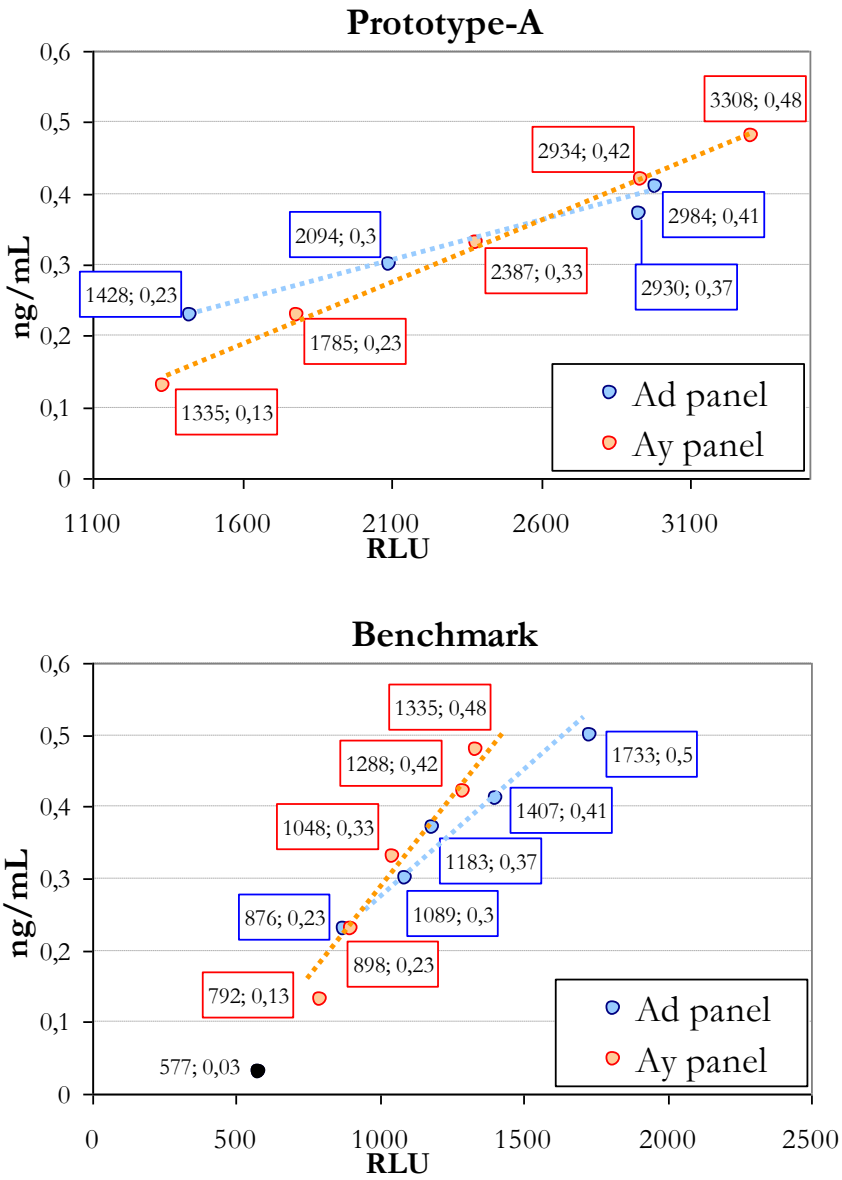


Figure 24. Prototype A and Benchmark obtained values against the increasing HBsAg concentrations for Ad and Ay panels.

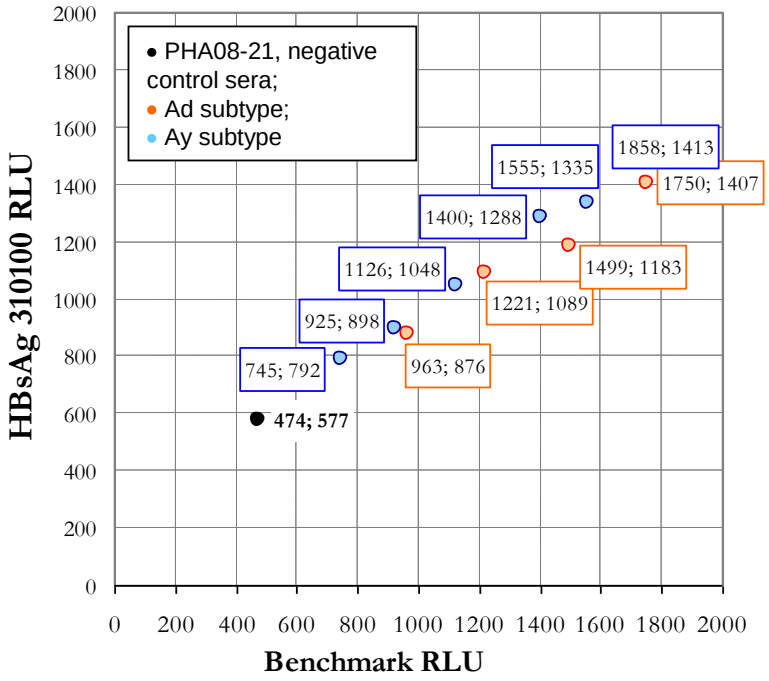


Figure 25. Comparison between RLU obtained with Benchmark and the commercial kit. They look similar but the high doses are lower for Benchmark.

4.3. New ABEI derivative conjugate

A new chemiluminescent molecule deriving from the ABEI molecule has been produced from the Milano-Bicocca University and test as signal source for the prototype assessment. The process to obtain this ABEI derivative is under patent at now; the peculiarities of this molecule are its ability to be obtained with high purity (98%) and to be more stable than the ABEI active ester as demonstrated through a stability test (not shown). Moreover it has different incorporation characteristics than the ABEI molecule; the improved incorporation characteristics allows to lower amount of probe to generate the same signal reached by using a higher amount of ABEI. Due to this the derivative enables a better sample discrimination as a result of the ability to decrease the amount of probe/antibody and tracer/probe necessary per reaction (figure 26 and table 8) allowing to have a lower average of negative samples with an acceptable standard deviation. This helps the system to maintain specificity performances

and avoid outliers outcomes. Here the prototype refers to the Benchmark by substituting during the conjugation the ABEI molecule with the ABEI derivative 10x (10 ABEI derivative molecules per antibody mole) instead of the 30x for the previous Benchmark and 35x for the commercial kit.

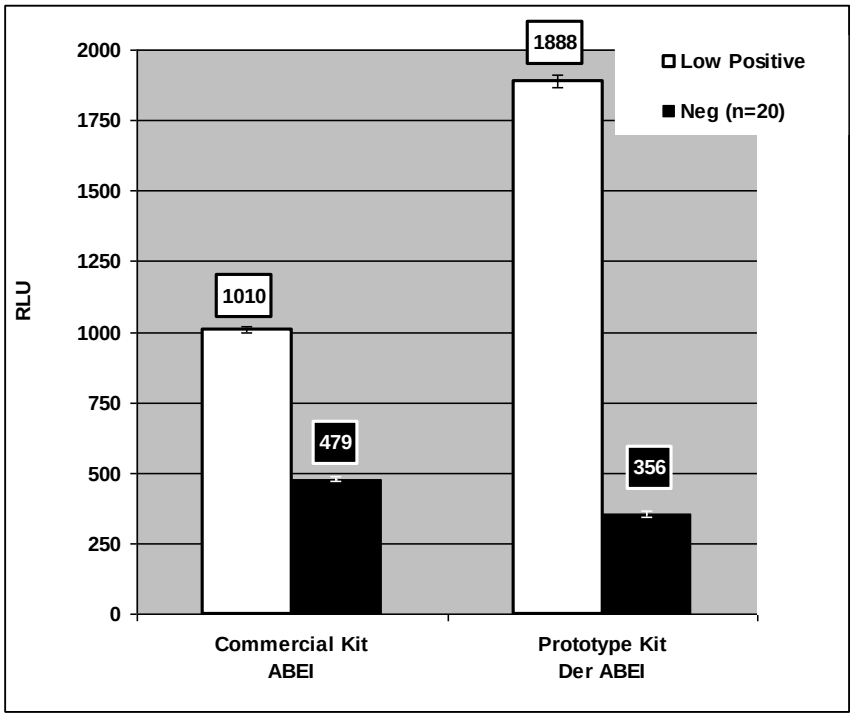


Figure 26. The ABEI derivative tracer/probe used for the prototype is more reactive against a low positive sample (0.025 PEI) maintaining a lower negative signal; a higher positive to negative ratio is obtained even though a 3.5 times lower molar conjugation ratio is employed of the ABEI derivative when compared with the currently used ABEI conjugate. The mean of three replicates of low positive sample and of the negative samples (n=20) with the relative standard deviation has been reported.

