Development of a Microtitre Plate Assay and a Rapid Membrane-Based Assay for the Detection of Antibodies to Hepatitis C Virus (HCV) in Human Serum

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by

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DECLARATION

I hereby certify that the material, which I now submit for assessment on the programme of study leading to the award of Master of Science, is entirely my own work and has not been taken from the work of others save to the extent that such work has been cited and acknowledged within the text of my work.

Signed ....Grainne Lynon..... Date...12th Sep 2000...

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ABBREVIATIONS

[H]	Reduction reaction
α-interferon	alpha interferon
ε	Molar coefficient
µl	Microlitres
3′	3 terminal
5′	5 terminal
A	Absorbance at 280nm
Ab	Antibody
Ag	Antigen
ALT	Alanine aminotransferase
AMP	Amino-methyl-proponal
Anti-D	anti- immunoglobulin D
AP	Alkaline phosphatase
AST	Aspartate aminotransferase
BBI	Boston Biomedica Incorporated
BCIP	Bromo-chloro-indolyl-phosphate
BGG	Bovine gamma globulin
BSA	Bovine serum albumin
BTSB	Blood Transfusion Service Board
c	Concentration
C	Core
CCB	Carbonate coating buffer
CFLP	Cleavase fragment length polymorphism
DAGS	Double antigen sandwich assay
DNA	Deoxyribonucleic acid
E	Envelope
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ET-NANB	Enteric non-A, non-B hepatitis
FBS	Foetal bovine serum
FDA	Food and Drugs Association
gp	Glycoprotein

GS	Goat serum
HSA	Human serum albumin
HAV	Hepatitis A virus
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcAg	Hepatitis C virus core antigen
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HGV	Hepatitis G virus
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ID	Identification
IEI	International Enzymes Incorporated
IFA	Immunofluorescence assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Ind.	Indeterminate
Int.	Intrepretation
kDa	Kilo daltons
KPL	Kirkegaard and Perry Laboratories
l	Light path
LH	Luteinizing hormone
M	Matrix
M	Molarity
m.w.	Molecular weight
mRNA	Messenger ribonucleic acid
NANBH	non-A, non-B hepatitis
NCR	Non-coding region
Neg.	Negative
NS	Non-structural
NS1	Non-structural protein 1

NS2	Non-structural protein 2
NS3	Non-structural protein 3
NS4	Non-structural protein 4
NS5	Non-structural protein 5
NSB	Non-specific binding
OD	Optical density
OPD	o-Phenylenediamine
ORF	Open reading frame
P/N	Positive to negative ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
POC	Point-of-care
Pos.	Positive
R	Protein chain
R′	Secondary protein chain
RdH	Riedel de Hein
Rh	Rhesus
RIA	Radioimmunoassay
RIBA	Rapid immunoblot assay
RNA	Ribonucleic acid
RS	Rabbit serum
RTD	Rapid test device
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Seconds
S	Structural
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sens.	Sensitivity
SOD	Superoxidedismutase
Spec.	Specificity
TBS	Tris buffered saline
TMB	Tetra methyl benzene
v/v	volume per volume
w/v	weight per volume

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ABSTRACT

Hepatitis C virus (HCV) was discovered in 1989 and was identified as the causative agent of non-A, non-B hepatitis. Third-generation ELISA techniques, incorporating the use of antigens specific to all hepatitis C viral regions, are used to diagnose HCV infection by serum antibody determination. Rapid immunoblot assay (RIBA) testing identifies antibodies specific to structural and non-structural viral proteins. RT-PCR is established as the gold standard of HCV RNA detection. A positive result on the developed assays does not indicate that the hepatitis C virus is present and replicating. A confirmatory test is needed to assess current HCV status and to monitor treatment.

The development of qualitative anti-HCV immunoglobulin G antibody tests is described in this study. A microtitre plate assay, suitable for multiple sample screening was optimised for use in standard laboratories. The rapid membrane-based assay, developed for use at point-of-care, requires no sample preparation. It is a user-friendly assay requiring no laboratory equipment or operator training. Both formats utilise bound synthetic peptides specific for non-structural and core regions of the HCV genome. Optimisation of assays was based on testing with characterised sera. Performance evaluation of the plate assay was carried out on 530 samples achieving 97.9% specificity and 93.6% sensitivity. A total of 751 samples evaluated on the rapid assay resulted in 99.4% specificity and 98.9% sensitivity.

Section 1-

Introduction

1.1 History of Viral Hepatitis

1.1.1 Blood Borne and Food Borne Hepatitis

Hepatitis results in the abnormal function of the liver due to the action of specific pathogens, causing necrosis and inflammation of hepatic cells by multiple causative agents such as bacteria, drugs, toxins, excessive alcohol intake and a number of viruses. Research has revealed that viral hepatitis encompasses six major viral agents with distinctive serologic characteristics and specific epidemiological attributes each with their own infective implications. The viruses so far identified are hepatitis B, hepatitis A, hepatitis D (delta hepatitis), hepatitis C, hepatitis E and hepatitis G. There are two primary types of viral hepatitis, food-borne hepatitis (hepatitis A and E) and blood-borne hepatitis (hepatitis B, C, D and G) (Koop, 1998a).

In 1970, electron microscopy was used to identify hepatitis B virus (HBV); a 42mm double-shelled round particle (Dane *et al.*, 1970), formally called serum hepatitis. HBV is much more prevalent and more infectious than HIV and is transmitted through blood and body fluids such as seminal fluid, vaginal secretions, breast milk, tears and saliva. It contains a partially double stranded DNA genome that replicates via a single stranded full length mRNA reverse transcribed into a minus strand genomic DNA (Will *et al.*, 1982). Hepatitis A (infectious hepatitis) was discovered by immunoelectron microscopy in 1973 and is a single stranded RNA virus (Feinstone *et al.*, 1973). It is transmitted by contaminated drinking water and food and generally depends on the hygienic and sanitary conditions in a given area. Hepatitis D virus (delta hepatitis, HDV) first described in 1977, is a particle of about 36nm diameter with a HBsAg envelope derived from HBV, and a ribonucleic genome (Rizzetto *et al.*, 1977). HDV encodes its own protein, the delta antigen but cannot form infectious particles by itself. It depends on the helper functions of HBV to replicate and survive. The disease is usually self-limited, and due to its co-dependence on HBV, HDV infection is prevented via the HBV vaccine. Hepatitis C virus (HCV) was identified in 1989 as a single stranded RNA molecule with a diameter between 40nm and 80nm (Choo *et al.*, 1989). It accounts for the majority of non-A, non-B hepatitis cases. In 1988 hepatitis E was shown to be an enteric non-A,

non-B hepatitis (ET-NANB). It is a non-enveloped spherical single stranded RNA virus, commonly found in the Indian Ocean, Africa and in underdeveloped countries and is associated with waterborne hepatitis epidemics in these countries (Bradley, 1988). The symptoms and modes of transmission of hepatitis E resemble those of hepatitis A. Hepatitis G virus (HGV) is an RNA virus and was identified from the plasma of a patient suffering from chronic hepatitis (Linnen *et al.*, 1996). It is, therefore, known to be a causative agent of acute and chronic hepatitis and is distantly related to hepatitis C virus.

1.1.2 Discovery of Hepatitis C Virus (HCV)

Accumulated knowledge of the structures of HBV and HAV in the early 70's and 80's, respectively, led to the development of reasonably sensitive and accurate serodiagnostic tests. This greatly reduced the transmission of transfusion-associated hepatitis. However, it became apparent that the majority of patients with hepatitis associated with transfusion were seronegative for HBV and HAV. Cases of viral hepatitis that cannot be attributed to Hepatitis A or B viruses or other known hepatotropic agents, such as Cytomegalovirus, were referred to as non-A, non-B hepatitis (NANBH). The agent causing NANBH eluded identification for almost twenty years, and unlike other hepatitis viruses, was identified after a study aimed at its discovery. Serological and immune electronmicroscopy, successful in identifying HAV and HBV, failed to detect this NANBH agent identified in 1989 as hepatitis C virus (Choo *et al.*, 1989).

Transmission experiments in chimpanzees confirmed the presence of at least two transmissible agents of NANBH, one of these being HCV, the other agent which is chloroform non-sensitive has yet to be identified but is a sporadically occurring, community acquired non-A, non-B hepatitis. Through these transmission studies in chimpanzees, it was established that the main agent of post-transfusion NANBH was likely to be an enveloped virus with a diameter between 40-80nm (Bradley *et al.*, 1987). These studies involved the pooling of chimpanzee plasma known to contain a relatively high titre of the agent. Most of these sera were shown to contain less than 1,000 infectious particles per ml of serum and only after serial passaging of HCV in animals was it possible to obtain sera with an unusually high titre (1,000,000 particles/ml) (Bradley *et al.*, 1983, 1985; Choo *et al.*, 1989).

In order to clone the viral genome, the virus was first pelleted from the plasma pool. A denaturation step was included prior to the synthesis of complimentary DNA so that either DNA or RNA could serve as a template. This was done because it was not known whether the genome was DNA or RNA. The resulting cDNA was inserted into a bacteriophage expression vector and led to the detection of a clone, using serum obtained from a patient with chronic non-A, non-B hepatitis. This clone was found to bind antibodies present in the sera of several individuals infected with the agent (Choo *et al.*, 1989). This clone was used as a probe to detect a larger, overlapping clone. It was possible to demonstrate that these sequences hybridised to a positive-sense RNA molecule of around 10,000 nucleotides which was found to be present in the livers of infected chimpanzees but was not detectable in uninfected individuals. It was possible to use newly detected overlapping clones as hybridisation probes to further select virus-specific clones. Thus, the entire HCV genome was sequenced and the complete nucleotide sequence was determined as shown and is presented schematically in Figure 1a (see section 1.2).

1.2 Structure of the Viral Genome

In spite of the considerable amount of knowledge that has so far been accumulated the physical structure of HCV remains essentially unknown. Hepatitis C virus is only about 50 nanometres in diameter and is so small its colour cannot be determined. 200,000 hepatitis C viruses placed together would measure a single centimetre. The transmission studies described demonstrates the HCV genome to be a single stranded RNA molecule of positive polarity containing approximately 10,000 nucleotides which encode a polypeptide of about 3,000 amino acids (Figure 1a) (Choo *et al.*, 1989).

1.2.1 Anatomy of the Virus

The low density of the virus and its sensitivity to organic solvents suggest that the virus has a lipid containing envelope. A core of genetic RNA is surrounded by a protective shell of protein and further encased in the lipid envelope. These features suggest that the genetic organisation of HCV is similar to that of viruses of the Flaviviridae family, such as the flaviviruses and pestiviruses. The fact that the genetic material is RNA, rather than the more stable DNA, gives hepatitis C its reactivity and dangerous ability to mutate which has important consequences in the life cycle of the virus. Mutations occur frequently and randomly across the entire length of the viral RNA producing mutated forms, which are so different from their ancestors that the immune system cannot recognise them (Koop, 1998b). Hepatitis C requires only a small amount of RNA to encode its core information giving fewer common characteristics that can be identified by the immune system.

The quantity of polypeptides encoded by the positive stranded RNA genomes is similar to the Flaviviridae family being 3,000-4,000 amino acids (Figure 1a). Also, the HCV proteins are encoded in a single large open reading frame (ORF) that is preceded at the 5′ terminal end by an untranslated highly conserved sequence (Crivelli, 1991). This region is resistant to denaturation, possibly because of protein linkage. The polyprotein encoded by the flaviviruses is processed into structural (S) proteins and non-structural (NS) proteins. The structural proteins form the viral particle whereas the non-structural proteins are involved in replication of the virus. Similar characteristics have been

identified for the HCV genome (Choo *et al.*, 1991; Takamizawa *et al.*, 1991). The HCV genome ORF codes for a polypeptide containing structural proteins at the 5′ terminus and non-structural proteins at the intermediate and 3′ regions. The HCV genome sequence encodes structural proteins designated E (envelope), M (matrix) and C (nucleocapsid or core) followed by non-structural proteins NS1, NS2, NS3, NS4 and NS5 originating from the 3′ terminal. Therefore, based on similarities between the HCV genome and that of the flavivirus and pestivirus family, HCV is classified as a separate genus within the flaviviradae (Heinz *et al.*, 1992).

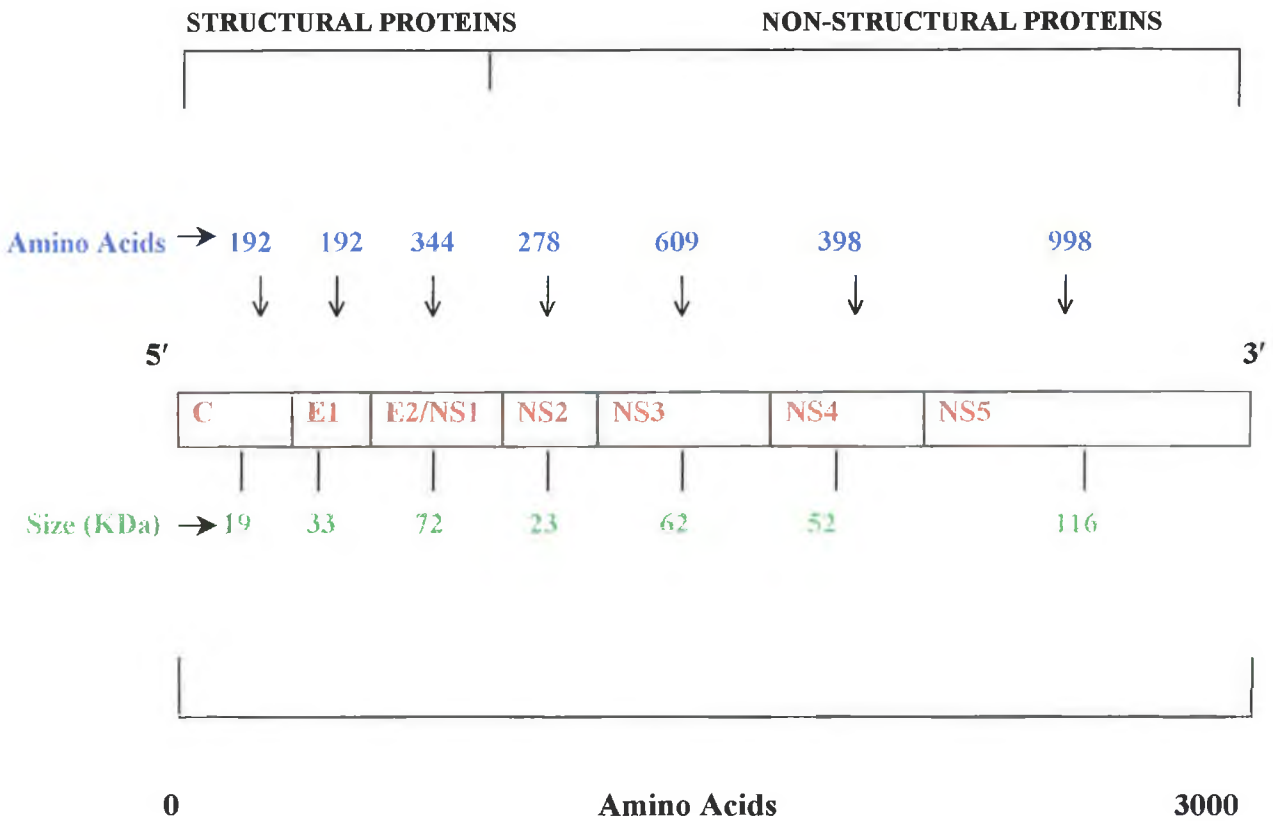


Figure 1a. Structure of the HCV genome, showing the size (green) and quantity (blue) of the amino acids forming the structural and non-structural proteins (red).