	Commercial	Prototype
	RLU	
low positive	1010	1888
2 PEI	26234	63398
Average neg (n=20)	479	356
Std.Dev. Neg	8%	12%

Table 8. The employment of the ABEI derivative, even with low incorporation amount, allows to double the signal for a positive sample.

This conjugation technology is easier to handle than the dextran matrix used in the prototype A, which could be difficult to industrialize, and is very reproducible due to the fact that the ABEI derivative is more stable than dextran and can be obtained with high purity increasing the incorporation rate on the target molecule. Analyzing the dextran molecules by HPLC (data not shown), there is not a single peak corresponding to the exact molecular weight but there is a Gaussian distribution of molecular weights near to the right one; this could make some problems arising for the reproducibility. As a consequence, in order to maintain the required specificity, the final prototype A conjugate concentration in reaction should be reduced obtaining RLU values on positive samples comparable to current kit. This conjugate approach also requires difficult synthesis steps, which even if reproducible, can be difficult for industrialization. For these reasons the prototype A will not be further investigated for improvements as the ABEI derivative represents at this moment the best choice to follow; the major specificity and sensitivity studies as well as the optimization efforts will be focus on the novel derivative.

4.3.1. First assessments

Various ABEI derivative incorporation factors has been tried to assess the good amount of chemiluminescent molecule per antibody. An example has been reported (figure 27) to highlight the fact that increasing the ABEI derivative ratio the discrimination between the negative and low positive samples decreases due to the higher background noise. Using 24x there is too much dispensation of RLU in the reaction mixture, resulting in high background and a risk of outliers without improving significantly the ratio between low positive sample and normal sera. The use of conjugates with 10x moles of ABEI derivative on ALSI IgGs allows the dispensation of lower to

comparable amounts of total RLU in reaction than commercial kit avoiding outliers outcome, with comparable or higher performance on low positive samples resulting in a better separation between positive and negative samples.
Assessments in the conjugate diluent buffer are to be made in order to “clean” the background. A simple buffer, referred as basal, has been used in this case with only human negative serum in phosphate buffer saline (PBS) and 10% BSA, in order to reduce the not specific binding but the diluent of the benchmark should be improved with the appropriate adjustments. Different components have been added, singularly at first and then in mixtures and after various formulations what has been found is shown in the following figure 28: the addition of both sheep and bovine sera to the human one help to reduce the background by increasing the discrimination between the low positive and negative samples and the presence of Tween 20 and Poly-vinyl-pyrrolidone as detergent/surfactants aid to reduce the not specific interactions. From this moment this buffer formulation will be used for the experiments to come.

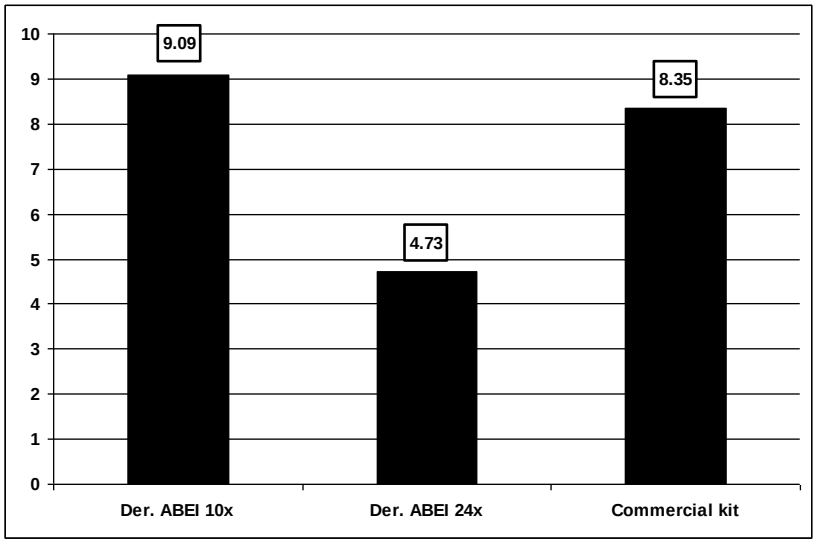


Figure 27. In this example the same concentration of tracer has been compared (800 ng/ml as the commercial kit) but with a lower concentration the same positive to negative ratio of the commercial kit can be obtained with ABEI derivative 10x.

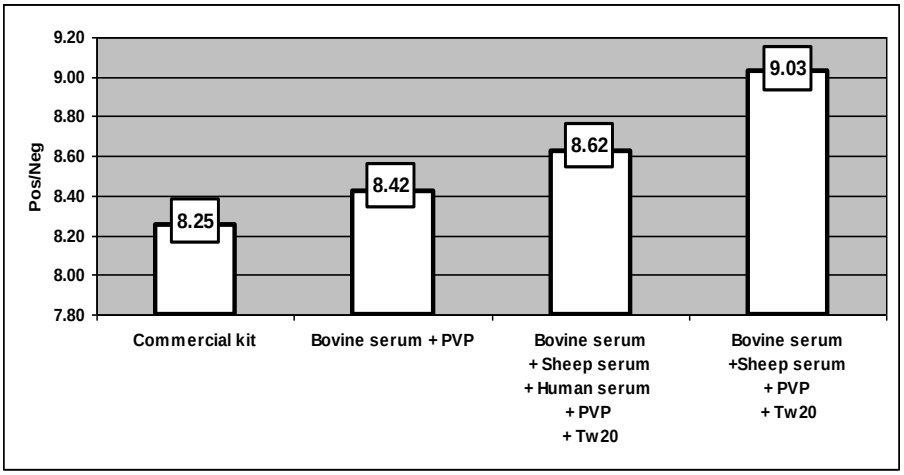
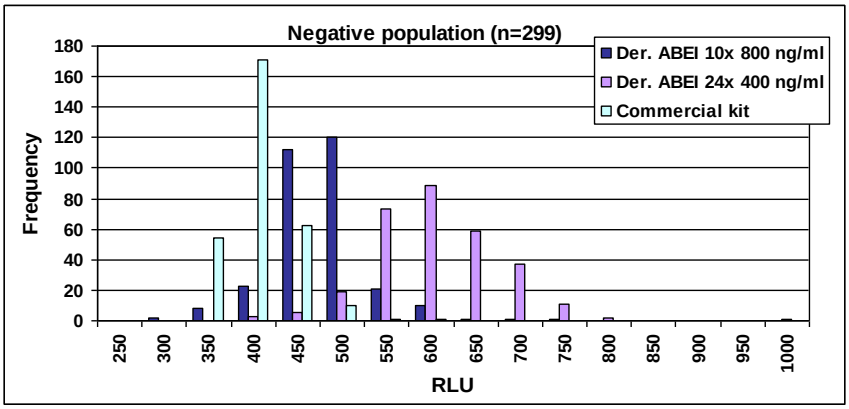


Figure 28. Study on the conjugate diluent composition tested over positive samples at 0.05 PEI (n=5) and negative samples (n=5). The mean of positive and negative values are calculated and the positive to negative ratio has been reported for each formulation.

The new conjugates have also been tested against a normal sera population to evaluate if with the improved conjugate buffer the background noise could be reduced also with high incorporation factor (figure 29 and table). With the ABEI derivative 24x the low positive sample is almost comparable with the ABEI derivative 10x although the half of concentration but the average of negative samples is higher (the highest negative is around 970 RLU against 715 RLU with 10x) as well as the standard deviation. A lower incorporation factor seems to be more under control, for this the tracer and benchmark prototype will have this conjugation assessment.



	Der. ABEI 10x 800 ng/ml	Der. ABEI 24x 400 ng/ml	Commercial kit
Mean neg	452	584	382
Std.Dev. Neg	54	72	36
Low pos	1320	1241	976

Figure 29. New conjugate evaluation over a normal sera distribution. Increasing the incorporation factor the negative signal is shifted to higher values which could be dangerous for outliers outcome. The values reported in the table refer to 0.025 PEI/ml for the low positive sample (n=5) and negative sera with relative standard deviation.

A comparison has also been done between the ABEI derivative benchmark prototype and the benchmark tracer conjugated with the simple ABEI 40x (very close to 35x of the commercial kit). As shown in figure 30, with the ABEI derivative a good normal distribution with a lower negative average and higher value for the lowest positive sample can be obtained due also to an incorporation factor which is four time less.
Even a last comparison with the prototype A has been performed testing a negative population (figure 31) to highlight that the prototype A negative average is still too high with the presence of outliers well above the low positive value, causing a too high standard deviation (149 vs 56 of the ABEI derivative prototype) and a reduced specificity. The lowest positive sample at 0.012 PEI is above, even for a little, to the higher negative sample for the ABEI derivative prototype while it is not the same for the prototype A which continue to have higher background noise. These data definitively establish that it is not worth

to evaluate the prototype A anymore and the benchmark prototype with the ABEI derivative is the best candidate to become the new HBsAg kit to replace the already existing commercial kit.