As discussed, cloning of the HCV genome led to its proposed classification, but identification of all the native proteins of the long ORF can only be assumed by the proposed homology with the other flaviviradae family members. This is because it has not been possible to infect cell cultures or animals other than chimpanzees, and natural infections generally produce only low viral titres lacking precipitating antibodies capable of concentrating virions for visualisation by electron microscopy.

1.2.2 Proteins of the Viral Genome

As can be seen in Figure 1a, the first of the structural proteins originating from the highly conserved amino N-terminus is the core protein, C, consisting of 192 amino acids. Possibly the first 70 of these is the matrix protein which is based on the homology with members of the flaviviradae (Takamizawa *et al.*, 1991). The core protein complexes with the genomic RNA to form the nucleocapsid. The amino acid sequence of this protein seems well conserved among different HCV isolates. The next product is a 33kDa glycoprotein found in the viral envelope and is known as E1 or gp35 (Takamizawa *et al.*, 1991). A second envelope glycoprotein E2, may be generated after cleavage by a protease within the viral polyprotein. This 72kDa glycoprotein, gp72 or E2/NS1, comprising 344 amino acids, present at the NS1 position makes HCV more similar to the pestiviruses rather than to flaviviruses. Significant variability has been found among different HCV isolates and 60% of the amino acid changes have been attributed to the heterogeneity observed in the hypervariable domain at the junction between the E1 and NS1/E2 region gp33 being more conserved than gp72 (Takeuchi *et al.*, 1990; Weiner *et al.*, 1991).

The non-structural regions of the viral genome show greater homology with flaviviruses and pestiviruses. This more variable genomic region includes five proteins. NS1 partly encodes the gp72 glycoprotein while NS2 containing approximately 278 amino acids and NS4 containing 398 amino acids, are thought to be membrane-binding proteins. NS3, with 609 amino acids is thought to be a viral helicase and contains an N-terminal protease. NS5, a 116kDa protein comprising 998 amino acids it thought to be the replicase, an RNA-dependent RNA polymerase (Miller and Purcell, 1990).

1.2.3 Genotype Determination

Following the discovery and identification of HCV, world-wide viral isolates were sequenced and much information on HCV diversity was accumulated. The unstable nature of the RNA molecule provides a mutagenic factor allowing the virus to develop new genetic variants of itself. The isolates were separated into distinct genotypes with different genotyping methods proposed and attempted. The importance of the determination of HCV genotypes has been established for a number of reasons. Viral genotype has emerged as a clinically significant variable prognostic for the stage of

development of HCV infection (Young *et al.*, 1993). Several genotypes have unique geographical origins, making them convenient markers in epidemiological studies (Zein *et al.*, 1996).

After the discovery of HCV, viral isolates from different geographical locations all over the world showed it to be a highly diverse virus. Different research groups have separated these isolates into distinct genotypes. Each major genotype may differ in their biological effects in terms of replication, mutation rates, type and severity of liver damage and detection and treatment options. The use of various genotyping methods and genomic regions for genotyping raised the question of whether a direct comparison could be made between results from one system and another. To resolve the issue, a standard nomenclature system was drawn up and agreed upon by HCV research groups (Table 1a, Column 7).

Okamoto/ Mori	Chayama	Simmonds	Kohara	Enomoto	Houghton/ Chaa	Proposed system
I	I	1a	I	PT	I	1a
II	II	1b	I	K1	II	1b
						1c
III	III	2a	II	K2a	III	2a
IV	IV	2b	II	K2b	III	2b
			II		III	2c
V		3			IV	3a
VI	V				IV	3b
		4				4a
					V	5a
						6a

Table 1a. Commonly used HCV genotype nomenclature systems based on standardisation by the following research groups: Tsukiyama et al., 1991; Chaa et al., 1992; Mori et al., 1992; Okamoto et al., 1992; Chayama et al., 1993; Simmonds et al., 1993; Simmonds et al., 1994.

Due to its sequence conservation, the 5′ non-coding region (5′ NCR) is the target for most HCV detection assays (Young *et al.*, 1993). However, because this region contains genotypically variable sequence positions, techniques for genotyping HCV have relied upon the examination of a limited number of diagnostic sequence polymorphisms in variable regions throughout the 5′ NCR (Marshall *et al.*, 1997). Restriction Fragment Length Polymorphism (RFLP) analysis (Constantine *et al.*, 1995), line probe assay (LiPA) analysis (Stuyver *et al.*, 1993), a second generation LiPA and RT-PCR amplification of the HCV 5′ NCR, with genotype-specific primers from the capsid encoding region, are four examples of such techniques.

An example of a second generation LiPA is the commercially available INNO-LiPA HCV II®. It is a fast, easy and specific DNA hybridisation test based on the 'reverse hybridisation' principle. The six major types of HCV can be readily distinguished in this assay, which features completely new sets of probes for types 4, 5 and 6 and new subtyping probes for types 1 and 2. Genotype-specific oligonucleotide probes are immobilised as parallel lines on ready-to-use membrane strips and are hybridised with target material labelled with alkaline phophatase.

All of these techniques have shown good concordance with DNA sequencing and are considerably simpler to execute than direct sequencing. However, they are restricted in terms of the number of types and subtypes detected and would be difficult to apply in geographic areas such as the Middle East and Far East where diverse genotypes predominate. Recent reports indicate such diversity is emerging in the U.S. and Europe as a result of considerable immigration and travel, necessitating a more broadly based means of genotyping samples (Zein *et al.*, 1996).

Routine direct sequencing, as described above, is not often feasible in a clinical laboratory. The high cost and low throughput of DNA sequencing for the routine analysis of the 5′ NCR prompted the development and application in 1997 (Third Wave Technologies, Inc., Madison, Wis.) of a novel DNA-based technology called Cleavase Fragment Length Polymorphism (CFLP). The CFLP technology relies on the formation of unique secondary structures that result when DNA is allowed to cool following brief heat denaturation (Orita *et al.*, 1989). These structures serve as substrates for the structure-specific Cleavase I enzyme, which can be used to generate a set of cleavage products from any given DNA fragment (Brow *et al.*, 1996). Formation of these

secondary structures is exquisitely sensitive to nucleotide sequence and the presence of sequence polymorphisms results in the generation of unique collections of cleavage products or structural fingerprints, for each sequence analysed. CFLP analysis exhibited complete correlation to nucleotide sequence data and was able to distinguish viral genotypes differing by as little as a single nucleotide. Blinded analysis of CFLP patterns compared to patterns from samples of known genotype resulted in assignments that were 100% consistent with DNA sequencing at the type and subtype levels. Furthermore, CFLP scanning detected variations within a given genotype, indicating that this method is suitable for identifying the appearance of new or rare HCV variants over other more costly and cumbersome genotyping approaches (Marshall *et al.*, 1997).

1.2.4 The Life Cycle of Hepatitis C

The hepatitis C virus must attach itself to and infect liver cells in order to reproduce. To do this it uses particular proteins present on its lipid coat to adhere to a receptor site. The protein core of the virus penetrates the plasma membrane and enters the cell. This is done by merging the lipid coat of the virus with the cell's outer membrane. The membrane then engulfs the virus and the viral core enters the cell. Enzymes present in liver cells aid in dissolving the protein coat to release the viral RNA in the cell.

The viral RNA then uses the cell's ribosomes to begin the process of viral reproduction. Viral RNA itself can be directly read by the host cell's ribosomes functioning like mRNA to synthesise RNA transcriptase. Once there is adequate RNA transcriptase, the viral RNA will make the new genetic material. Some of this RNA will contain mutations. The protective protein coat of the virus is produced by the ribosomes and assembles around the new viral RNA forming new viral particles. The viral particles form a spherical shell, called a capsid, that fully encapsulates the RNA. The completed particle is called a nucleocapsid.

The newly formed viruses travel to the inside portion of the plasma membrane and attach to it. The plasma membrane encircles the virus, then releases it, providing the virus with a protective lipid coat which it will use to attach to another liver cell. Each surviving virus can produce thousands of offspring resulting in significant damage to the liver (Koop, 1998c).

1.3 Clinical Infection of Hepatitis C Virus

Currently, there are approximately 200 million people world-wide who are infected with HCV. Chronic liver disease is the tenth leading cause of death among adults in the United States, and accounts for approximately 25,000 deaths annually, or approximately 1% of all deaths. According to the U.S.A. February 1999 consensus statement, HCV accounts for 20% of cases of acute hepatitis, 70% of cases of chronic hepatitis, 40% of cases of end-stage cirrhosis, 60% of cases of hepatocellular carcinoma and 30% of liver transplants (Shaw, 1999a).

HCV infection is the most common chronic bloodborne infection in the United States (Alter *et al.*, 1998). In combination with hepatitis B, it now accounts for 75% of all cases of liver disease globally. Most HCV-infected persons are aged 30-49 years (McQuillan *et al.*, 1997), this group of infected persons reaching ages at which complications from chronic liver disease typically occur. It is not surprising that the number of deaths attributable to HCV-related chronic liver disease could increase substantially during the next 10-20 years. Currently, 8,000 to 10,000 deaths each year in the U.S. are a result of HCV and it is estimated that by the year 2010, 30,000 to 40,000 patients will die annually of HCV related chronic liver disease.

HCV appears to be a weak RNA virus lacking the properties of retroviruses that survive in infected hosts by integration into cellular DNA. Nevertheless, it is believed to account for the majority of NANB hepatitis cases, causing 60% of acute cases and over 90% of chronic cases. The pathogenicity of HCV is peculiar, its mechanism capable of escaping host immune elimination and persisting in infected hosts causing various degrees of liver damage. The extent of liver damage is measured by the levels of liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the plasma. The presence of HCV-RNA in the liver and serum play a major role indicating that the virus is present and replicating. The spectrum of HCV infection ranges from the acute phase, with possible progression to chronic hepatitis causing cirrhosis and hepatocellular carcinoma (HCC) (Crivelli, 1991).

1.3.1 Acute HCV Infection

Acute hepatitis C represents a clinically mild phase of infection. Research has shown that 75% of cases of acute HCV infection are relatively asymptomatic (Alter *et al.*, 1989; Aach *et al.*, 1991). 60% have no discernible symptoms; 20-30% may have jaundice and 10-20% may have non-specific symptoms such as anorexia, malaise or abdominal pain (Alter *et al.*, 1998). The incubation period of HCV appears to be from 15 to 160 days after administration of HCV-contaminated blood or blood derivatives, with a mode of 45 to 55 days. In the case of patients who have received clotting-factor concentrates, incubation periods of less than 30 days have been reported (Bamber *et al.*, 1981). However, a more realistic and common time factor of 2 - 26 weeks is reported between exposure to HCV and the development of clinical acute hepatitis (Alter *et al.*, 1989). The detection of serum antibodies indicates that seroconversion has taken place, the mean time estimated to be 2.3 weeks (Mattson *et al.*, 1992). In approximately 20% of cases the onset of symptoms might precede anti-HCV conversion.

In patients with transfusion associated hepatitis C, the time between HCV exposure and the onset of hepatic viral replication depicted by HCV-RNA has been found to be as short as one week (Farci *et al.*, 1991). In the cases of patients who do not develop chronic hepatitis but have acute self-limiting infections, seroconversion occurs much less frequently (Alter *et al.*, 1989; Nishioka *et al.*, 1991). Anti-HCV antibody can be detected in 80% of patients within 15 weeks after exposure, in approximately 90% within 5 months and in 97% by 6 months after exposure. Rarely is seroconversion delayed until 9 months after exposure.

Fluctuating serum ALT levels are a characteristic feature of hepatitis C infection (Alter *et al.*, 1989; Mondelli & Colombo, 1991). The significant association that exists between serum HCV antibody and HCV-RNA suggests that antibody may be considered an indirect marker of HCV infection. The presence of anti-HCV antibodies indicates that an individual may have been infected with HCV, may harbour infectious HCV and may be capable of transmitting HCV infection. However, the level of this antibody has been found to significantly reduce in patients recovering from acute hepatitis C (after 1 - 4 years). Its persistence indicates chronic hepatitis infection (Alter *et al.*, 1991).

1.3.2 Chronic HCV Infection

After acute infection 15-20% of patients appear to resolve their infection. Statistics show that 80 - 85% of acute hepatitis C sufferers follow a chronic course of infection (Drenstag *et al.*, 1983). No clinical or epidemiological features among patients with acute infection have been found to be predictive of either persistent infection or chronic disease (Alter *et al.*, 1998).

Regardless of the route of infection, approximately 60% of chronic patients progress to cirrhosis and possibly to hepatocellular carcinoma (HCC), resulting in significant morbidity and mortality rates. Chronic HCV is therefore associated with a wide spectrum of liver disease ranging from the asymptomatic carrier state to severe liver damage. These findings indicate that HCV is not necessarily pathogenic and genetic heterogeneity of HCV could be a major cause of such variability. To distinguish between patients with chronic HCV and carriers with unrelated liver disease, evidence of viral infection and the presence of virus replication markers is necessary.

In many patients, development of chronic liver disease is heralded by persistent elevations in serum ALT activity, presence and persistence of serum antibodies and continued presence of HCV-RNA not only in patient serum, but also in liver tissues. In most patients, progression of hepatitis C to cirrhosis averages around 20 years (Kiyosawa *et al.*, 1990) and the association of persistent ALT levels with chronic HCV infection remains unclear. In 1995, it was shown that 20 - 40% of patients in a study with viremia and chronic hepatitis gave normal liver test results with normal aminotransferase levels on multiple analysis over a decade (Hayashi *et al.*, 1997). Therefore, ALT determination cannot be used to exclude ongoing hepatic injury and long-term follow-up of infected patients is necessary to determine their prognosis. Some investigators have recently reported persistent hepatitis C viremia associated with both normal aminotransferase levels and normal or minimal changes on liver biopsy (Prieto *et al.*, 1995).

Various clinical and epidemiological factors influence the severity of chronic hepatitis C and the rate of its progression to cirrhosis. Such factors include age at infection, sex, duration of disease, serum ALT levels, co-occurrence of HBV or HIV infection and

alcoholism. Even the intake of moderate amounts of alcohol in patients with chronic hepatitis C might enhance disease progression. More severe liver injury observed in persons with alcoholic liver disease and HCV infection possibly is attributable to alcohol-induced enhancement of viral replication or increased susceptibility of cells to viral injury. The serum titres of HCV-RNA have been found to be higher in immunosuppressed patients than in HIV seronegative patients (Wright *et al.*, 1992). Also, recent studies conducted on patients with chronic HCV infection have found that distinct genotypes of HCV are associated with distinct manifestations of the disease (Marshall *et al.*, 1997).

1.3.3 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours in the world, particularly in Africa, China and South East Asia. In these high incidence areas, most cases of HCC are associated with HBV infection whereas in low incidence areas such as the United States, less than 25% of cases of HCC appear to be related to HBV infection (Di Bisceglie *et al.*, 1991).