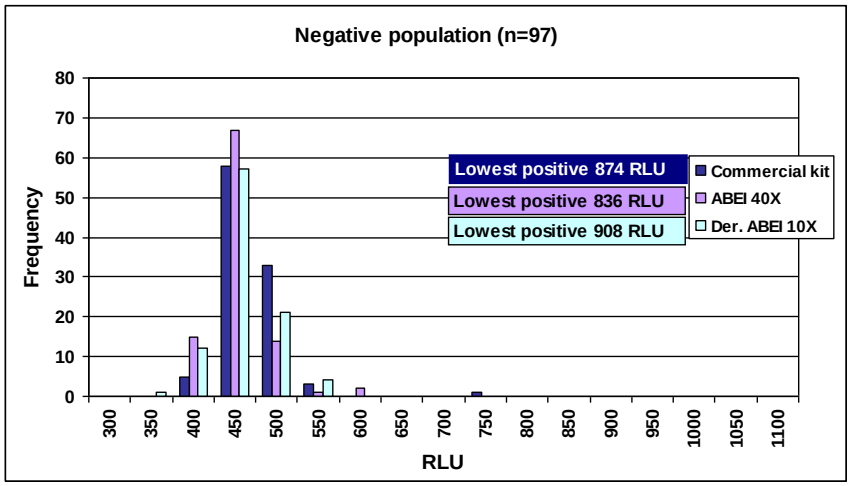


Figure 30. Comparison of normal sera distribution of the prototype, the commercial kit and the prototype labeled with normal ABEI. The lowest positive sample is 0.012 PEI.

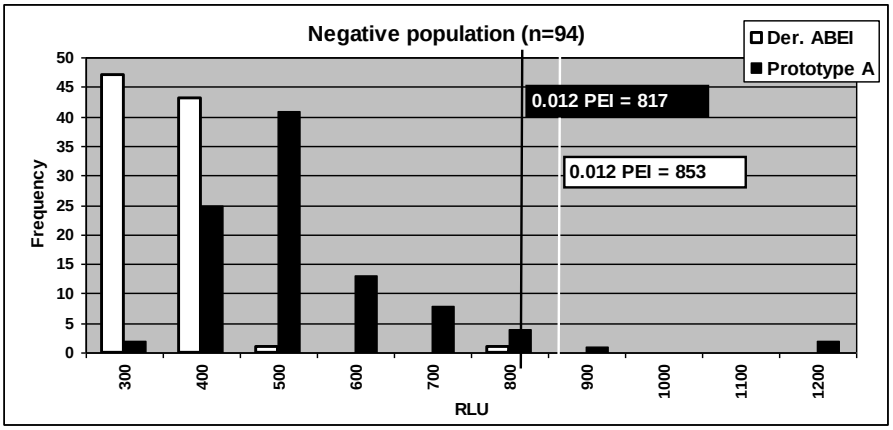


Figure 31. Last prototypes comparison: the prototype A will be definitively discarded.

For what is concerning the positive samples detection, data obtained through the analysis of the standard curve (expressed in PEI units) have been reported to show how the benchmark prototype has a good correlation with the antigen concentration similar to the

commercial kit but higher immunoreactivity (figure 32) even these results have to be confirmed and further improved.

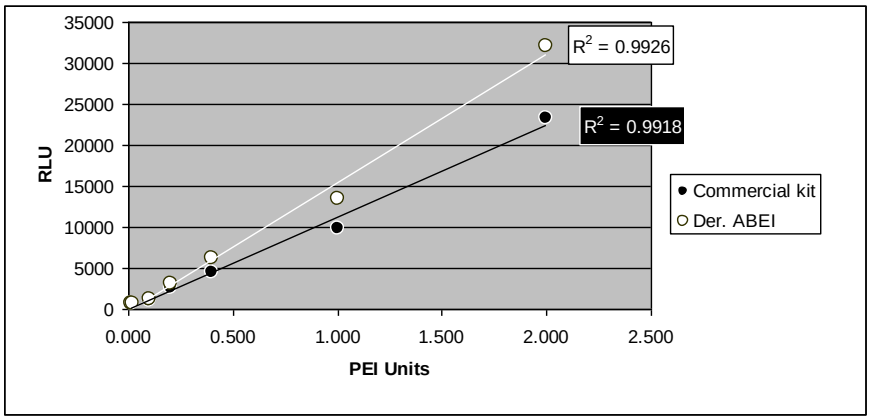


Figure 32. Correlation analysis between PEI units and RLU of the prototype compared to the commercial kit: the prototype correlation is slightly better and the RLU values are higher.

4.3.2. Improvements for a quantitative detection

The sequential one step approach used up to now has been judged useful to give an interpretation about the adequacy of raw materials.
The new immunoassay kit should be able to quantitatively detect the amount of antigen present in the sample. Due to this, a two steps protocol is required to gain a good linearity between signal and dose to the increasing positivity of samples.The quantitative format of the assay will allow the customers to carry out the quantitative determination of the HBsAg, as suggested by recent publications, at the start of treatment and during the follow up.
The two steps format foresees two wash steps instead the only one present in the commercial kit. As before, in the first step the sample is allowed to react with the solid phase and the ALSI buffer to dissolve the lipid bilayer constituent the envelope allowing the transmembrane region to react with the antibodies coated onto the solid phase. At the end of incubation a wash cycle needs to be added to remove the unbound material. A second incubation foresees the addition of the tracer. The tracer reacts with the antigen present in the previous formed immuno-complexes. A second wash cycle is needed before measuring the light signal in a way that it could be directly related to the total amount of antigen present in the sample with more precision than in a one step protocol. Starting to maintain the same first incubation time as in the previous sequential protocol (5 TIC which

means about 10 minutes), a series of kinetics incubation studies have been performed to set the best incubation times which allow to reach the equilibrium in the two separated immuno-reactions. The minimum time required in the first incubation corresponds to not less than 10 TIC (20 minutes) to enable the ALSI assay buffer to unfold the HBsAg membrane and reveal the internal epitopes making them accessible to the monoclonal antibodies. Once fixed the first incubation, the kinetics of the second one has been reported (figure 33): at 15 TIC time the lowest positive sample to negative mean ratio is the highest. This incubation time allows to achieve the requested through-put (100 tests per hour) while with 10 TIC as second incubation the through-put could be better (170 tests per hour). The lengthening of the tracer incubation time could affect the specificity of the system (table 9); for this a titration of tracer concentration has been performed (figure 34): decreasing the tracer amount the specificity can be recovered.

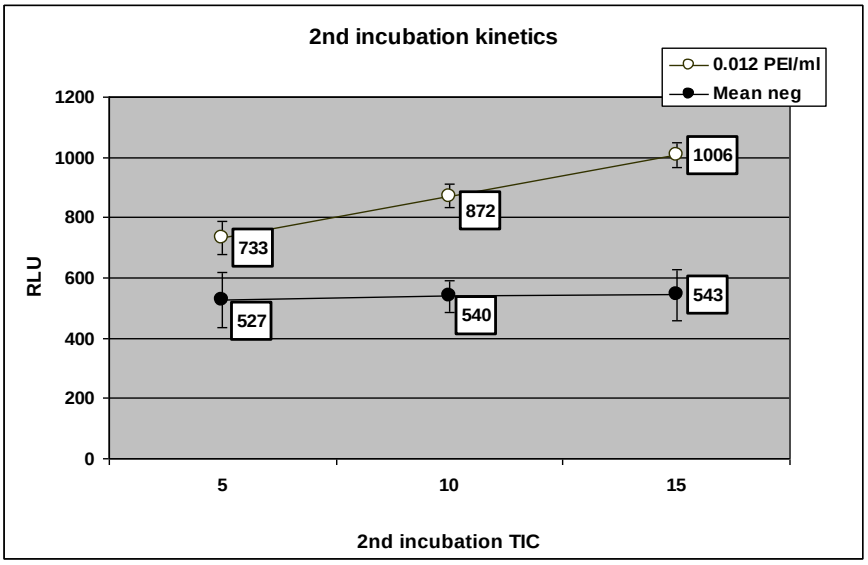


Figure 33. Second incubation kinetics study: increasing to 15 TIC the specificity between negative and the lowest positive sample increased. The mean of three replicates with the relative standard deviation has been reported.

	Prototype pos/neg	Commercial kit pos/neg
One Step	2.2	1.6
Two Steps	1.8	

Table 9. Different positive to negative ratio between one and two steps.

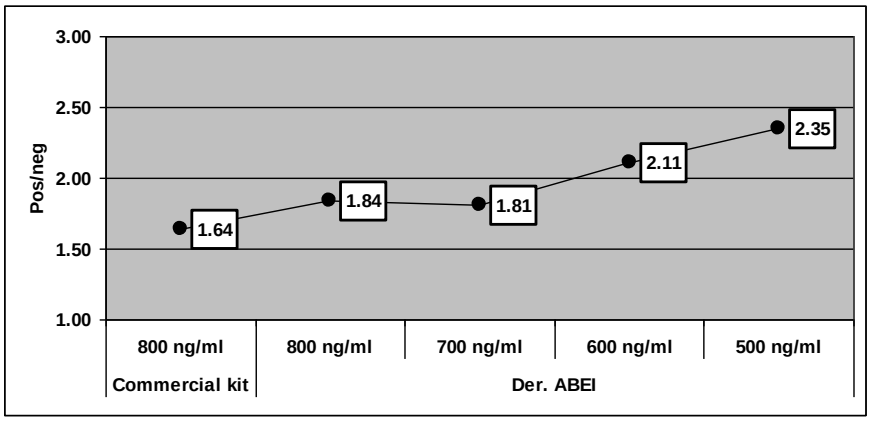


Figure 34. Improving the specificity of the system with tracer titration. The ratio between the lowest positive 0.012 PEI/ml (n=6) and the negative samples (n=10) has been reported.

Other assessments will be done to further improve as much as possible the prototype which from now will use a lower tracer concentration of 500-600 ng/ml instead of 800 ng/ml with a two steps assay format to quantitative discriminate the antigen in the sample.
First of all a solid phase improvement has been carried out through a different approach in the coating procedure with the aim to decrease the amount of raw materials and/or reagent consumption: in the previous studies the streptavidinated beads used for the coating were at 50 μg/ml of streptavidin with 20 μg/ml for the mixture of the biotinylated Fabs; 40 μg/ml of mixed Fabs have now been coated onto 50 μg/ml and 100 μg/ml streptavidinated beads. The last solution gave a higher positive to negative ratio when compared with the previous condition and with the increased amount of Fabs coated on the same streptavidin concentration (figure 35); 50 μg/ml of streptavidin are too low for the doubled Fabs concentration. Doubling both of these parameters the reactivity is higher, even almost similar, than the condition used till now but this enables to double the final dilution of the beads (0.125% instead of 0.25%) during the reaction

with a lower consumption even the amount of Fab per reaction remains equal to the commercial kit (0.1 μg of antibodies/Fabs per reaction in 20 μl). To increase the final beads dilution maintaining the quantity of Fabs consumption, the beads concentration during the coating is raised at 2%: it provides a lower, or similar, raw material consumption maintaining or improving the performances (figure 36). By coating with 2% beads, if the same final amount of ligand is maintained at 0.1μg per reaction (0.25% final beads dilution ready to use) the reactivity progresses while if a double dilution occurs (0.125% final beads dilution ready to use) the performance is maintained but the final consumption of ligand is halved (0.05μg per reaction).

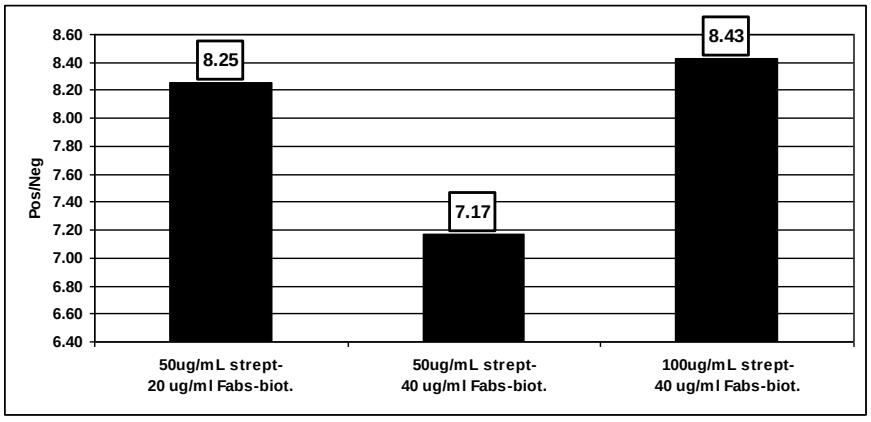


Figure 35. Effects of new coating procedures over a mean of low positive samples (n=5) and negative samples (n=5): the positive to negative ratio has been reported.

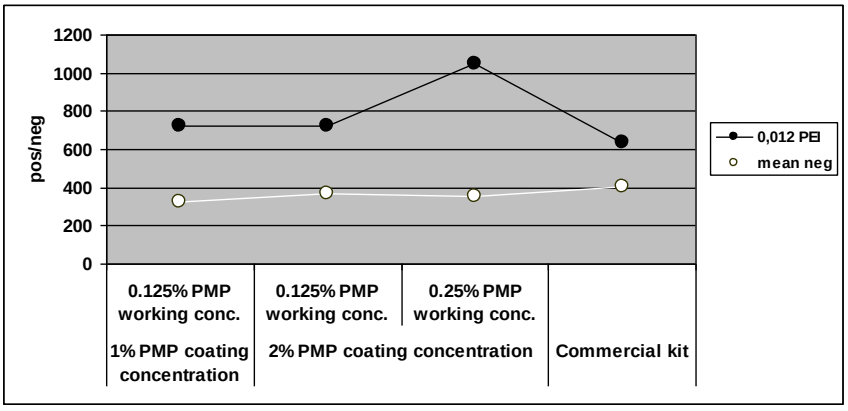


Figure 36. Effects of increasing beads concentration during the coating step. The mean of three replicates with the standard deviation has been reported for both positive and negative samples.

By performing a series of beads dilution to test with a tracer concentration titration the optimal condition corresponding to a 0.167% final beads dilution (0.067μg ligand amount per reaction) with 600 ng/ml tracer concentration has been found as shown in figure 37. Lower beads and FAb concentrations in the reaction allow to reduce the risks of poor performance both in terms of specificity and interferences.

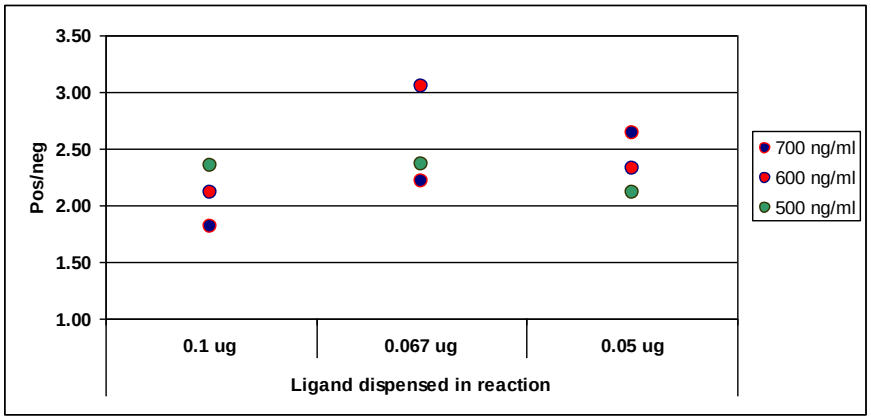
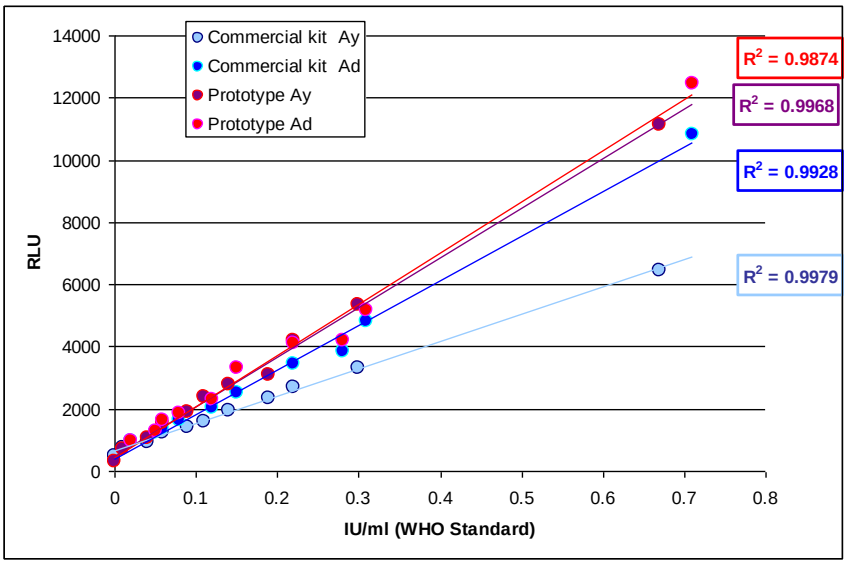


Figure 37. Effect of tracer concentration and beads dilution on specificity employing the lowest positive samples. The positive to negative ratio has been calculated and reported.

Higher beads coating concentration than 2% and with the same or higher Fabs concentration do not seem to improve the assay sensitivity. Beads have been observed using an optical microscope and showed increased aggregation when using concentrations higher than 2% of beads during the coating procedure. Coating at 2% showed to have low (from 5 to 10 PMPs per clump) or no aggregation.