The identification of HCV and analysis of HCV infection have shown an association between chronic HCV infection and HCC. In recent studies, it was found that HCC developed only in patients with abnormal ALT levels and so persistence of ALT elevations may prove to be a convenient marker for identifying increased risk of HCC in chronic HCV patients. Most studies have reported that cirrhosis develops in 10-20% of patients with chronic hepatitis C over a period of 20-30 years, and HCC develops in 1-5% with striking geographic variations in rates of the disease. Early studies for anti-HCV found evidence of infection in up to 75% of cases of HCC in Europe and Japan (Bruix *et al.*, 1991) whereas among HCC patients in the U.S., the prevalence of anti-HCV is generally low, ranging from 13 - 29% (Yu *et al.*, 1990). Therefore, HCV infection appears to play a less important role in the pathogenesis of HCC in the U.S. than in southern Europe and Japan. Positive strand HCV-RNA has been found in the liver tissue of patients with HBsAg-negative anti-HCV positive HCC confirming the association of HCV-RNA with HCC sufferers (Horiike *et al.*, 1993).

1.4 Treatment of HCV Infection

1.4.1 Disease Progression

Medical intervention and treatment of acute HCV infection is limited, as most cases are asymptomatic. For more than six months following initial infection, the disease is virtually undetectable. The symptoms are progressive in nature and often very mild. The most common symptom, commencing sometimes years after initial infection is fatigue. The signs and symptoms associated with symptomatic acute infection include mild fever, nausea and vomiting, rashes, abdominal pain, loss of appetite, painful muscles and joints and sometimes diarrhoea. Many cases are undiagnosed because such symptoms are suggestive of a flu-like illness. A minority of patients report dark urine and light coloured stool, followed by jaundice and itching of the skin.

Low level infection, in which the infected individual is virtually asymptomatic but still highly contagious, may continue for years, even decades before progressing significantly. The course of chronic liver disease is usually insidious, progressing at a slow rate without symptoms or physical signs in the majority of patients during the first two or more decades after infection. Frequently, chronic hepatitis C is not recognised until asymptomatic patients are identified as HCV-positive during blood-donor screening, or elevated ALT levels are detected during routine physical examination.

1.4.2 Acute HCV Treatment

In the case of excessive fatigue, frequent rest is advised. It is advised to avoid increasing stress on a damaged liver and so alcohol should be avoided. In cases of bleeding due to impaired synthetic liver function, replacement of coagulation factors takes place.

The use of antiviral agents has been chosen by hepatologists for the treatment of acute hepatitis C to prevent the progression to chronic hepatitis. Acyclovin has been used with little success and clinical trials have shown that natural β-interferon was effective in controlling disease activity (Omata *et al.*, 1991). Recombinant α-interferon showed no benefit for the long-term outcome of the disease.

1.4.3 Chronic HCV Treatment

Due to the severity of chronic HCV infection, effective treatments were looked at to reduce progression to cirrhosis and HCC. Those at greatest risk of such progression include anti-HCV positive patients with persistently high ALT levels, detectable HCV RNA and a liver biopsy indicating moderate necrosis or inflammation.

Ribavirin is a synthetic nucleotide, which proves effective against both DNA and RNA viruses (Fernandez *et al.*, 1986). It has been successful in lowering transaminase levels in chronic hepatitis C sufferers, although when used alone, it does not eradicate HCV-RNA from serum and so is not effective against hepatitis C viral replication. It produces side effects such as anaemia, a metallic taste, dry mouth, flatulence, dyspepsia, nausea, headaches, irritability, skin rashes and myalgia.

A more effective therapy was observed with the administration of the antiviral agent alpha interferon, (α-interferon). Also called leukocyte interferon, it is composed of as many as sixteen different proteins linked or twined together (Edelhart *et al.*, 1982). The object of anti-viral treatment of Hepatitis C virus infection is to eliminate virus replication and, thus, prevent the progression to HCV related chronic liver disease. Interferons are proteins normally produced by the immune system in response to a viral infection. The human body produces over 20 different types of interferons. They do not attack or destroy cells, or have much effect on the cell that stimulates its production. Once triggered by viral-RNA in an infected cell, it acts as a warning to surrounding cells. The protein part of the molecule, called interferoid, travels through the cellular fluid to the cell wall promoting the production of antiviral proteins. These antiviral proteins prevent the virus from replicating.

Patients with persistently normal levels of ALT or pregnant women should not be treated with interferon as treatment may actually induce liver enzyme abnormalities. The FDA (Food and Drugs Association) does not approve interferon for the treatment of persons between the ages of 18-60 years (Alter *et al.*, 1998). Contraindications to treatment with interferon include major depressive illness, apathy, irritability, cytopenias, hyperthyroidism, renal transplantation and evidence of autoimmune disease. Flu-like symptoms experienced in the first 2-3 weeks of therapy but diminish with continued treatment. Symptomatic therapy with Paracetomol of Ibrufen prior to the injection and

every 6 to 8 hours as needed, is usually sufficient to control the flu-like side effects, as does drinking plenty of water and regular exercise. Mild depression should be treated with anti-depressants while the patient receives interferon. Patients with severe or manic-depression or recent suicidal ideation should not be treated with interferon. Prior to the administration of interferon therapy, careful assessment should be made and the risks and benefits discussed with the patient, as often the treatment is more painful than the disease symptoms and for this reason is discontinued in 5-15% of cases. High iron levels in the liver may reduce the efficacy of interferon. Interferon can suppress the bone marrow and decrease the number of white cells and platelet cells in the blood. Close follow up with frequent monitoring is needed for early detection of bone marrow suppression (Shaw, 1999b). However, the majority of patients taking interferon are able to continue to work full-time and carry on their normal daily activities.

In 1986, prior to the identification of HCV, the first report on the use of α-interferon showed that most patients with chronic NANB hepatitis experienced rapid ALT normalisation when treated with interferon (Hoofnagle *et al.*, 1997). It succeeded in clearing HCV-RNA from serum in 33% and improving liver histology in 50% of patients treated. This was based on a thrice-weekly dosage of between 1MU/ml and 10MU/ml for 12 months (Davis *et al.*, 1989). However, the relapse rates after the completion of treatment was high (approx. 50%) with increased ALT levels and reappearance of HCV-RNA. Only about 25% of patients showed a long-term response to therapy. By using higher doses of α-interferon and continuing treatment for longer periods of time, a higher response rate is observed but the rate of relapse upon cessation of treatment is not significantly different. A study carried out in 1986 (Crivelli, 1991) established that response to α-interferon treatment is dependant on the dosage used and the duration of treatment. It also showed that a positive response is seen in parentally transmitted hepatitis C along with community-acquired cases. This was also evidence that patients with histology of chronic persistent hepatitis are those who had the highest response rate (67%) while those with cirrhosis had the lowest (48%), indicating that interferon response cannot easily be predicted in chronic HCV patients (Hoofnagle *et al.*, 1997).

It has also been observed that some subtypes of anti-HCV antibody may be useful for monitoring interferon therapy. While the antibodies against structural and some non-structural antigens are not predictive of a response, a reduction in NS4-specific antibodies and their disappearance were found to be associated with complete response

to interferon without relapse. Other antiviral antibodies are less helpful as they remain detectable even in the cases of complete interferon response without relapse.

1.4.4 Combination Therapy

Patients who have persistently abnormal ALT levels and detectable serum HCV-RNA after 3 months of interferon treatment are unlikely to respond to treatment and should be discontinued. In the case of such patients, a combination therapy has been introduced. The administration of interferon α-2b and ribavirin has been shown to offer a sustained response and eradication of HCV-RNA in chronic hepatitis patients (Schvarcz *et al.*, 1995). Clinical studies of patients treated with this combination have demonstrated a substantial increase in response, drug administration was discontinued in 8% of patients after 24 weeks and in 21% of patients treated for 48 weeks (Shaw, 1999b).

Combination therapy has been approved by the FDA for the treatment of chronic hepatitis C in patients who have relapsed following interferon treatment alone and may be approved in the future for patients who have not been treated previously. Further trials have yet to be conducted using the combination therapy to determine if this treatment can completely eradicate the infection or alternatively sustain the suppression of viral and liver disease activity. Side effects of combination therapy with interferon and ribavirin include those expected from the use of interferon along with those unique to ribavirin. The most common potentially dangerous side effect from ribavirin use is anaemia. Patients with pre-existing anaemia, borderline red blood cell counts or known heart disease should not be treated with ribavirin or should be closely monitored.

In the cases of chronic hepatitis C sufferers who relapse after the administration of combination therapy, a certain number proceed to develop cirrhosis and, to a lesser extent, HCC. Liver transplantation may be life-saving at this stage of liver disease but is costly and involves continuing health care. This treatment option is further complicated by a shortage of liver donors. For HCV-positive patients undergoing transplantation, re-infection is almost universal.

At present there is no effective vaccine for hepatitis C. This is largely due to the great variation in genotypes of hepatitis C along with its frequent mutation. Once the host has become infected, the virus creates different genetic variations of itself within the body of the host. Thus, if the immune system begins to succeed against one variation, the mutant

strains quickly take over and become new, predominant strains (Koop, 1998d). Thus, there is no guarantee that a treatment, test or vaccine against one strain will be effective against all of them. As a result, hepatitis C is usually not self-limited.

However, the result of extensive research has led to the possible development of a HCV vaccine. In the study, four HCV epitopes from conserved regions of the HCV genome were shown to be associated with strong T-cell responses in HCV-infected patients who fully recover from the disease (Shaw, 1999e). The epitopes were selected based on their conserved amino acid sequences among known HCV strains, reducing the likelihood of mutant strains escaping T-cell responses. Such epitopes, along with other epitopes previously reported to stimulate T-cell responses, represent candidates for the construction of a therapeutic T-cell based vaccine against HCV. T-cells are believed to play an important role in controlling HCV infection. Patients who have recovered from HCV infection have mounted T-cell responses whereas those who develop chronic infections have weak responses. To be effective the HCV vaccine must provide protection against multiple strains of the virus and must induce T-cells directed to conserved viral regions, otherwise the hepatitis C virus may be able to escape the immune system's attack.

In areas of high incidence of HCV infection, the total administration of a HCV vaccine could potentially be more cost-effective than serological antibody multiple screening. Such screening would, inevitably, be followed by the administration of the vaccine to patients with a negative screening response. In low incidence areas, the total administration of a HCV vaccine may be a better alternative than sample screening. The administration of a vaccine to low-risk people such as police and prison officers would also be of benefit. The effects of vaccine administration to recovered HCV patients would be limited, due to previous exposure.

Further research has revealed that a ribozyme, used to directly attack HCV-RNA, was shown to inhibit viral replication (Shaw, 1999f). The enzyme, which demonstrated highly specific and dramatic inhibition of hepatitis C viral replication, has been shown to have potent antiviral activity and is expected to be effective against all known HCV genotypes. When combined with the anti-viral drug, interferon, the ribozyme has been reported to demonstrate greater inhibition. The insight into the structure of the enzyme complex critical to the replication of HCV, points to novel targets for drug development.

1.5 Epidemiology and Modes of Transmission of HCV

1.5.1 Global Epidemiology

The existence of HCV infection has been recognised in virtually all parts of the world in which serological testing has been achieved. It is a global disease. Of the 200 million infected people world-wide over four million of those are in the United States and five million in Western Europe with the prevalence even higher in Eastern Europe. It occurs among persons of all ages, but the highest incidence of acute hepatitis C is found among persons aged 20-39 years and males predominate slightly (Alter *et al.*, 1990).

Hepatitis C shows significant genetic variation in worldwide populations, evidence of its frequent rates of mutation and rapid evolution. The basic genotypes and subtypes vary in prevalence across different regions of the world. The prevalence of hepatitis C is lowest in Northern European countries, including Great Britain, Germany and France. The prevalence of HCV antibodies in blood donors averages less than 1% for this region. Higher rates have been reported in Southeast Asian countries, including India (1.5%), Malaysia (2.3%) and the Philippines (2.3%). The incidence in Japan was 1.2%. Alarming rates were reported for many African nations, reaching as high as 14.5% in Egypt. These studies suggest that over 200 million people around the world are infected with hepatitis C, an overall incidence of around 3.3% of the world's population. Statistically, as many, if not more, people are infected with HCV as are with HIV.

The HCV carrier state appears to be more common at low socio-economic levels, probably because of higher rates of other cofactors. A higher prevalence of infection is found among the African Americans and Hispanics than is found among the white population. In the United States, anti-HCV was detected in 0.5% of white blood donors. In contrast, anti-HCV was detected in 7.1% of Hispanics and 8% of African Americans (McQuillan *et al.*, 1997). In the general population, the highest rates of infection are found within the ages of 30-49 years. High proportions of haemophiliacs are also infected, although current processes to inactivate the HIV virus also destroy the hepatitis C virus. This results in the reduced risk of uninfected haemophiliacs contracting the disease.

The greatest variation in the prevalence of HCV infection occurs among those with different risk factors for infection (Alter *et al.*, 1995). The highest prevalence is found among those with direct and repeated exposures to blood or blood products. The following table indicates these high-risk groups and highlights the various risk groups (Alter *et al.*, 1998).

High-risk	Moderate-risk	Low-risk
Intravenous drug users	Long-term haemodialysis patients	Health care workers/ Household contact
Haemophiliacs	Persons with evidence of multiple sex partners	Military personnel
Those treated with clotting factor concentrates	Persons with a history of sexually transmitted diseases	Volunteer blood donors
Recipients of transfusions	Persons with abnormal ALT levels	Infants born to infected mothers
Transplant patients		Homosexual men

Table 1b. List of various risk groups associated with HCV infection.

1.5.2 Transmission Modes of HCV

HCV is transmitted primarily through large or repeated direct percutaneous exposures to blood or blood products such as clotting factors or immunoglobulins. However, unlike many other blood borne viruses such as the HIV virus virtually any source of blood or blood products seems to be capable of carrying the virus, even an indirect source such as a used razor. This makes hepatitis C virus far more transmissible than most other blood borne viruses including HIV. The virus is also present in saliva and semen, but the level of infectious agent in these secretions does not appear to be high enough to pose a significant risk (Alter *et al.*, 1998).

The relative importance of the two most common exposures associated with transmission of HCV, blood transfusion and intravenous drug use, has changed over time (Alter *et al.*, 1997). In the United States 15 years ago, infection of HCV by blood transfusion

accounted for 20% of cases. Today, it rarely accounts for recently acquired infection. In contrast, injecting-drug use has consistently accounted for a substantial proportion of HCV infections and currently accounts for 60% of HCV transmission. A high proportion of infections continues to be associated with injecting-drug use, but for reasons that are unclear, the dramatic decline in incidence of acute hepatitis C since 1989 correlates with a decrease in cases among intravenous drug users.

Other modes of transmission of HCV include employment in patient care or clinical laboratory work, exposure to a sex partner or household member who has had a history of hepatitis, exposure to multiple sex partners, and low socio-economic level (Alter *et al.*, 1982). These studies reported no association with military service or exposures resulting from medical or dental procedures, tattooing, acupuncture, ear piercing or foreign travel. If transmission from such exposures does occur, the frequency might be too low to detect (Alter *et al.*, 1998).

The role of breast-feeding in transmission of HCV was investigated in a study on 42 healthy anti-HCV negative mothers and 65 asymptomatic mothers with anti-HCV antibody. Blood samples were collected from all infants at birth and at 1,3,6,9 and 12 months. All infants were breast-fed. By three months, five of the sixty-five mothers developed symptomatic liver disease and three of their infants developed acute viral hepatitis. None of the remaining infants had evidence of infection up to 1 year and all 42 mother-infant pairs from the control group remained negative. These results indicate that breast-feeding among symptomatic HCV mothers should be avoided (Shaw, 1999c). A study carried out in Germany in 1991 showed that children with HCV had a greater ability to fight the infection than do adults. The infection cleared without treatment in 50% of children who acquired HCV through heart surgery, whereas that number was only 20% in adults (Shaw, 1999d).

1.6 Diagnosis of HCV Infection

Methods of detection of HCV infection rapidly became available after the identification of HCV and its viral genome. The detection of serum antibodies and HCV-RNA has formed the basis for diagnosis of infection and of viral replication. Basic screening tests using the enzyme immunoassay (EIA) format involve the utilisation of recombinant and synthetic HCV antigens for the detection of anti-HCV antibodies in serum. Continued research and development produced an improved recombinant immunoblot assay (RIBA) which utilised individual structural and non-structural viral proteins. In a more expensive and cumbersome test, serum HCV-RNA is detected by a method known as reverse transcription polymerase chain reaction (RT-PCR), which is used as a confirmatory test if evidence is pointing towards viral infection (Crivelli, 1991).

1.6.1 Molecular Methods of Diagnosis

In chronic hepatitis C cases the detection of HCV-RNA is essential to determine if HCV replication is taking place. Serum and liver HCV-RNA is usually associated with the presence of anti-HCV antibodies but in a few individuals HCV nucleic acid can represent the only detectable HCV marker (Weiner *et al.*, 1991). The persistent detection of HCV-RNA has proven to be the only marker to differentiate acute from chronic carriers hence highlighting the importance of HCV-RNA detection. Polymerase Chain Reaction (PCR) can be used for the detection of HCV-RNA in serum, liver and mononuclear cells. The use of a thermostable polymerase allows the dissociation of newly formed complimentary DNA or RNA and subsequent hybridisation of primers to the target sequence with minimal loss of enzymatic activity.

The use of PCR involves a series of steps. The first of these steps is the synthesis of a complimentary DNA copy of the target region of the RNA genome using reverse transcriptase primed by the antigenomic PCR primer. The 5′ NCR has been shown to be highly conserved and so is the preferred target region for PCR. The concentration of virus in serum samples is often very low, so that the mass of product from the PCR reaction is insufficient for visualisation on a stained gel. This necessitates a second round

of amplification, which utilises Southern hybridisation to detect the primary product. The efficiency of detection of HCV-RNA is dependent on the conservation of samples, the amount of specimen available and the specific primers used.

Amplification of viral nucleic acid by PCR provides a highly sensitive antibody independent mode of detecting viral infection and, therefore, has become the gold standard in laboratory diagnosis. HCV-RNA occurs in the blood before other markers and often within days of infection. Results of PCR tests for HCV-RNA have been compared to those of antibody tests from second and third generation assays.

In a study carried out in 1991 the PCR-based test was compared to supplementary anti-HCV assays (Widell *et al.*, 1991), Ortho anti-HCV (C100-3) ELISA™, Abbot anti-HCV (C100-3) ELISA™, RIBA-1, RIBA-2 and an antibody consumption test referred to as a 'neutralisation' assay. Results confirmed that the presence of HCV-RNA by PCR correlated well with anti-HCV reactivity indicated by the above testing methods. The pattern of HCV antibody markers and HCV-RNA during infection is represented in Figure 1b.

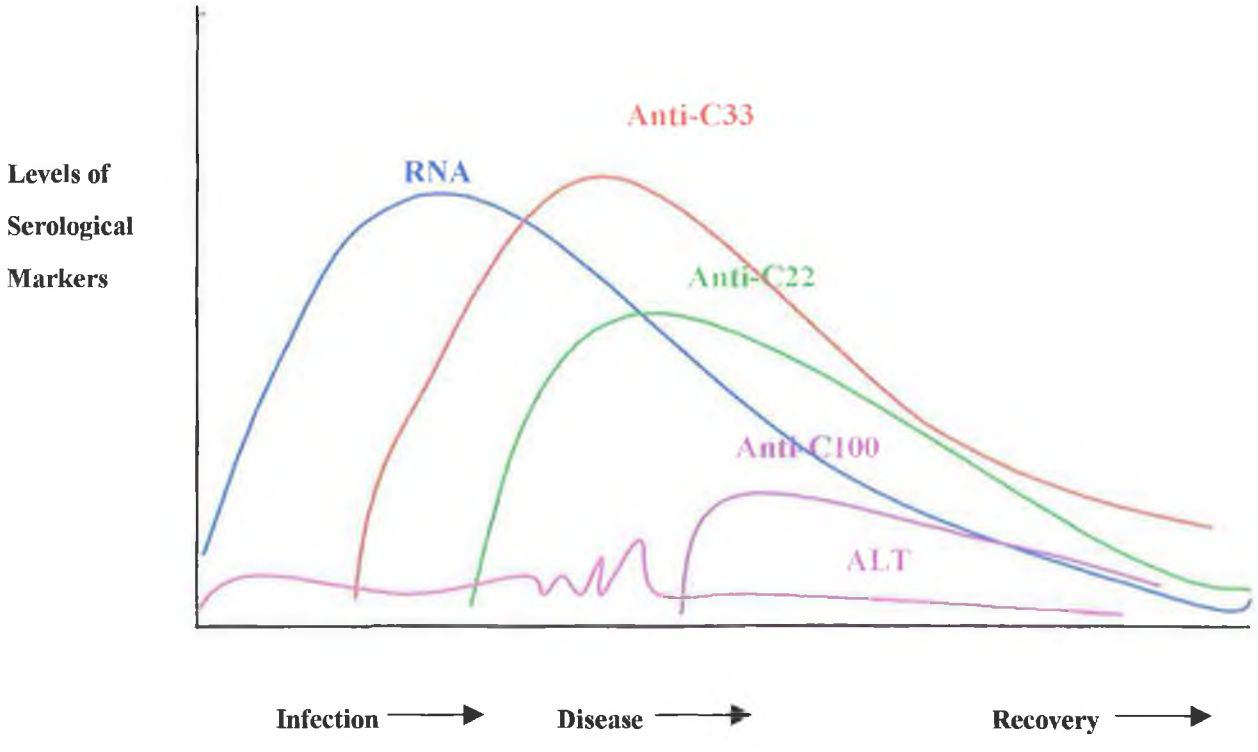


Figure 1b. The pattern of various HCV serological, RNA and ALT enzyme markers during HCV infection, disease and recovery (Crivelli, 1991).

The presence of HCV-RNA detected by PCR has been used in monitoring patient response to treatment. In patients undergoing interferon therapy, HCV-RNA disappeared from the serum of responders, with persistently normal liver function tests checked monthly for 24 months after cessation of treatment. HCV-RNA was detected continuously in the serum of individuals who did not respond to treatment. These findings indicate the role of PT-PCR for monitoring antiviral therapy in chronic HCV infection.

The success of PCR for the detection of serum HCV-RNA has been established, but PCR has also detected HCV-RNA in liver biopsy samples from patients with chronic hepatitis C and confirmed by Southern blotting (Shieh *et al.*, 1991). The presence of minus-strand HCV-RNA has been detected in liver specimens of chronic hepatitis C sufferers, but not in serum samples indicating that HCV-RNA replication predominates in the liver. While the detection of HCV-RNA in liver tissue has succeeded in determining the site of HCV-RNA replication, the role of serum marker assays is invaluable as a simple method used for the early detection of anti-HCV antibodies.

The most accurate way of assessing the severity of liver damage is by liver biopsy. This will establish the activity of the disease and whether or not permanent damage has occurred. Ultasound examination of the liver and CT scan are not reliable to assess liver damage. HCV infection affects the liver by causing inflammation leading to death and destruction of cells. If the virus is controlled, inflammation can disappear and the liver can regenerate cells. Infection also causes damage by producing fibrosis. Both inflammation and fibrosis can be determined by liver biopsy, a biopsy with mild inflammation and no fibrosis indicates that progression to severe liver disease is unlikely. On the other hand a biopsy with the presence of fibrosis indicates a likely progression to cirrhosis (Shaw, 1999b).

1.6.2 Serological Methods of Diagnosis

In the previous section, the reactivity of the PCR-based test was compared to that of a number of commercially available anti-HCV screening assays (Figure 1b). The routine testing for the presence of anti-HCV antibodies by these methods was described in the late 1980's. The technology used in the development of these tests is the enzyme immunoassay (EIA) and was a successful diagnostic tool for almost 20 years prior to its

utilisation in the first anti-HCV screening test. This technology and the various types of EIA, along with more modern rapid assays will be described in Section 1.7.

1.6.2.1 First Generation Assays

In 1989, the first tests became available and tested for anti-HCV in serum. A recombinant C100-3 antigen was derived in yeast and is specific for antibodies for the NS4 region of the viral genome. The captured anti-HCV is then detected by secondary anti-human immunoglobulin (IgG) labelled with enzyme (Kuo *et al.*, 1989). Screening for this anti-C100-3 greatly reduced the number of post-transfusion NANBH, up to 60% in some countries. This test also detected anti-HCV in approximately 60% of HCV RNA-positive blood donors (Watanabe *et al.*, 1993).

Studies of anti-C-100-3 kinetics during acute post-transfusion hepatitis suggest that the antibody is usually absent during the incubation period and acute phase, becoming detectable 2 to 52 weeks after the first liver enzyme elevation (Alter *et al.*, 1990). Not all cases of self-limiting acute hepatitis are detectable by this assay, due to the fact that anti-C-100-3 may rapidly disappear or never even reach detectable levels. Anti-C100-3 positivity correlates with donor ALT level. Its prevalence was found in high-risk groups including intravenous drug users (48-81%) and in patients who had been multiply transfused. Anti-C100-3 was detected in 64-85% of haemophiliacs who received untreated blood or blood products from many donors. Serum anti-C-100-3 correlates with HCV-RNA detection in both serum and liver.

Despite the initial success of this test, problems were observed. A study carried out on chronic hepatitis patients showed that false positive results were obtained (Mc Farlene *et al.*, 1990). This low specificity resulted from the cross reactivity of human IgG from another pathogen or the adhesion of a component to the solid phase resulting in the binding of IgG. It is also believed that multiple freeze-thaw cycles of stored specimens also contributed to the low specificity of first generation tests (Mc Farlene *et al.*, 1990).

Contributing to the specificity problem was the intriguing finding of the presence of anti-C-100-3 antibody in the serum of a high proportion of patients with autoantibodies, particularly in those with antibodies against liver/kidney microsomes and in patients with hepatocellular carcinoma (Mishiro *et al.*, 1990). The specificity of these findings suggests that HCV infection significantly affects the host's immune response.

1.6.2.2 Second Generation Assays

An attempt was made to increase assay specificity with a second-generation test known as recombinant immunoblot assay. This supplementary test increased specificity by offering different test conditions and presenting the antigen in a slightly modified form. The use of additional recombinant antigens improved sensitivity compared to that based on C100-3 protein alone (Widell *et al.*, 1991). New antigens from the NS3 region (C33c protein) and the nucleocapsid (core) region (C22 protein) were used along with the C100-3 antigen, which was expressed in *E.coli*. These recombinant proteins were immobilised on to cellulose strips, and then visualised as discrete bands with superoxidedismutase (SOD) used as a control.

The sensitivity and specificity achieved in the second-generation assays presented a great improvement over the first generation tests as new HCV infections and positive serum specimens were detected earlier by these assays. More than 98% of RNA positive samples were detected compared to 60% in first generation tests (Watanabe *et al.*, 1993).

1.6.2.3 Third Generation Assays

The incorporation of additional antigens to further improve sensitivity and specificity formed the basis for the third generation assay. These tests included proteins derived from the core, NS3, NS4 and NS5 regions of the genome, as in the Murex Anti-HCV (Version III) ELISA®. The Ortho HCV 3.0 ELISA® utilised the antigens C22-3, C200 expressed as one polypeptide comprising C100-3 and C33c, and NS5 recombinant proteins. A more recently developed third generation EIA is the Innogenetics Innotest HCV Ab III®. This microtitre plate format boasts optimal coverage of the immunodominant HCV epitopes. The use of these additional proteins allows for earlier detection of seroconversion following HCV infection. The significantly lower sensitivity of the first generation assays could be attributed to highly divergent HCV-RNA nucleotide sequences that make a single antibody (C100-3) assay unable to detect.

1.6.3 Detection of HCV Antigen

Despite the high sensitivity and specificity of these third generation tests they are unable to confirm viral infections during periods in the early phase of infection before the production of anti-HCV antibodies (Aoyagi *et al.*, 1999). In addition, antibody tests cannot distinguish between persons with anti-HCV antibodies who have recovered and patients exhibiting an active infection. The detection of HCV antigen is, therefore, necessary. It is detectable as a HCV marker in liver tissues and has been detected immunohistochemically using fluorescein isothiocyanate-labelled immunoglobulin G (IgG) fractions from sera strongly reactive with recombinant proteins of structural and non-structural HCV.

In recent years, methods for detecting viral antigen were developed by applying a monoclonal antibody to the HCV core antigen (HCVcAg). This method is reported to have a low sensitivity for detecting HCVcAg and required the concentration and fractionation of HCV to detect the antigen. Other such detection methods were reported to be useful for monitoring interferon therapy however their low sensitivity and the complicated specimen pre-treatment process make it difficult to apply them to the mass screening of blood donors. These problems were overcome in a more recent study by introducing an efficient specimen pre-treatment method to the EIA for HCVcAg (Aoyagi *et al.*, 1999). The developed one-step method consists of a 30-minute incubation of the specimen with a solution containing three different types of detergents (Triton X-100, CHAPS and sodium dodecyl sulphate). This method sufficiently inactivated the interfering anti-core antibody in the sample and the presence of HCVcAg could be successfully detected. This provided an important breakthrough in the early diagnosis and monitoring of HCV infection.

1.7 ELISA and Membrane Technology

In recent years, the routine use of immunoassays for the detection and quantitation of biologically active substances has become increasingly widespread. The combination of sensitivity and specificity offered by immunoassay techniques ensures their use as well-established diagnostic tools. There are several known techniques, which can be used in immunodiagnostic analysis. Such techniques include enzyme immunoassays (EIA), radioimmunoassays (RIA), immunofluorescence assays (IFA) first introduced in the early 1940's, blot assays and agglutination assays such as latex, particle and haemagglutinin (Coons *et al.*, 1976). The strategy of the EIA consists of a reaction between the immunoreactants, the antibody with the corresponding antigen, and the detection of that reaction using enzymes, labelled to the reactants, as indicators.

All immunoassay techniques are based on the same principle involving antibody-antigen reactivity; the amount of complex formed is measured relative to a standard or negative sample. The presence of the complex is determined by fluorescence (IFA), radioactivity (RIA) or a substrate colour change (EIA). The technique involves the immobilisation of an antibody or antigen onto a solid phase such as a microtitre plate, glass tubes, plastic beads or a synthetic membrane.