4.3.3. Specificity and sensitivity assessments

Continuous tests with the sensitivity panels and negative populations are carried out to monitor the prototype sensitivity and specificity respectively. Before all the last improvements the sensitivity panel analysis for both Ad and Ay subtypes from BBI showed the following results (figure 38):



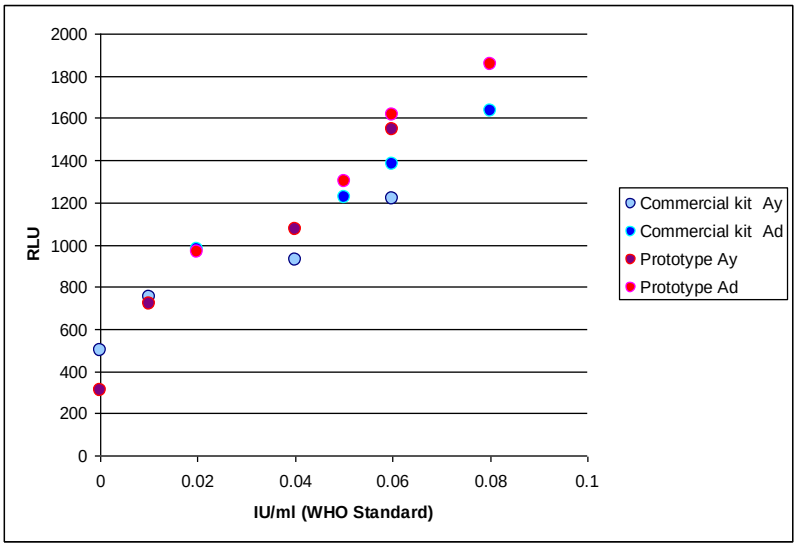


Figure 38. Prototype and commercial kit tested over the Boston Biomedica Inc. sensitivity panel for both subtypes; the prototype is more sensitive or equal to the commercial kit and the correlation is very similar. The panel concentration is expressed in IU/ml or WHO standard units where the 0.05 IU/ml represents the sensitivity targets.

The prototype shows a good correlation comparable with the commercial kit through the BBI panel standard curve expressed in IU/ml and from the target sensitivity dose fixed at 0.05 IU/ml the prototype for both subtypes is more sensitive than the commercial kit. To gain better sensitivity even at very low concentration the improving assessments seen before are to be used. Higher specificity, and also sensitivity, can be gained by adding a little amount of ALSI buffer (10%) to the tracer during the second step of the assay as shown in figure 39. This enables to further separate the negative mean from the lowest positive sample mean; oddly, the same effect was not reached during the assessing of the amount of ALSI buffer to be added to the sample during the first reaction step; the mechanism at the basis of this effect is not very clear but due to the results it seems to positively act on the tracer antibodies in combination with the sample rather than on sample alone. Due to all these improvements, the two steps protocol reached better performances than the beginning (figure 40), and is better and more specific than the one step format assay.

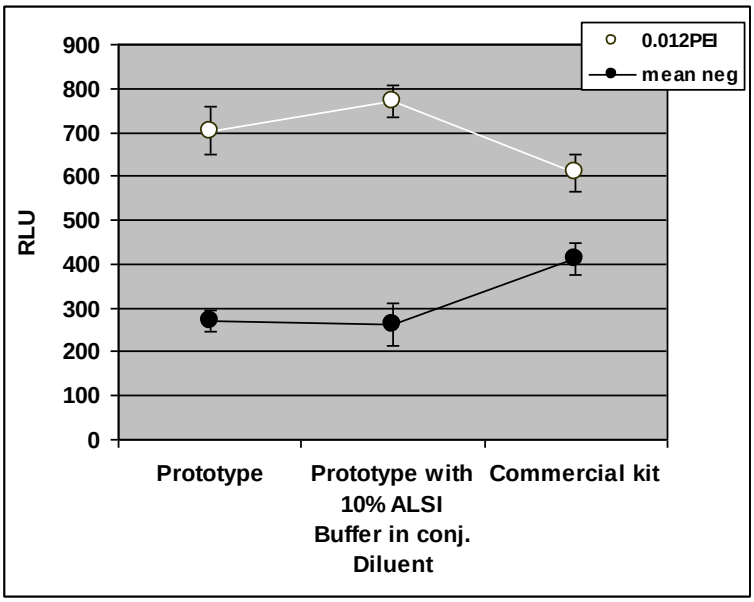


Figure 39. Improving specificity by addition of ALSI buffer in the tracer. The mean of three replicates with the relative standard deviation has been reported.

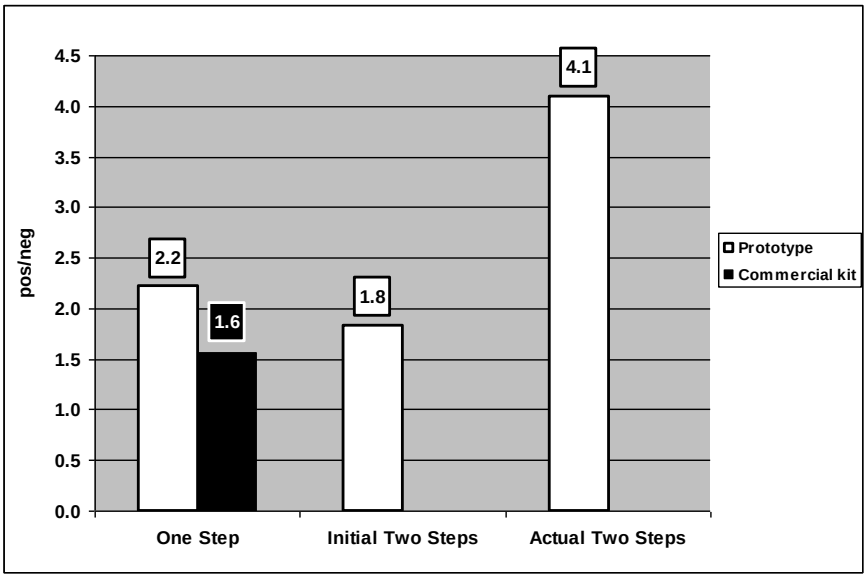


Figure 40. Comparison of two steps after all the assessments with the initial to steps and the one step. The positive to negative ratio has been calculated and reported.

Employing the solutions found up to now, the following results are obtained with an increase of the prototype sensitivity evaluated over the BBI sensitivity panel (figure 41) for both subtypes. The prototype has now a really good sensitivity far enough from the negative average of a normal population (not shown). The cut off is far from the lowest positive detection. A further sensitivity data derives from the WHO sensitivity panel and sample NIBS 00/588 (0.05 IU/ml). There is a big correlation between doses and RLU and good slope even at low doses (figure 42). The target sensitivity requirement results to vary between 1300-1500 RLU with a negative average around 400-500 RLU with a positive to negative ratio equal around 3.

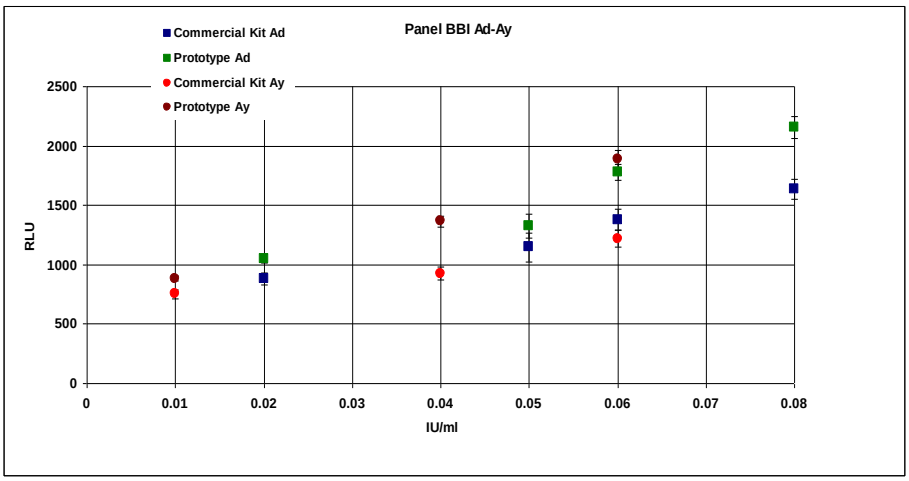


Figure 41. Prototype improvement tested over the Boston Biomedica Inc. sensitivity panel for both subtypes; the prototype is more sensitive than the commercial kit. The panel concentration is expressed in IU/ml. The mean of three replicates of each sample with the relative standard deviation has been reported.