1.7.1 Application of Protein Coupling and Purification in Immunoassay

Proteins have evolved in nature to carry out a remarkable variety of functions, many of which are complex interactive roles of subtlety and specificity. This evolutionary process has resulted in proteins, which offer a high level of performance where a huge number of molecules exist; the human genome alone is thought to code for between 30,000 and 50,000 different proteins (Aslam and Dent, 1998).

One of the main features of a protein's behaviour is its ability to form complexes with other molecules. More often, the more useful applications have resulted from the coupling of proteins to these molecules or to a second protein. The complex formed is termed a conjugate, in the case where both partners are soluble molecular species. Fields such as

clinical diagnostics and immunology rely on the use of these coupled protein reagents for successful protein applications.

Immunoassay antibody-antigen interactions can be measured by coupling proteins to labels forming conjugates, which can be read by fluorescence, luminescence or another suitable detection method. Over the last few years, two improved methods of signal generation have been achieved which use DNA fragments as the label (Aslam and Dent, 1998). In the first in 1995, detection is subsequently achieved by employing the label to express a luminescence-catalysing enzyme and in the second, also in 1995, the product of PCR amplification of the label is detected by staining after gel electrophoresis.

The application of protein-protein coupling in ELISA techniques produces a colourimetric end-point that can be measured by simple cheap instrumentation, which is readily available in most laboratories. The enzymes used in ELISA assays should be stable and pure, as performance characteristics are dependent on the reaction parameters of the immunoreagents.

1.7.2 Enzyme-linked Immunosorbent Assays

In 1971, the enzyme-linked immunosorbent assay (ELISA) was first described (Avrameas and Guilbert, 1971; Engvall and Perlmann, 1971; Van Weeman and Schuurs, 1971). It incorporates similar features to the IFA and RIA but offers many advantages over these older techniques. These advantages are achieved by substituting the radioactive isotope of the RIA or the fluorochromes of the IFA with an enzyme as a label in the immunoassay. Several enzymes, including alkaline phosphatase, horseradish peroxidase, glucose oxidase and glucoamylase have been coupled to antigen or antibody by the use of glutaraldehyde, sodium periodate or by other procedures.

The EIA relies on the extremely high catalytic power and specificity of enzymes, which are usually detectable with great ease thus allowing the quantitation of small amounts of enzyme. Enzymatic activity is quantified in a simple fashion such as spectrophotometry. The ELISA is a highly sensitive procedure in which a nanogram or lesser amounts of antigen or antibody can be accurately quantitated. The advantages of the ELISA over the older methods of RIA and IFA are outlined in Table 1c.

Enzyme Immunoassays (EIA)	*RIA and IFA*
Very high sensitivity, detectability, and specificity are possible	Relatively high specificity and lower detectability levels.
Relatively cheap standard laboratory equipment is required	Expensive equipment is required to measure radioactivity and fluorescence
Reproducibility is high and evaluation is objective	Relatively similar reproducibility.
No radiation hazards	They represent potential health hazards and RIA often requires licensing.
Reagents are relatively cheap and generally of longer shelf life	Reagents have a short shelf-life.
Easy disposal of waste products	Necessitates special disposal of radioactive wastes.

Table 1c. Comparison of EIA with RIA and IFA as immunoassay techniques; advantages and disadvantages of the techniques.

The aforementioned serological methods of diagnosis utilise this ELISA technology. Third generation assays such as the Ortho HCV 3.0 ELISA™, and the Murex Anti-HCV (Version III) ELISA are currently used worldwide for the detection of anti-HCV antibodies in human serum and plasma. The features which benefit these ELISA tests over molecular methods of diagnosis such as PCR-based methods include speed and simplicity of sample preparation, the use of standard laboratory equipment, cost saving, time saving and clear interpretation of results.

The use of ELISA tests also reduces the likelihood of sample contamination, which often occurs with the PCR-based method. The reagents used in these ELISA assays are safe and relatively inexpensive with a long shelf-life. Microtitre plates offer the advantage of simultaneous analysis of many samples. ELISA has shown sensitivity greater than agglutination assays and equal to RIA tests while being safer tests to carry out.

1.7.3 ELISA Assay Types

There are five main types of ELISA tests used in immunodiagnostics, all incorporating the formation of antigen-antibody interactions. They include the indirect ELISA (Figure 1.7.1), the double antibody sandwich ELISA (Figure 1.7.2), the competitive ELISA (Figure 1.7.3), the double-antigen sandwich assay (DAGS) (Figure 1.7.4) and the class-specific antibody capture assay (Figure 1.7.5). The anti-HCV ELISA described in the previous section is an example of an indirect ELISA. The different ELISA types and are now described and employ following drawing keys as shown below.

Microtitre plate well

Specific antigen

Specific antibody

Capture antibody

Enzyme conjugate label

Substrate reagent

Anti-human IgG antibody

Total class antibody

1.7.3.1 Indirect ELISA (Figure 1.7.1)

This is used mainly where the analyte is serum antibody as in the anti-HCV assay. The microtitre well is coated with purified antigen or recombinant or synthetic proteins. The test specimen is incubated on the coated wells, any antibody present forming a complex with the immobilised solid phase antigens. Following a wash step, a conjugate containing labelled anti-species immunoglobulin (IgG or IgM) is then added to the plate. Unbound conjugate is washed away and the complex formed is developed with an appropriate substrate. The measured amount is proportional to the quantity of specific antibody present. Such a test is represented by the Innogenetics Innotest HCV Ab III® Assay.

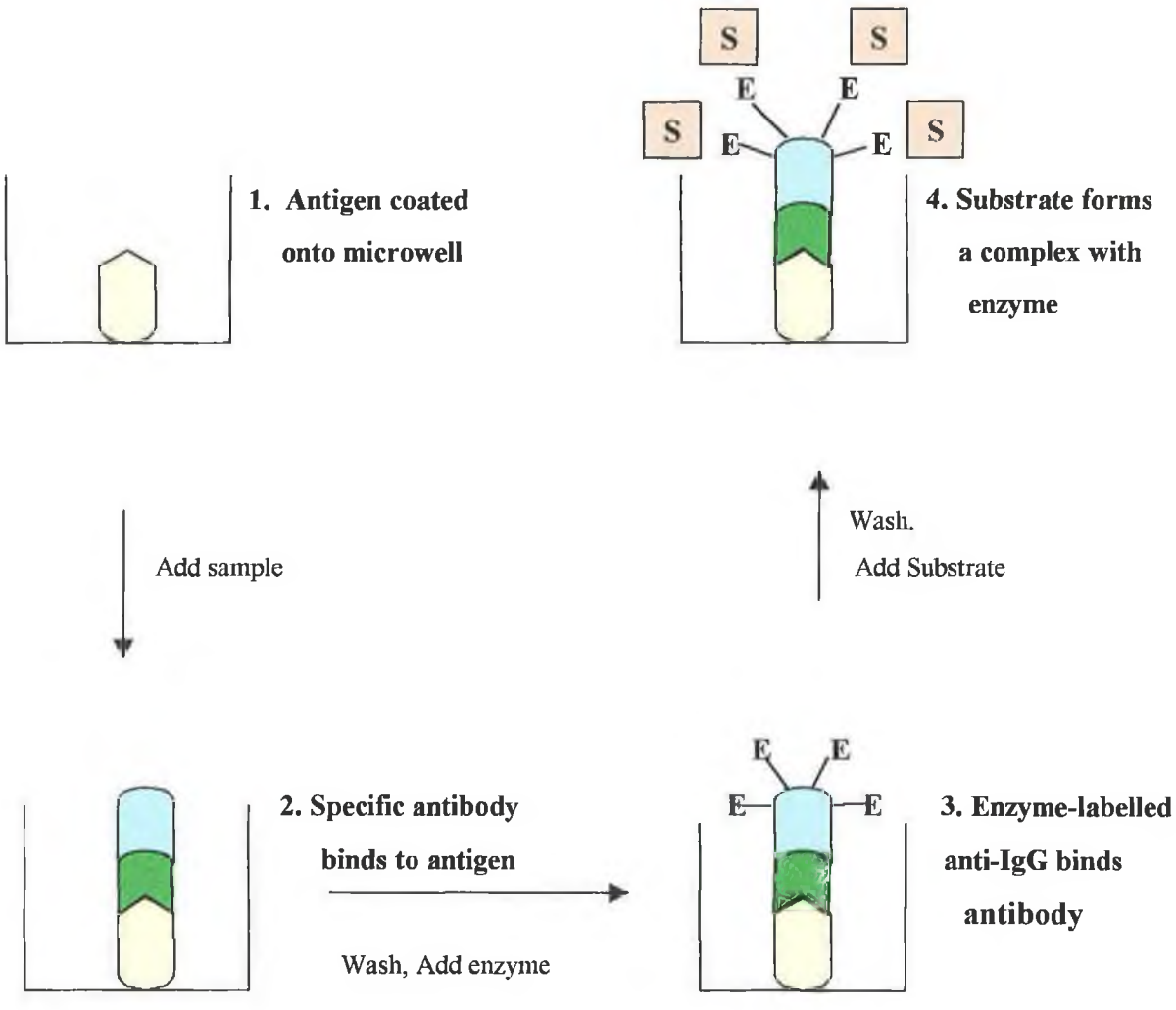


Figure 1.7.1. Indirect ELISA assay protocol.

1.7.3.2 Double Antibody Sandwich ELISA (Figure 1.7.2)

This format involves the capture of the analyte, the antigen, between two specific antibodies. One purified antibody is coated on to the microtitre well and the test sample incubated. A wash step then removes unbound sample and the well is incubated with anti-species secondary antibody labelled with enzyme. After washing, the Ab-Ag-Ab-conjugate complex is incubated with substrate and the developed colour indicates the quantity of antigen present in the test sample. The Trinity Biotech Rotavirus™ EIA represents such a test.

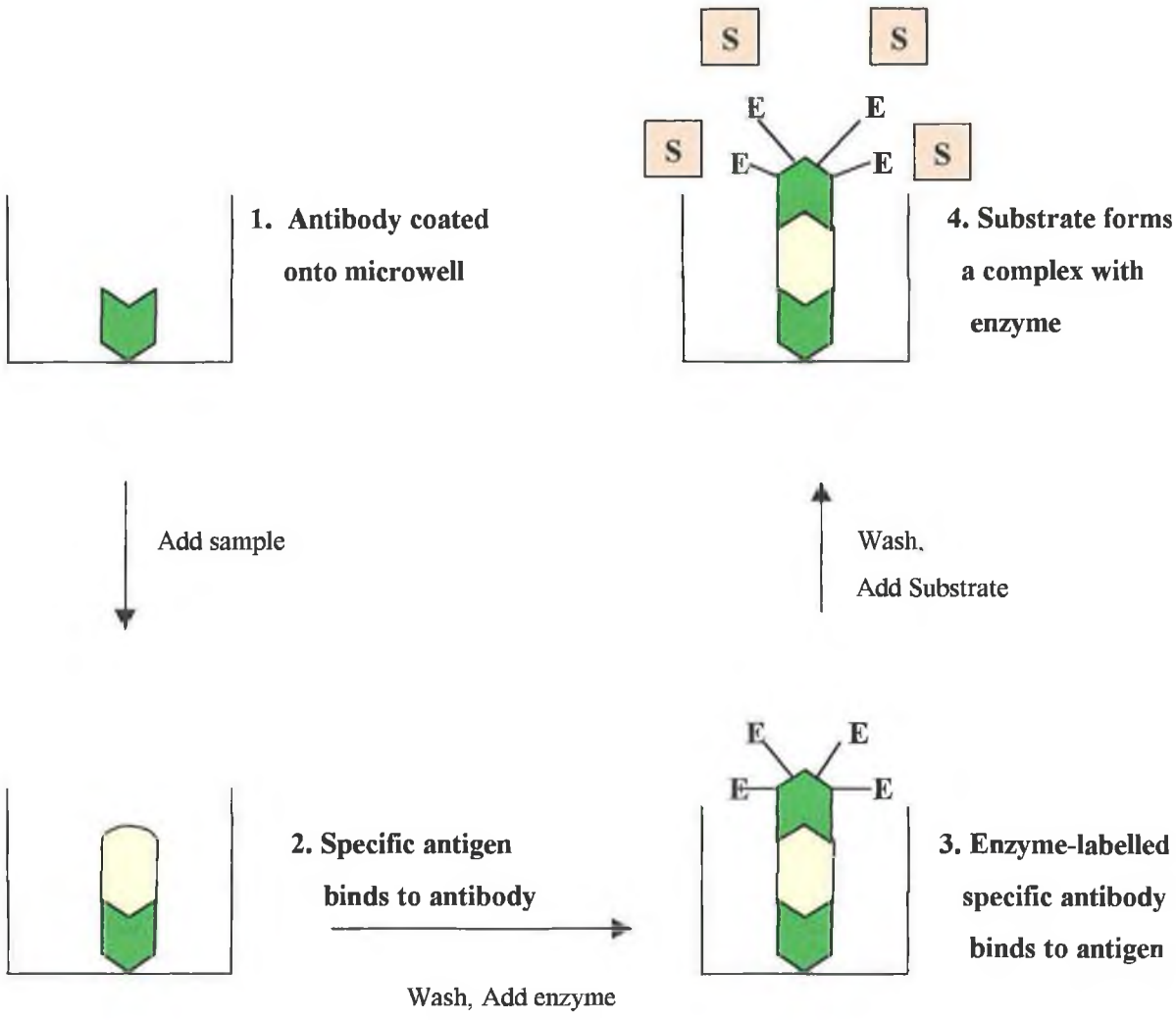


Figure 1.7.2. Double Antibody Sandwich ELISA assay protocol.

1.7.3.3 Competitive ELISA (Figure 1.7.3)

The first EIA described belonged to this group. The principle behind this test differs from the sandwich assay and the indirect assay. Unlike those tests, the development of colour in a competitive assay is indirectly proportional to the quantity of analyte in the sample. The wells are coated with antibody and simultaneous incubation of free antigen in the sample and enzyme-labelled antigen results in competition of sample and conjugate for reactive sites on the solid phase. Since a restricted number of antibodies are available the enzyme activity is lowered. The development of colour upon substrate incubation indicates the binding of conjugate rather than positive sample and so a negative sample is interpreted. The Trinity Biotech Osteocalcin EIA™ Test is an example of a competitive assay.