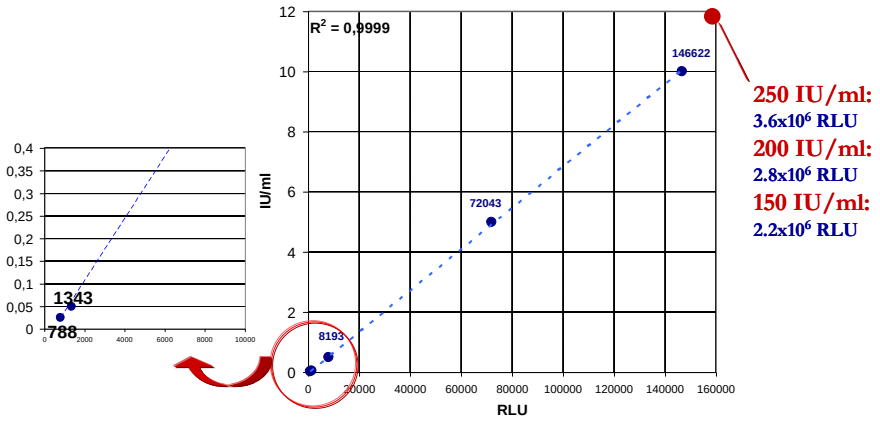


Figure 42. A further sensitivity data derives from sample NIBS 00/588 (0.05 IU/ml, 1343 RLU). There is a big correlation between doses and RLU and good slope even at low doses. RLU values at higher doses has also been reported.

The diagnostics sensitivity has been also evaluated over ten HBsAg seroconversion panels from BBI which demonstrates comparable or better reactivity than the current commercial kit (not shown).
Concerning doses results, the final prototype will be calibrated against WHO standard and will give the HBsAg concentration expressed in IU/ml; the sensitivity limit from now will be referred to the WHO standard.
This almost final prototype tested over a negative population gives a really good specificity having the sample at 0.05 IU/ml as sensitivity limit at 1400 RLU which corresponds to the negative samples mean added to 27 times the relative standard deviation, more over than the acceptable target and is almost near to four times the negative mean (figure 43).
The estimated limit of detection is around 0.015 IU/ml (corresponding about to 960 RLU) so the target analytical sensitivity at 0.05 IU/ml should be fulfilled. The cut off should be set around 1300 RLU without equivocal zone as the commercial kit.

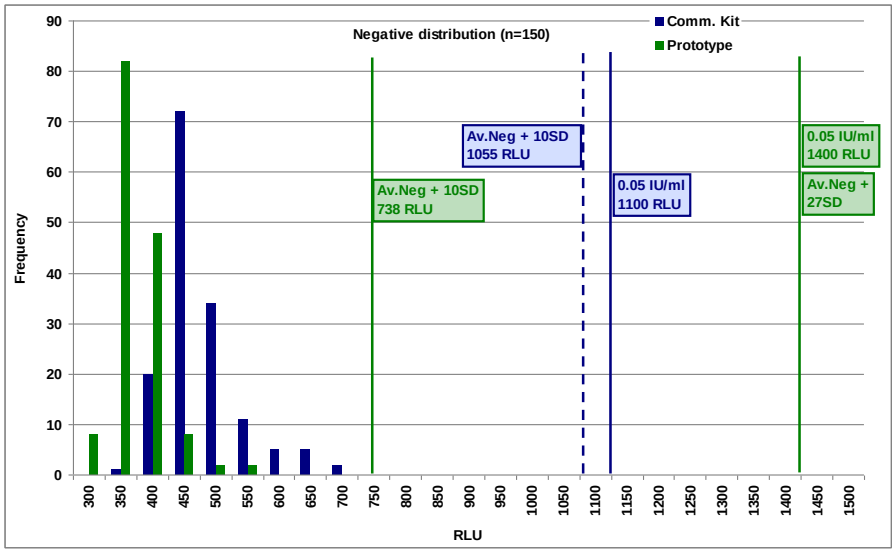


Figure 43. Normal samples tested with both the commercial kit and the prototype. To reach the RLU values at 0.05 IU/ml as sensitivity limit, the standard deviation must be added 10 times to the population mean for the commercial kit (in blue), against the 27 times for the prototype. This is due to the lower negative signals and standard deviation enabling a better discrimination between positive and negative samples.

Many other negative and positive population evaluations have been carried out and the diagnostic specificity has been evaluated to be

100% on positive samples and 99.75-99.87% on almost 800 negative samples, higher than the target requirement established at 99.5%.
Finally heat stress stability tests have been performed over the prototype assessment without affecting the reactivity. Here an accelerated stability test has been reported (figure 44) showing a slight decrease of positive signal and a slight increase on the negative one without any relevant impact on the reactivity.

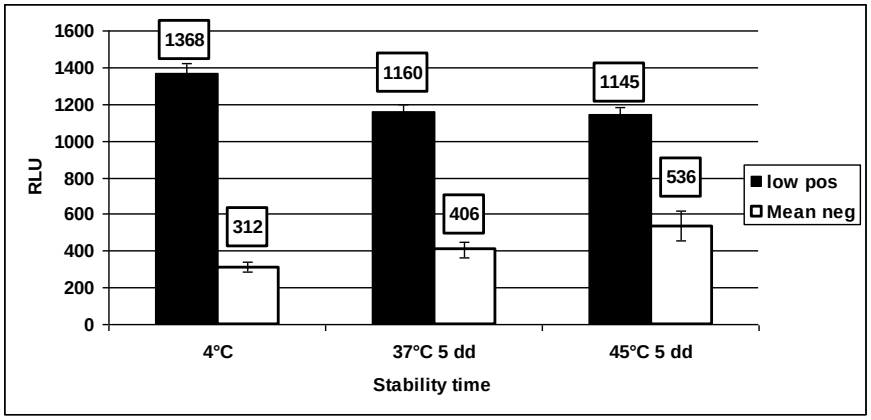


Figure 44. Heat stability evaluation of the prototype stability measured over the lowest positive samples (n=10) and negative samples (n=10). The mean of three replicates has been calculated and reported with the relative standard deviation.

4.4. Mutants detection

Many mutations which occur during the viral replication are highly detrimental or lethal and are not maintained in the virus population. A proportion of mutants may survive but only if they confer some advantage over pre-existing strains. The immune responses of the host as well as prophylactic and therapeutic interventions may select variants which arise during the process of replication.
In conventional detection systems, antibodies mainly recognize the “a” loop with difficulties in detecting escape mutants which commonly arise in this region; the employment of monoclonal antibodies directed specifically against the highly conserved inner region in combination with a buffer able to disrupt the lipid bilayer should help in their detection (figure 45).

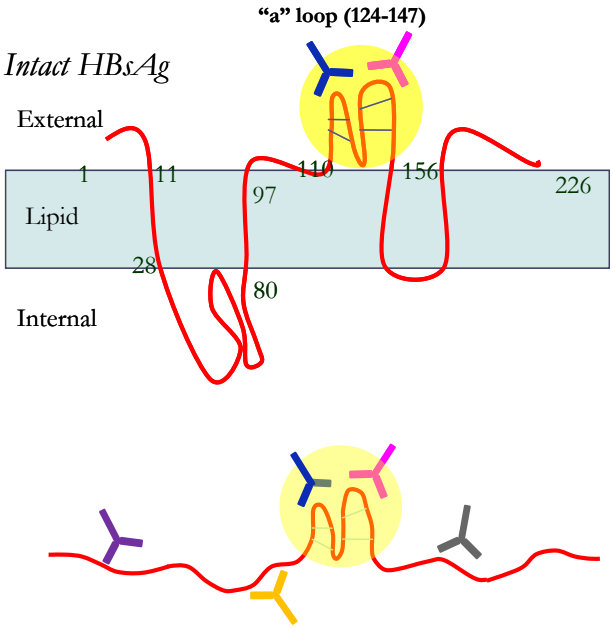


Figure 45. Schematic representation of intact HBsAg inside the virus membrane and after destruction of lipid bilayer which gives the transmembrane epitopes back available for the reaction.

Based on literature description of the most characterized natural mutations a panel of ten HBsAg recombinant mutants, not all detected yet by most of commercial immunoassay kits, has been produced in collaboration with the University of Novara. This library provides mutants of the "a" loop, of the surface region among 110-123 residues and combined.
The DiaSorin produced panel is the following:

Mutant	Target Region
G145R	
D144A	
P142L - G145R	**"a" loop 124-147**
P142S - G145R	
F/Y134H - P142L - D144E - G145R	
T123N	
T123N - T124S	**Surface**
122+DT	**110-123**
I110R - S117I - G119R - T123N - C124R	
G145R - 122+DT	**Combined**

Table 10. DiaSorin mutant panel with the target region affected by the relative mutations.

Every mutant will be checked with the prototype to verify an effective improvement against the commercial kit and with the main competitor's assays in order to compare sensitivity against each of them.
Data on table 11 confirm the ALSI IgGs ability to detect all of the HBsAg mutants from the panel than both the commercial kit for the Liaison system and ELISA assay.