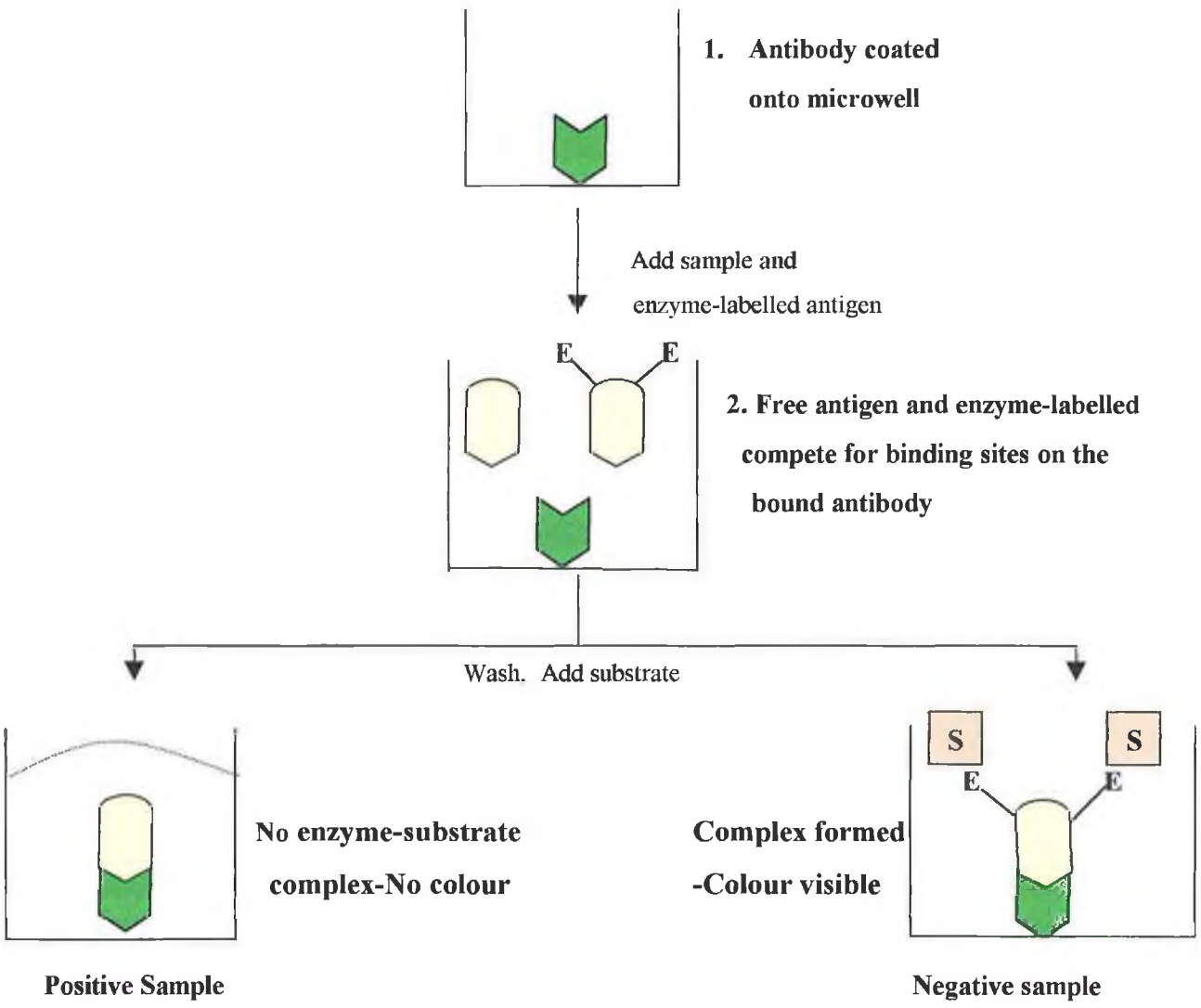


Figure 1.7.3. Competitive ELISA assay protocol.

1.7.3.4 Double-Antigen Sandwich Assay (DAGS) (Figure 1.7.4)

The double-antigen sandwich assay has recently been introduced as a format, which enables the detection of all classes of antibodies generally not detected by the indirect (anti-gamma globulin) format (Burgisser *et al.*, 1999). As in the case of the indirect assay (Figure 1.7.1), the microtitre well is coated with purified recombinant protein or antigen. All classes of specific antibody present in the test sample is bound to the immobilised antigen during the first incubation. Unbound material is removed by washing the wells and during the second incubation step bound antibodies are labelled with specific antigen-peroxidase conjugates. After a second washing step, the fraction of bound enzyme is determined by a chromogenic reaction. The intensity of colour is proportional to the presence of all classes of specific antibody in the sample. An example of such a test is the Orgenics HIV 1&2 DoubleCheck™ rapid EIA.

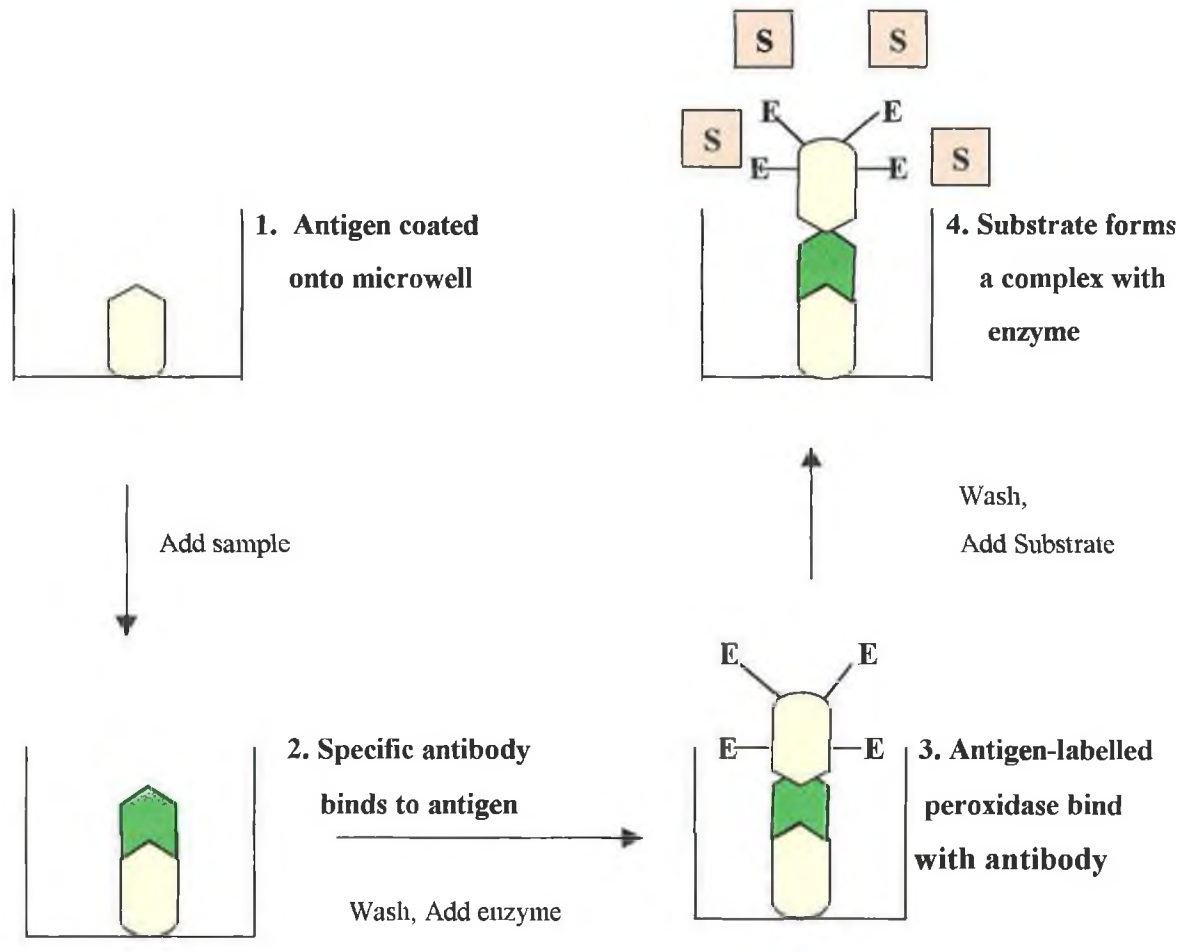


Figure 1.7.4. Double Antigen Sandwich ELISA assay protocol (DAGS).

1.7.3.5 Class-Specific Antibody Capture Assay (Figure 1.7.5)

This format is used to capture antibodies in a test sample, specific for an immunoglobulin class required. Plates are coated with capture antibodies (anti-IgG, IgM or IgA). The test antibody solution is then incubated on the coated wells and the total IgG, IgM or IgA antibody present in the sample is bound to the coated anti-immunoglobulin. A wash step removes all unbound material prior to a second incubation with specific antigen, which binds to any specific antibody bound to the solid phase. Following a further washing step is incubation with specific antibody conjugated to peroxidase. The colour intensity on addition of chromogenic substrate is proportional to the quantity of specific class antibody present in the test specimen. An example of a class-specific HCV IgM assay was developed in 1997.

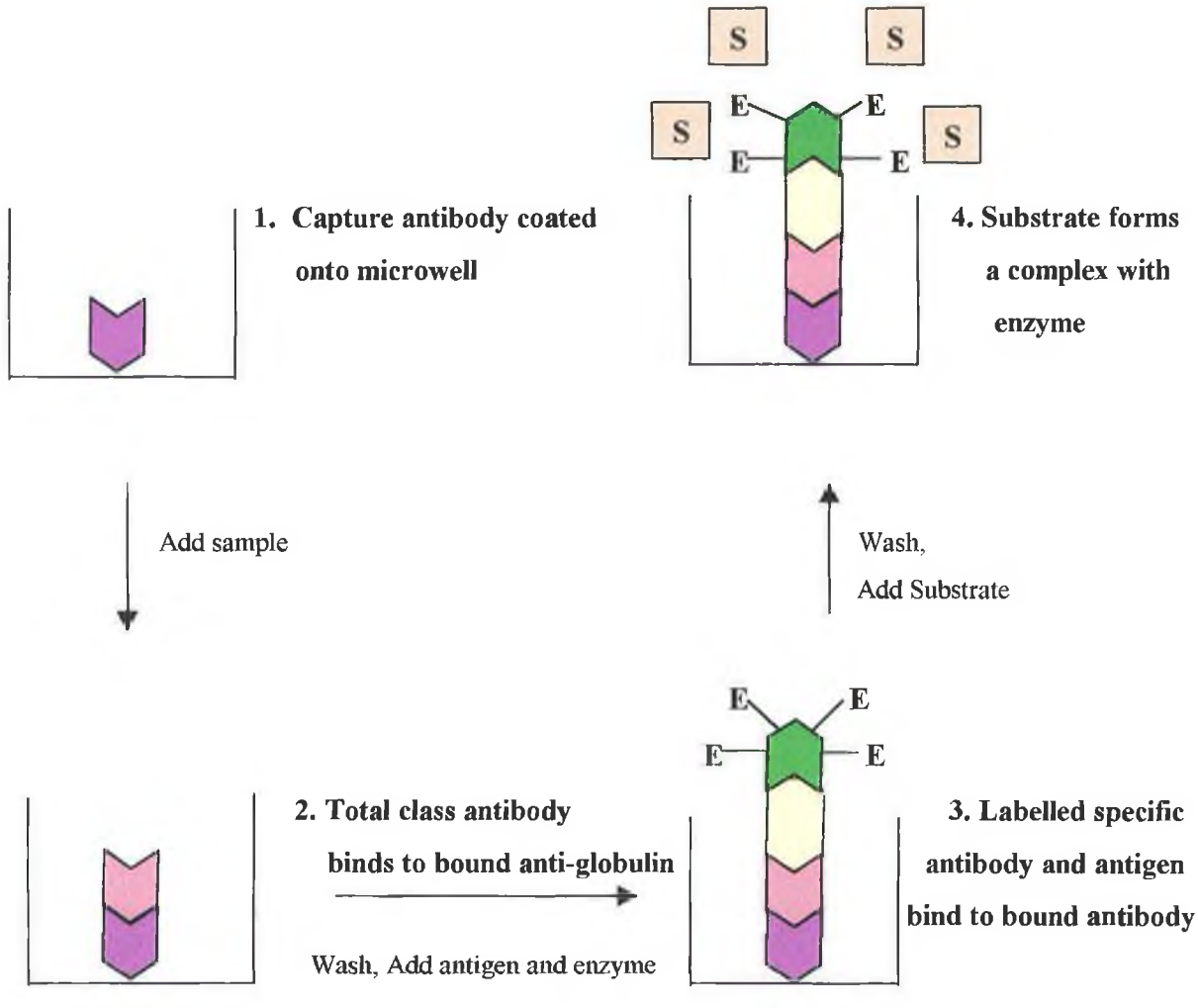


Figure 1.7.5. Class-Specific Antibody Capture ELISA assay protocol.

1.7.4 Rapid Technology

The need for simple, rapid and inexpensive diagnostic tools prompted the development, globally, of a range of new products. These rapid formats follow the same basic antibody-antigen interaction principle as the ELISA technique but differ in the nature of the solid phase and in the technology used for antigen or antibody detection.

1.7.4.1 Agglutination Reactions

An agglutination reaction occurs when antibody molecules combine with particulate antigens. The most commonly used particulate antigens are whole bacteria or red cells. Because of the great sensitivity of agglutination reactions, many soluble antigens have been placed on red cells or inert particles. These passively coated cells or particles agglutinate in the presence of specific antibody. Such inert particles are in the form of polystyrene latex beads in the case of the Cambridge Biotech CAPILLUS® HIV-1/HIV-2 Assay. This test employs the use of HIV-1 and HIV-2 polypeptides, which have been expressed and purified. These proteins are bound to polystyrene latex beads to form the basis of the direct latex agglutination assay for the detection of HIV-1 and HIV-2 antibodies.

The latex reagent and the test sample are mixed together on a thin flat slide and are drawn through the flow channel by capillary action. Samples, which are positive and contain antibodies to HIV, cause the antigen-coated latex to agglutinate. This capillary action enhances the binding of the specific antibodies to the latex, which promotes aggregation. The reaction is then read visually or with the use of a digital reader once the latex solution reaches a viewing window on the slide. A smooth appearance is considered non-reactive or negative whereas a clumpy aggregated solution indicates a positive result. This is a fast, effective and easy to use test.

1.7.4.2 Precipitation Reactions

When a soluble antigen and it's specific antibody are in contact in solution, the resultant antibody-antigen complexes may become insoluble and precipitate (White, 1978). The precipitation rate depends on the on the proportions of the reactants and on the temperature, salt concentration and to some extent, the pH of the solution.

The precipitation reaction can be induced by mixing antigen and antibody in test tubes or in Lang-Levy pipettes (White, 1978). It can also be obtained by carefully overlaying one reactant with the other in a narrow test tube so that diffusion can take place. The antibody-antigen interaction is then visible as a precipitation ring at the liquid surface. The Swift-Wilson-Lancefield precipitation test combines these techniques and is performed in capillary tubes. Precipitation is observed at the interface and also in the lower part of the tubes. A precipitation reaction will take place when antigen and antibody are allowed to diffuse in gels.

1.7.4.3 Lateral Flow Immunochromatography

This format involves the adhesion of antibody, antigen or recombinant protein onto a solid phase porous membrane in a technique known as immunochromatography. It is used in the detection of viral antibody and antigen and is a rapid, simple method, which does not require the use of laboratory equipment. Its speed and simplicity offers advantages over other methods of analysis without compromising test sensitivity and specificity. Lateral flow tests such as the Trinity Biotech HIV 1/2 SeroCard® utilises an enzyme-labelled conjugate but these tests may employ gold conjugates, latex tests as manufactured by Quidel or carbon tests from Medix Biotech.