Mutant	Commercial kit	DiaSorin ELISA	Prototype
T123N	POS	POS	POS
T123N-T124S	NEG	NEG	POS
P142L-F/Y143H-D144E-G145R	POS	POS	POS
I110R-S117I-G119R-T123N-C124R	POS	POS	POS
122+DT	POS	POS	POS
122+DT-G145R	NEG	NEG	POS
G145R	POS	POS	POS
D144A	POS	POS	POS
P142L-G145R	POS	POS	POS
P142S-G145R	POS	POS	POS

Table 11. Comparison of the prototype ability to detect DiaSorin mutant panel than the marketed Liaison and ELISA kits.

DiaSorin panel contains three interesting samples:

- **T123N-T124S**: this mutant is negative with Abbott Architect (competitor of reference), Modular Roche and the current DiaSorin commercial kit.
- **F/Y134H-P142L-D144E-G145R**: this mutant is negative with Abbott Architect and Modular Roche.
- **G145R-122+DT**: this mutant is negative with the current DiaSorin commercial kit.

Data on table 12 show a better performance of the prototype than the commercial kit and the two above mentioned competitors.
Other panels on which to focus the attention derive from competitors. The kit should be able to identify all the mutants present in the competitor's panels or at least be better than their detection. The panels to be evaluated are:

- the complete Abbott recombinant panel;
- samples found to be negative with DiaSorin ELISA assay (some samples from Roche and Bio-Rad recombinant panels)
- I110R - S117I - G119R - T123N - C124R (negative with Abbott Architect) and C121Y - R/K 122L - T123N - G130E - M133I - D144G - G145R native mutants.

The prototype is able to detect all of the aforementioned competitor's panels and the two native mutations not both detected by the competitors. Even Abbott is the best competitor able to detect HBs antigen mutations, the prototype is even better as it is able,

conversely to Abbott, to detect both the native mutations (table 13) and obviously the complete DiaSorin panel.
Further clinical validation studies on the prototype are still ongoing before the market launch.

Mutant	Prototype	Architect Abbott	Modular Roche	DiaSorin ELISA
G145R	POS	POS	POS	POS
D144A	POS	POS	POS	POS
P142L - G145R	POS	POS	POS	POS
P142S - G145R	POS	POS	POS	POS
F/Y134H - P142L - D144E - G145R	POS	NEG	NEG	POS
T123N	POS	POS	POS	POS
T123N - T124S	POS	NEG	NEG	NEG
122+DT	POS	POS	NEG	POS
I110R - S117I - G119R - T123N - C124R	POS	NEG	NEG	POS
G145R - 122+DT	POS	POS	NEG	NEG

Table 12. A better performance of the prototype than the commercial ELISA kit and two competitors to detect the DiaSorin mutant panel has been reported.

		Prototype		ETI-MAK-4		Architect		Modular	
		RLU	Label	Dia-Sorin		Abbott		Roche II	
Abbott panel	Glutamine 129 to Histidine	11240	POS	46,9	POS	1,57	POS	23,1	POS
	Methionine 133 to Leucine	8674	POS	37,6	POS	1,49	POS	19,8	POS
	Aspartic Acid 144 to Alanine	7764	POS	24,9	POS	0,67	POS	16,5	POS
	Glycine 145 to Arginine	10304	POS	29,5	POS	1,1	POS	4,2	POS
	Pro 142 to Leu + Gly 145 to Arg	8247	POS	25,2	POS	0,97	POS	3,2	POS
	Pro 142 to Ser + Gly 145 to Arg	8798	POS	21,1	POS	0,8	POS	2,9	POS
	Thr 123 to Ala	13127	POS	60,7	POS	0,32	POS	39,8	POS
	122 Asn Thr insertion	56540	POS	79,6	POS	0,2	POS	1,2	POS
	122 Arg Ala insertion	14653	POS	44,4	POS	0,24	POS	7,1	POS
Roche Panel	F8L/R24K/N40R/G43R/L94S/ M103I/113A114/M133T/P142L/D144G	11579	POS	0,4	POS	0,3	POS	7,54	POS
	I110L/S113T/T114S/T126I/N131T/ F134Y/T143S/G145R	9745	POS	0,7	NEG	0,4	POS	6,26	POS
	S132Y/P142S/G145R	11284	POS	0,1	NEG	1,2	POS	16,28	POS
	G145K	4241	POS	0,2	NEG	0,6	POS	9,78	POS
	P142L/G145R	8747	POS	0,2	NEG	0,8	POS	4,17	POS
Bio-Rad Panel	P120S/T125MP127T/S143L	1510	POS	6,1	POS	0,2	POS	0,6	NEG
	Y100S/T114S/T118V/N131T/ K133I/F134N/P142S/T143L	4674	POS	0,4	NEG	0,3	POS	2,2	POS
	T123N/C124R	30995	POS	12,6	POS	0,2	POS	0,3	NEG
	D144A	11174	POS	2,1	POS	0,1	POS	1,9	POS
Native undiluted	*COHEN:* I110R/S117I/G119R/T123N/C124R	48242	POS	6,7	POS	0,04	NEG	0,3	NEG
	Cont 2006 HBIG escape: C121Y/ R/K122L/T123N/G130E/ M133I/D144G/G145R	92495	POS	0,1	NEG	0,9	POS	0,4	NEG

Table 13. Better performance of the prototype also over other mutant panels and native mutants.

Discussion

The HBsAg detection is used for the diagnosis of acute and chronic hepatitis B virus infection and it indicates a potential infectivity. It is also useful as a follow up marker, since declining concentrations are observed in resolving hepatitis B.
The Liaison HBsAg assay is intended to be used as an aid in the diagnosis of HBV infection and as a screening test for donated blood and plasma. The HBsAg quantification has been demonstrated to be helpful to evaluate the response to treatment and for the early detection of drug-resistant strains, in addition to HBV DNA quantification.
The implementation of a certain HBsAg assay in the routine diagnostic work is guided by the ability to fulfill the following criteria: 1) high analytical sensitivity for the early recognition of HBsAg at the end of incubation period, 2) high sensitivity, 3) high specificity at screening, 4) high sensitivity for mutants recognition, 5) full automation with medium to high throughput.
The production of a more specific and sensitive assay able to detect mutants species is at the basis of this work. The Liaison HBsAg assay to be produced will be a chemiluminescent immunoassay (CLIA) for the quantitative detection of HBsAg in human serum and plasma.

Mutations of HBV S gene have been reported in infants born to carrier mothers or in individuals after liver transplant or blood transfusions despite the presence in each case of anti HBs antibodies. The “a” determinant, the surface conformational epitope spanning 101-159 amino acids, contains eight cysteine residues which form disulphide bridges to maintain the correct conformation of the “a” loop. Most of the antibodies found in patients sera are specific for the epitopes localized between 124-147 amino acids (90; 91). The mutations onset in this region (amino acid substitutions, insertions or deletions) can cause a conformational alteration which impairs the antibody-antigen interaction decreasing the vaccine efficacy; in these cases there is the development of the infection even in presence of anti-HBs antibodies
In addition to these ‘vaccine and immune escape mutants’, ‘diagnostic escape mutants’ have been described since most of the HBsAg detection assays are based on anti-HBs antibodies (4; 8). A number of HBsAg mutants which demonstrate different levels of reactivity depending on the commercial kit employed and the fact that certain mutants may be misclassified as negatives have been

reported in literature (105). Monoclonal antibodies against the wild-type “a” determinant used in conventional HBs antigen diagnostic methods fail to find occurring HBV infection (4; 91).
The importance to produce a novel quantitative immunoassay to detect with high sensitivity mutants form in blood patients screening is required; moreover the improvement of the sensitivity detection will aid to rescue the patients as soon as the infection onsets (125).
For all these reasons, in this work a quantitative determination of HBsAg with a direct two steps “sandwich” chemiluminescent immunoassay has been developed.
Comparable sensitivity against mutants detection is assured by using seven mouse monoclonal antibodies directed against epitopes on the surface, on transmembrane region (highly conserved and not subjected to mutations) and inner hydrophilic region.
Three of these seven antibodies are linked on paramagnetic particles used as solid phase and allowed to react with the sample for first. When this first immunocomplex has been formed, the remaining four antibodies are added as labeled probe to generate a chemiluminescent signal directly proportional to the HBsAg concentration present in samples. The antigen at this point is directly bound by antibodies from the solid phase on one side and the tracer on the other in a way of sandwich: from this, the assay format is a direct two step sandwich immunoassay with two incubations, each of them followed by a wash cycle to remove the unbound material to guarantee the antigen quantitative determination.
Abbott with the Architect system, the competitor reference method, and very recently Roche with Modular system, are the competitors that are currently available to offer to the customers the HBsAg quantification. All the other competitors are selling tests for qualitative determination of HBsAg.
The Liaison kit has been demonstrated to have sensitivity at 0.05 IU/ml as well as Abbott Architect but with an higher specificity: Liaison system has the sensitivity target limit around 1400 RLU while Abbott only 1100 RLU too near than our prototype to the negative sample distribution as demonstrated in our optimization laboratory (DiaSorin, Saluggia) during the final tests and comparisons with competitors. This could imply an higher, even if rare, risk to get near to an equivocal zone (negative-positive overlapping zone) in which high or false positive could be misclassified with low or false positive, as occurs with our actual marketed kit for which the specificity is 94-98% and has to be improved. This will be not foreseen by our system for which the specificity target requirement is reached and overcome (99.8/99.9% instead of 99.5% required) and the sensitivity target at 0.05 IU/ml, with a RLU value which is over the negative population

RLU average with ten time the standard deviation (the cut-off beyond which only positive samples are present), is reached. This better sensitivity increases the distance from the negative population reaching in this way a specificity close to 99.9%; this allows to reduce the overlapping zone between negative and positive populations distribution and thus the chance to find false positive or negative samples.