In recent times many diagnostic test kits became commercially available utilising the principles of immunochromatography. The first major target analyte for this test format was human chorionic gonadotropin (hCG) for the detection of pregnancy. This was followed by tests for detecting a wide range of analytes amongst which were luteinizing hormone (LH), Streptococcus, Chlamydia. Tests for better known infectious diseases were also developed including human immunodeficiency virus (HIV), as described in the Orgenics HIV 1&2 DoubleCheck™ rapid EIA, Hepatitis B antibody and antigen, as described in Chembio's Hepatitis B Stat-Pak™ rapid immunochromatographic assay for the detection of HBsAg, and also tests for Hepatitis C virus as described at a later stage.

The Uni-Gold™ HBsAg test utilises the principle of lateral flow. It is a rapid immunochromatographic test for the presence of hepatitis B surface antigen (Figure 1c). The method employs a combination of antibody-dye-conjugate, in this case colloidal gold, and solid phase antibodies specific to HBsAg. A conjugate pad is impregnated

with the colloidal gold-conjugated antibodies but they remain unbound to the pad. The sample flows through this absorbent pad during which time the labelled antibody binds to HBsAg if present. The conjugate is solubilised and the complex then passes on to a chromatrographic membrane strip. Specific antibodies are immobilised on to a region of this strip known as the test line and are referred to as capture antibodies.

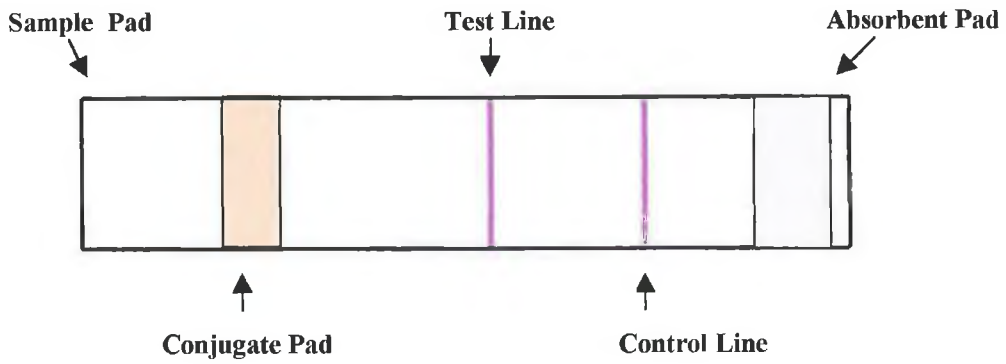


Figure 1c. Uni-Gold™ HBsAg test, a rapid, colloidal gold-based assay for detection of HBV surface antigen, is an example of lateral flow immunochromatography-a positive result is interpreted by colour on the test line along with the control line.

As the antigen-antibody complex moves along the strip, a line of immobilised complex forms on the test line. A control line is included in the test device, which consists of immobilised anti-gold antibodies. Any unbound colloidal gold will flow up the membrane and form a control complex. In both cases the results are interpreted visually by the appearance of a line of colour at the test line and the control line.

An example of an HCV-specific lateral flow test is the Innogenetics INNO-LIA HCV Ab III® test. It is a third generation line immunoassay consisting of a nylon strip coated with recombinant proteins and antigens specific for immunodominant HCV epitopes. It is used in the confirmation and differentiation of antibodies to HCV. A phosphatase-labelled conjugate detects the specific antibodies, which are represented as bands on the strip. The results can be determined visually.

1.7.4.4 Vertical Flow Immunochromatography

In certain chromatrographic tests the flow of the sample and antibody-antigen complexes occurs vertically. The Orgenics ImmunoComb®II *H.pylori* test is an example of such a test. The solid phase consists of a comb with projections, sensitised with antibodies to

human IgG on an upper spot and *H. pylori* antigens on a lower spot. A plate contains ready-to-use reagents for each stage of the test. Upon sample incubation *H. pylori* antibodies present in the sample bind to the specific antigens on the lower spot. Simultaneously, any IgG will bind to the anti-human IgG on the upper spot to act as a control. In the next step, captured anti-H pylori IgG will react with anti-human IgG alkaline phosphatase (AP), which is present in the plate. The bound AP then reacts with chromogenic components. The results are visible as grey-blue spots on the surface of the ImmunoComb.

1.7.4.5 Flow-Through Immunochromatography

The Cambridge Biotech Recombigen® HIV-1/HIV-2 Rapid Test Device (RTD) is an example of an assay in which the sample and test reagents flow through the test device. It is an indirect assay utilising recombinant proteins representing the immunodominant regions of HIV-1 and HIV-2. These recombinant antigens are separately coated onto microlatex particles and each is impregnated on the membrane surface of the device in separate spots (Figure 1d). A HIV-1/HIV-2 procedural control is also impregnated onto the membrane.

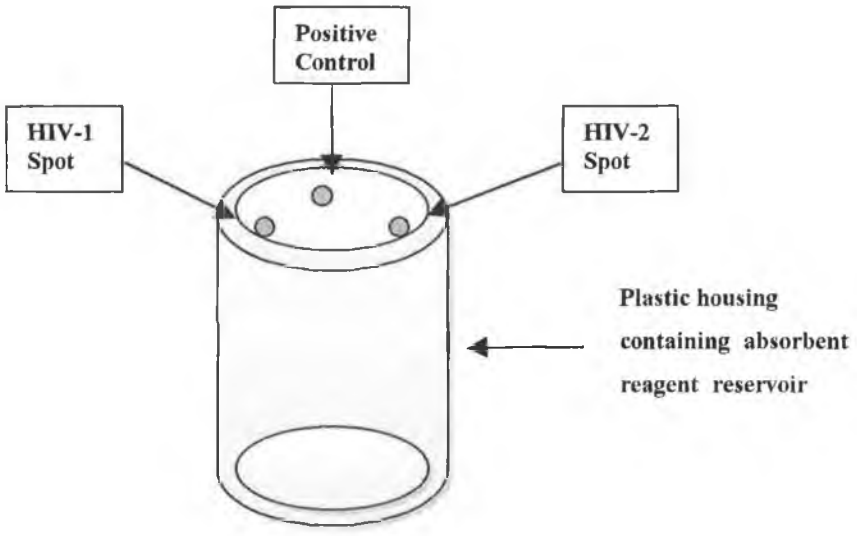


Figure 1d. Recombigen™ HIV 1/2 RTD device is an example of flow-through immunochromatography.

Diluted patient samples are applied to the device, which flow through the latex particles on the membrane surface. Antibodies to the virus, if present, bind to the antigen-coated latex particles. Enzyme-labelled, anti-human antibodies are added to the device and

allowed to flow through the membrane. A wash step removes unbound conjugate and the addition of substrate solution produces a blue dot, indicating the presence of HIV-1 and/or HIV-2 antibodies. The plastic housing as shown in Figure 1i contains an absorbent reagent reservoir.

1.7.5 Proposed Development of anti-HCV Antibody Tests

Both the microtitre plate assay and the rapid membrane-based assay are examples of the indirect assay, described in Figure 1c. The development of both assays followed a similar method and started with evaluation of peptides for use on the solid phase. This was done by testing with characterised sera. When an initial solid phase was chosen a number of different parameters were investigated. These included evaluation of enzyme conjugate dilution, enzyme conjugate diluent, sample dilution and incubation times of various stages of the assay.

During development, initial performance was evaluated with clinical samples of known status. Upon optimisation of tests, clinical trials were carried out to assess the performance of both tests off-site. The assay protocol used for both the microtitre plate test and the rapid test are outlined in section 1.7.5.1 and 1.7.5.2.

1.7.5.1 ELISA Test Development

The proposed method for the development of a qualitative microtitre plate assay follows the following protocol. The microwells are coated with purified antigens or recombinant proteins containing sequences from the various regions of HCV. During the first incubation at optimal temperature any specific anti-HCV antibody binds to the solid phase forming an antigen-antibody complex.

A washing step removes any unbound specimen and a second incubation with an enzyme-labelled anti-human IgG conjugate detects the presence, if any, of captured antibody. An antigen-antibody-anti-human antibody-enzyme complex is formed. A further washing step removes excess conjugate and is followed by incubation with a suitable substrate. Quantitation is based on a colour change relative to a negative control, the formation of colour being directly proportional to the presence of specimen

antibody. The reaction is stopped with an acidic agent and the interpretation is achieved visually or using a spectrophotometer.

1.7.5.2 Rapid Test Development

Another form of lateral flow membrane technology is used in the development of a rapid test for antibodies to HCV. The porous nature of this solid phase membrane necessitates the need for an additional step prior to adhesion of antigen to the membrane. The antigen is immobilised in carrier-bound form, the peptide coupled to a high molecular weight protein such as bovine serum albumin (BSA). A combination of carriers may be used provided the carrier does not interfere with the ability of the peptide to bind antibodies. This allows the adhesion of low molecular weight peptides and antigens to the porous membrane. The carrier-peptide complex is purified prior to immobilisation to the solid phase (Figure 1e).

The first step in the test is the addition of test sample to the membrane at the sample ports. The sample flows up the membrane to the reaction site where the immobilised antigen forms a complex with any antibody present in the sample.

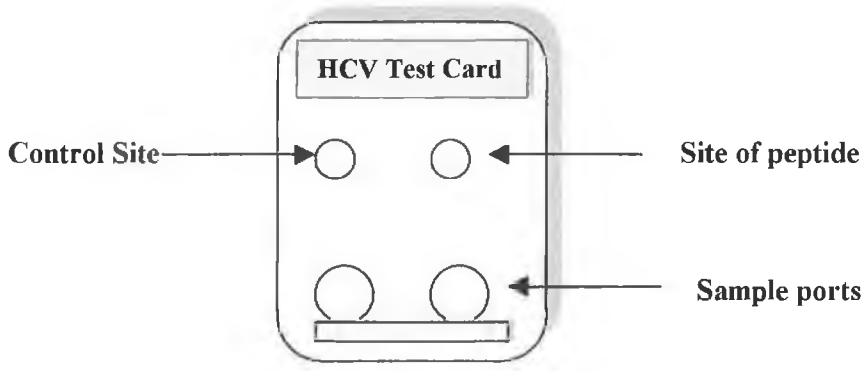


Figure 1e. HCV rapid SeroCard test device showing the site of bound peptides during spotting and of test specimen during assay procedure.

A wash step removes unbound sample prior to the incubation of an anti-human IgG labelled conjugate. This test does not involve coupling of antibody to latex or colloidal gold to form a conjugate. Following another wash step to remove free conjugate an appropriate substrate is added to the membrane and the resulting colour is read visually. The intensity of the colour is directly proportional to the quantity of antibody present in the test sample.

Section 2-

Materials and Methods

2.1 Raw Material Suppliers

2.1.1 Chemicals and Raw Material Suppliers

Table 2.1 outlines, in alphabetical order, the suppliers of the chemicals and raw materials used in the development of both the microtitre plate assay and the rapid assay.

Supplier	*Address*
Alex Mitchell Plastics	Unit 5, IDA Estate, East Wall Road, Dublin 3, Ireland
Biosynth AG	Rietlistr, 4, Postfrach 125, Staad 9422, Switzerland
Boston Biomedica, Inc.	375, West street, W. Bridgewater, MA 02379, United States of America
Cork Blood Bank	St. Finbarr's Hospital, Cork, Ireland
Dako	Denmark House, Angel Drove, Ely, Cambridgeshire, CB74ET, United Kingdom
Eurodiagnostica	Idoen SE-205, 12 Malmo, Sweden
Genzyme	50 Gibson Drive, West Malling, Kent, ME19 GHG, United Kingdom
Gibco BRL	c/o Life Technologies Ltd., P.O. Box 35, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, United Kingdom
Hi-Tech Products	8530 Roland St, Buena Park, CA 90621, United States of America
International Enzymes Inc.	c/o Aalto Bio Reagents Ltd., 14 Main Street, Rathfarnham, Dublin 14, Ireland
Kirkegaard and Perry Laboratories	2 Cessna Court, Gaithersgury, Maryland, United States of America
Lennox Laboratories	JFK Drive, Naas Road, Dublin 12 Ireland
NUNC Incorporated	2000 North Aurora Road, Naperille, Illinois 60566, United States of America
Pro-Pac Ltd.	Unit 4, Turnpike Industrial Estate, Ballymount, Dublin 22, Ireland
Riedel de Hein	c/o R.B. Chemicals Ltd., Hoechst House, Cookstown Industrial Estate, Tallaght, Co. Dublin
Safe-Pak	Dunboyne Industrial Park, Dunboyne, Co. Meath, Ireland
Silgelac	171 Avenue Georges, Clemenceu 92024, Nan Terro, Cedex, France
Sigma Aldrich	Airton Road, Tallaght, Dublin 24, Ireland

Table 2.1.List of suppliers of raw materials for use in the ELISA and rapid assays

2.2 Materials Used in ELISA Test

2.2.1 Chemicals used in ELISA Test

Chemical	Supplier	Cat. No.	Grade
Bovine Serum Albumin	Sigma	A-7030	Minimum 98%
Cytochrome C (from Horse Heart)	Sigma	C-7752	Minimum 95%
Hydrochloric Acid (Conc.)	Sigma	H-7020	ACS Reagent
Sodium Azide	Sigma	S-2002	Minimun 99.5%
Sodium Carbonate	RdH	S-6139	ACS Reagent
Sodium Chloride	Sigma	S-7653	Sigma Ultra
Sodium Hydrogen Carbonate	RdH	31437	ACS Reagent
di-Sodium Hydrogen Phosphate	RdH	30435	Reagent
Sodium di-Hydrogen Phosphate	RdH	04269	Extra Pure
Sodium Hydroxide (Pellets)	Sigma	S-0899	ACS Reagent
Sucrose	Sigma	S-5016	ACS Reagent
Sulphuric Acid	RdH	S-1526	ACS Reagent
Tetra Methyl Benzidine (TMB)	Dako	S1599	Reagent
Thimerosal	Sigma	T-5125	Minimun 95%
Trizma Base	Sigma	T-1503	Reagent Grade
Trizma Hydrochloric	Sigma	T-3253	Reagent Grade
Triton X-100	Sigma	X-100	Laboratory Grade
Polyoxyethylenesorbitan Monolaurate (Tween 20)	Sigma	P-1379	Reagent Grade

2.2.2 Buffers and Reagents used in ELISA Test

0.1M 1x Phosphate Buffered Saline, pH 7.2

Sodium Chloride	8.7g
25X Phosphate Buffered Saline	40ml
Sodium Azide	0.5g
1M Sodium Hydroxide	as required
1M Hydrochloric Acid	as required

Adjusted to pH 7.2 ± 0.1 and made to 1000ml with deionised water

0.1M 25X Phosphate Buffered Saline (PBS)

Potassium Phosphate Monobasic	2.72g
Potassium Phosphate Dibasic	27.9g
1M Sodium Hydroxide	as required
1M Hydrochloric Acid	as required

Adjusted to 1000ml with deionised water and adjusted to pH 7.8 ± 0.2

1M Sodium Hydroxide

Sodium Hydroxide	40g
Deionised Water	1000ml

1M Hydrochloric Acid

Hydrochloric Acid (Conc)	83ml
Deionised Water	917ml

EIA Blocking Buffer

Sucrose	50g
Bovine Serum Albumin	5g

Adjusted to 1000ml with Carbonate Coating Buffer

25X Wash Reagent

Sodium Chloride	198.69g
Tris Base	60.57g
Tween 20	25ml
Thimerosal	0.1g
Hydrochloric Acid (Conc)	as required
1M Sodium Hydroxide	as required