In addition to this, the Liaison quantitative HBsAg will be able to avoid false negative results deriving from HBsAg variants without compromising the analytical sensitivity or the diagnostic specificity. To assure the ability of the monoclonal antibodies to react with their transmembrane epitopes, a particular buffer was developed and patented (91) to destroy the lipid bilayer of the antigen. It is then used during the monoclonal antibodies screening to select those able to resist in this harsh environment. This represents an added value of the new assay to be produced over the already existing in-house and competitor assays.

A collection of ten recombinant HBsAg mutants was produced in-house to have utility in assessing the performance characteristics of the existing commercial assays and the prototype under development. Abbott Architect and Modular Roche as the two main competitors as well as the marketed Liaison HBsAg assays are not able to detect every component of this panel; the same situation occurs against other mutants panels which are not completely detected by the aforementioned commercial systems. The new assays would be the only one able to detect all variants due to the presence of its monoclonal antibodies.

The improvements achieved with this new prototype are in part due to the use of a particular ABEI derivative which allows one to have a considerable light signal in synergy with the employment of seven very specific monoclonal antibodies able to detect also mutant species. The novel chemiluminescent derivative will be briefly under patent and has the feature to be obtained in a purer form around 98% contrary to the 60-70% of purity of the ABEI molecule which is also less stable. The ABEI derivative is more stable at storage conditions and after two months its purity is still 90% while the simple ABEI tends to convert in its correspondent acid form. Due to this, the lower incorporation factor, with four times less consumption of probe molecules, needed to generate high light signal enables to have a “cleaner” background, lower negative values and higher positive values enhancing the separation between negative and positive populations. This allows also to strongly reduce the risk of outliers outcome as it happens sometimes with the marketed kit.

Generally this novel immunoassay is easy to produce and

industrialize and all its component are highly reproducible.
In conclusion, the results obtained in this work demonstrate the high potential and value of the new HBsAg kit and the efficacy in detecting all the variants tested, contrary to other commercial assays of competitors, with high sensibility and specificity; it is the best candidate to replace the actual Liaison immunoassay.
Many validation tests, foreseen by the European legislative decree 2009/565/CE, which determines the requirements to be achieved by a diagnostic test to be commercialized within the European community, are ongoing. This implies a minimum number of positive and negative specimens, standard and seroconversion panels to be tested to check the analytical sensitivity, the diagnostic sensitivity and the specificity.
As soon as the last validation and clinical tests will be performed and checked the new Liaison quantitative HBsAg II in CLIA format will be marketed.

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RIASSUNTO

L'infezione da virus dell'epatite B (HBV) è molto diffusa a livello mondiale: 4,5 milioni di persone vengono infettate ogni anno con un tasso di mortalità di mezzo milione. L'infezione da HBV è prevalente in Asia, Africa, Europa del Sud e Sud America. Il 5-10% delle persone infette non è in grado di produrre anticorpi per debellare il virus e l'infezione diventa cronica; i portatori cronici dell'HBV presentano un elevato rischio di cirrosi e carcinoma epatico.

L'HBV è un virus a DNA e codifica per tre proteine antigeniche: l'antigene di superficie (HBsAg, il costituente principale dell'involucro virale), l'antigene "e" (HBeAg) e l'antigene del core (HBcAg). L'organismo infettato produce anticorpi diretti contro queste proteine; anticorpi e antigeni (in particolare l'HBsAg) costituiscono i marker serologici per la diagnosi dell'infezione. Recentemente, alcuni saggi immunologici quantitativi per la rilevazione di HBsAg e HBeAg hanno dimostrato che l'utilizzo di questi marker è molto idoneo per la valutazione dell'efficacia e della risposta delle terapie antivirali.

L'individuazione del virus in donne in gravidanza o in donatori di sangue, inoltre, eviterebbe la trasmissione dell'HBV attraverso le trasfusioni e ridurrebbe drasticamente la trasmissione dalla madre al feto.

L'antigene HBs è una proteina di membrana di 226 aminoacidi con quattro domini transmembrana; il determinante "a" è un epitopo conformazionale compreso tra gli aminoacidi 101-159: in questa regione sono presenti otto cisteine impegnate nella formazione di ponti disolfuro che consentono il mantenimento della corretta conformazione del determinante detto anche "a" loop. La maggior parte degli anticorpi presenti nel siero di pazienti infetti da HBV, sono diretti in modo specifico contro epitopi localizzati nella regione compresa tra gli aminoacidi 124-147. Le mutazioni che insorgono in questa regione (siano esse sostituzioni aminoacidiche, inserzioni o delezioni) possono provocare alterazioni conformazionali che sfavoriscono l'interazione dell'antigene con gli anticorpi. Questi mutanti non sono responsivi ai vaccini e l'infezione si sviluppa ed evolve anche in presenza di anticorpi anti-HBsAg (i cosiddetti "vaccine escape mutants"). Molti anticorpi monoclonali utilizzati nei convenzionali kit diagnostici sono diretti contro la regione wild-type

del determinante '"a" e per tale ragione questi saggi non sono in grado di diagnosticare l'infezione da HBV di forme mutanti (definiti "diagnostic escape mutants").
Mutazioni sull'antigene di superficie sono state individuate in bambini nati da madri portatrici o in individui sottoposti a trapianti o trasfusioni nonostante la presenza di anticorpi o l'assenza di infezione rilevabile nei donatori.
Risulta evidente la necessità di ottenere kit diagnostici quantitativi altamente sensibili in grado di rivelare la presenza del virus nei primissimi stadi della malattia e che consentano di seguire l'evolvere della malattia e la risposta ai trattamenti antivirali nonché la capacità nel diagnosticare la presenza di forme mutanti del virus.
Presso i laboratori DiaSorin è stato sviluppato un saggio immunochimico completamente automatizzato attraverso l'utilizzo della piattaforma Liaison® che sfrutta il fenomeno della chemiluminescenza. La tipologia di saggio prevede l'utilizzo di una fase solida (microparticelle paramagnetiche) su cui vengono immobilizzati tre anticorpi monoclonali diretti contro l'HBsAg: il primo riconosce una regione del determinante "a", il secondo una regione transmembrana e il terzo una regione idrofilica interna alla particella virale. In una prima incubazione il campione reagisce con la fase solida in presenza di un buffer in grado di distruggere la membrana lipidica dell'involucro virale consentendo alle regioni interna e transmembrana di interagire con i rispettivi anticorpi. Il saggio prevede inoltre un tracciante che in questo caso è costituito da altri quattro anticorpi monoclonali marcati con una molecola chemiluminescente che genera un segnale proporzionale alla quantità di antigene presente nel campione in esame. Il segnale luminoso è misurato da un fotomoltiplicatore ed espresso in unità di luce relativa (RLU). Due degli anticorpi costituenti il tracciante sono diretti contro il determinante "a", un terzo verso un dominio idrofilio interno e il quarto verso una regione molto conservata adiacente l'"a" loop.
La molecola chemiluminescente è un derivato dell'amino-butil-etil-isoluminolo (ABEI) con buone caratteristiche di purezza, stabilità e riproducibilità che hanno permesso di ottenere un miglioramento nella sensibilità e specificità del saggio rispetto all'utilizzo del vecchio ABEI. Questa molecola in combinazione con l'utilizzo di sette anticorpi monoclonali molto specifici hanno permesso di ottenere un

prototipo di saggio quantitativo per l'HBsAg molto sensibile in grado di identificare la presenza di mutanti. Questo consente di dare al nuovo prototipo un valore aggiunto rispetto ai competitori presenti sul mercato che, se da una parte hanno caratteristiche di specificità e sensibilità simili al nuovo prototipo, dall'altra non sono in grado di rilevare la presenza di tutti i mutanti testati compresa una libreria di mutanti prodotta da DiaSorin per valutare le prestazioni sia del nuovo saggio che di quelli già presenti sul mercato.
Non appena le fasi di validazione e le prove cliniche previste dalla normativa europea per la commercializzazione di un saggio diagnostico all'interno della comunità CE verranno ultimate, il nuovo saggio quantitativo diretto contro l'antigene dell'epatite B potrà essere commercializzato.