Adjusted to 1000ml with deionised water and adjusted to pH 7.4 ± 0.2

Carbonate Coating Buffer

Sodium Carbonate	1.22g
Sodium Hydrogen Carbonate	3.22g
Thimerosal	0.1g
1M Hydrochloric Acid	as required
1M Sodium Hydroxide	as required

Adjusted to pH 9.6±0.1 and made to1000ml with deionised water

Sample Diluent

10X Tris Buffered Saline	100ml
Bovine Serum Albumin	50g
Bovine Gamma Globulin	10g
Thimerosal	0.1g
Goat Serum (Heat Inactivated)	50ml

Adjusted to 1000ml with deionised water

1X Wash Reagent

25X Tris Wash Reagent	40ml
Deionised Water	960ml

10X Tris Buffered Saline

Tris HCl	117.2g
Tris Base	31.06g
Sodium Chloride	87.65g
Hydrochlorc Acid (Conc)	as required
1M Sodium Hydroxide	as required

Adjusted to pH 7.2 -7.5 and made to 1000ml with deionised water

Enzyme Conjugate Reagent

Triton X-100	0.5ml
Foetal Bovine Serum	100ml
Goat anti-human IgG-HRP Conjugate	as required
Cytochrome C Conjugate Diluent	899.5ml

Cytochrome C Conjugate Diluent

di-Sodium Hydrogen Phosphate	17.8g
Sodium di-Hydrogen Phosphate	15.6g
Bovine Serum Albumin	5g
Cytochrome C	0.25g
Thimerosal	0.1g

Adjusted to pH 6.6±0.1 and made to 1000ml with deionised water

4N Stopping Reagent

Sulphuric Acid	108.75ml
Deionised Water	891.25ml

2.2.3 Raw Materials and Equipment used in ELISA Test

Raw Materials

96-Well Plates	NUNC Inc.
1g Indicating Desiccant	Silgelac
Ziplock Foil Pouch	Safe-Pak
Plate Sealers	Kennilworth

Equipment Used in Plate Coating

Multi-Channel Pipettes, 50-300µl	Eppendorf
Single-Channel Pipettes, 2-20µl, 10-1000µl	Eppendorf
Multidrop Plate Coater	Labsystems
ELx50 Auto-Strip Washer	Bio-Tek Instruments
MR 5000 Plate Reader	Dynatech
LX-850 Printer	Epson

Additional Equipment

pH Meter 245	Corning
PM 400 Balance and LC-P45 Printer	Mettler Toledo
HR60 AND Analytical Balance	Mettler Toledo
AD1311 Printer	Mettler Toledo
Laminar Air Flow Cabinet NU-425-600E	Nuaire
Incubating Chambers D-6450	Heraeus Instruments
Waterbath	Grant
Magnetic Stirrer SM 1	Stuart Scientific
Peristaltic Filtering Pump 313S	Watson Marlow
Hotplate Magnetic Stirrer 34532	Snijders
Autovortex Mixer SA2	Stuart Scienfic
Autoclave SAL Type UCC 16 MP 6D1	

2.3 Materials Used in Rapid Test

2.3.1 Chemicals used in Rapid Test

Chemical	Supplier	Cat. No.	Grade
Amino-methyl-propanal (AMP)	Sigma	A-6518-2	Minimum 95%
BCIP	Biosynth	AGB-7500	Minimum 99.2%
Bovine Gamma Globulin (BGG)	Sigma	G-5009	99% Purity
Bovine Serum Albumin (BSA)	Sigma	A-7030	Minimum 98%
Casein	Lennox	440202C	Reagent
Glutaraldehyde 25%(v/v)	Sigma	G-5882	Grade 1
Hydrochloric Acid (Conc)	Sigma	H-7020	ACS Reagent
Imidazole	Sigma	I-0125	99% Purity
Magnesium Chloride	Sigma	M-9272	ACS Reagent
Potassium Phosphate, (Dibasic)	Sigma	P-3786	ACS Reagent
Potassium Phosphate, (Monobasic)	Sigma	P-5379	Minimum 99%
Sephacryl S-200 Gel	Pharmacia	17-0584-01	Reagent
Sodium Azide	Sigma	S-2002	Minimum 99.5%
Sodium Chloride	Sigma	S-7653	Sigma Ultra
Sodium Cyanoborohydride	Sigma	S-8628	Minimum 90%
Sodium Hydroxide (Pellets)	Sigma	S-0899	ACS Reagent
Sucrose	Sigma	S-5016	ACS Reagent
Thimerosal	Sigma	T-5125	Minimum 97%
Trizma Base	Sigma	T-1503	Reagent
Trizma Hydrochloric	Sigma	T-3253	Reagent
Triton X-405	Sigma	X-405	70% Aqueous
Zinc Chloride	Sigma	Z-3500	ACS Reagent

2.3.2 Buffers and Reagents used in Rapid Test

0.1M 1x Phosphate Buffered Saline, pH 7.2 (Column Elution Buffer)

Sodium Chloride	8.7g
25X Phosphate Buffered Saline	40ml
Sodium Azide	0.5g
1M Sodium Hydroxide	as required
1M Hydrochloric Acid	as required

Adjusted to pH 7.2 ± 0.1 and made to 1000ml with deionised water

0.1M 25X Phosphate Buffered Saline (PBS)

Potassium Phosphate Monobasic	2.72g
Potassium Phosphate Dibasic	27.9g
1M Sodium Hydroxide	as required
1M Hydrochloric Acid	as required

Adjusted to 1000ml with deionised water and adjusted to pH 7.8 ± 0.2

1M Sodium Hydroxide

Sodium Hydroxide	40g
Deionised Water	1000ml

1M Hydrochloric Acid

Hydrochloric Acid (Conc)	83ml
Deionised Water	917ml

2X Phosphate Buffered Saline (PBS)

Sodium Chloride	8.7g
25X PBS	80ml
Deionised Water	960ml

Control Dilution Buffer

10X Tris Buffered Saline (TBS)	100ml
Bovine Serum Albumin	50g
Bovine Gamma Globulin	10g
Thimerosal	0.1g

Adjusted to 1000ml with deionised water

10X Tris Buffered Saline

Tris HCl	117.2g
Tris Base	31.06g
Sodium Chloride	87.65g
Hydrochlorc Acid (Conc)	as required
1M Sodium Hydroxide	as required

Adjusted to pH 7.2 -7.5 and made to 1000ml with deionised water

Blocking Buffer

25X PBS	40ml
Sucrose	20g
Sodium Chloride	8.75g
Casein	7.5g
Triton X-405	0.5ml
Thimerosal	0.1g

Adjusted to 1000ml with deionised water

1M Magnesium Chloride with Azide

Magnesium Chloride	203.3g
Sodium Azide	1g

Adjusted to 1000ml with deionised water

Enzyme Conjugate Reagent

10X TBS	100ml
Bovine Serum Albumin	20g
1M Magnesium Chloride with Azide	1ml
0.1M Zinc Chloride with Azide	1ml
Sodium Azide	0.95g
Goat anti-human IgG Alk. Phos.	as required
Hydrochloric Acid (Conc)	as required
1M Sodium Hydroxide	as required

Adjusted to pH 7.4±0.1 and made to 1000ml with deionised water

0.1M Zinc Chloride with Azide

Zinc Chloride	13.6g
Sodium Azide	0.095g
Deionised Water	1000ml

Wash Reagent

Imidazole	13.61g
Sodium Chloride	8.77g
Sodium Azide	0.95g
Casein	7.5g
Hydrochloric Acid (Conc)	1.83ml

Adjusted to pH 7.6-8.4 and made up to 1000ml with deionised water

1M Magnesium Chloride

Magnesium Chloride	203.3g
Thimerosal	0.1g

Adjusted to 1000ml with deionised water

Substrate Reagent

AMP	96ml
Hydrochloric Acid (Conc)	48ml
1M Magnesium Chloride	1ml
Sodium Azide	0.95g
BCIP	1g
5N Sodium Hydroxide	as required

Adjusted to pH 9.8±0.1 and made to 1000ml with deionised water

5N Sodium Hydroxide

Sodium Hydroxide Pellets	200g

Adjusted to 1000ml with deionised water

2.3.3 Biological Components used in ELISA and Rapid Tests

Component	Supplier	Cat No.	Grade
HCV Core peptide	Eurodiagnostica	N/A	N/A
HCV 52 (NS4) peptide	Eurodiagnostica	N/A	N/A
HCV 15 (Core) peptide	Eurodiagnostica	N/A	N/A
HCV NS3 peptide	Eurodiagnostica	N/A	N/A
HCV NS5 peptide	Eurodiagnostica	N/A	N/A
E47 (Envelope) peptide	Eurodiagnostica	N/A	N/A
E51 (Envelope) peptide	Eurodiagnostica	N/A	N/A
MS94-23 (Core) peptide	Eurodiagnostica	N/A	N/A
PS9401-1 (NS3) peptide	Eurodiagnostica	N/A	N/A
PS9401-2 (NS3) peptide	Eurodiagnostica	N/A	N/A
PS9401-12 (NS3) peptide	Eurodiagnostica	N/A	N/A
PS9307-8 (NS5) peptide	Eurodiagnostica	N/A	N/A

PS9307-9 (NS5) peptide	Eurodiagnostica	N/A	N/A
NS4a peptide	Eurodiagnostica	N/A	N/A
NS3 Recombinant Protein	Eurodiagnostica	N/A	N/A
Goat anti-human IgG-AP conjugated	Genzyme	RIA 4334	Affinity Purified
Goat anti-human IgG-HRP conjugated	KPL Laboratories	074-1002	Affinity Purified
Goat Serum	Gibco BRL	16210-072	Mycoplasma Screened
Foetal Bovine Serum	Gibco BRL	10108-165	Mycoplasma Screened
HCV-Positive Serum	IEI	2017	N/A
	Boston Biomedica	PHV 901	N/A
HCV-Negative Human Serum	Cork Blood Bank	N/A	N/A

2.3.4 Equipment and Raw Materials used in Rapid Test

Assembled Cards

Moulded 2-position Test Cards	Alex Mitchell Plastics
Cut Absorbent Pads	Hi-Tech Products
Glass Cut Membrane	Hi-Tech Products
White Backing Label	Hi-Tech Products
0.25g Indicating Desiccant	Silgelac
Foil Pouch	Pro-Pak Ltd.

Equipment Used in Card Spotting

Card Spotter Model 1 JVADGS001-2	JV&A Design Group
Card Blocker Model 1JVADGB001-2	JV&A Design Group
Robotics Pouchmaster XII	About Packaging
Vacuum Chamber Z696	Severn Science

Equipment Used in Conjugation of Peptide

Concentration Units B15	Amicon Inc.
Sprectrophotometer UV/VIS 6105	Jenway
Conjugation System consisting of	Pharmacia Biotech
LKB Pump P-1	
LKB Optical Unit	
LKB Control Unit	
LKB Redifrac (Fraction Collector)	
LKB REC 101 (Chart Recorder)	

Additional Equipment

pH Meter 245	Corning
PM 400 Balance and LC-P45 Printer	Mettler Toledo
HR60 AND Analytical Balance	Mettler Toledo
AD1311 Printer	Mettler Toledo
Laminar Air Flow Cabinet NU-425-600E	Nuaire
Incubating Chambers D-6450	Heraeus Instruments
Waterbath	Grant
Magnetic Stirrer SM 1	Stuart Scientific
Peristaltic Filtering Pump 313S	Watson Marlow
Hotplate Magnetic Stirrer 34532	Snijders
Autovortex Mixer SA2	Stuart Scienfic
Autoclave SAL Type UCC 16 MP 6D1	
0.2µm filters to filter reagents as necessary	
Clean, dry glassware	
Syringes	
Latex gloves	
Face masks	
Safety glasses	

2.4 Methods Used in ELISA Test

2.4.1. Coating of Peptide onto Microtitre Wells

This procedure bound the peptides for evaluation on to the plastic wells of the microtitre plate for use as the solid phase.

1) At a chosen coating concentration, the peptide was allowed to mix in 1M 1X PBS, pH 7.2 (coating buffer).
2) 100µl of coating solution was applied to each well using a multi-channel 8-head pipette or an automated coating machine.
3) The plate was sealed and incubated for 2 hours at 37°C or overnight at 4°C.
4) The coating solution was then washed off the plate.
5) Following washing, the plates were then blocked by dispensing 250µl of EIA blocking buffer onto each well.
6) An incubation of 2 hours at 37°C follows before the blocking buffer was tapped out of each well . The plate was allowed to dry uncovered overnight at 37°C.
7) The blocked plate was then placed in an airtight bag or pouch containing a desiccant and stored at 4°C for use.

2.4.2 Procedure for Washing Microtitre Plates

During the coating of the plates and during the assay protocol, this procedure was carried out to wash away unbound material from the solid phase.

1) The wash buffer was prepared to working strength by diluting 25X wash buffer with deionised water.
2) Using an automated plate washer or an appropriate multi-channel pipette, 300µl of wash buffer was dispensed into each well and allowed to soak for approximately 10 seconds.
3) The buffer was aspirated either by suction or manually by inverson of the plate.
4) The above procedure was repeated until the plate has been washed and aspirated a total of five times.
5) Following the final aspiration the plate was dried by repeated tapping on to a dry tissue.

2.4.3 Procedure to Optimise the Solid Phase

This procedure was followed when choosing a peptide suitable for coating on to the microtitre plate.

1) A number of peptides specific for various regions of the HCV genome were sourced externally (see Biological Components, section 2.3.3).
2) The peptides were evaluated individually, and also were pooled prior to coating to form different peptide combinations. This is described in section 3.1.2.
3) The coating solution was made from a stock solution known to contain a 1.0mg/ml solution of each peptide. This 1.0mg/ml stock was made as follows. For every mg of peptide added to a clean glass container 1ml of 1X PBS, pH 7.2 was added. For example, if 1.4mgs peptide is weighed, 1.4ml 1X PBS is added.
4) From this stock 1.0mg/ml solution of the desired coating concentrations were made ie. *x*ng/ml and were coated according to the coating procedure in section 2.4.1.
5) The different solid phase combinations were known to contain peptides from various viral regions and were tested with characterised sera.

2.4.4 Assay Protocol for ELISA Test

The procedure for running the HCV EIA test followed the following protocol.

1) 10µl of sample or control was added to the microtitre well using a calibrated pipette capable of delivering this volume, such as a 2-20µl multipipette.
2) To the well was added 90µl of sample diluent giving a sample dilution of 1 in 10.
3) The sample was mixed by gently tapping the plate.
4) The plate was covered with a plate sealer and placed in an incubator set at 37°C for a period of 30 min.
5) The sample was then tapped into a biological waste container and the wells washed five times according to the procedure outlined.
6) Any bound antibodies were then incubated with 100µl of enzyme conjugate. This conjugate was goat anti-human IgG antibody bound to HRP.
7) The plate was incubated at 37°C for a further 30 min.
8) Following this incubation the plate was washed five times to remove any unbound conjugate.

9) The next step was the addition of TMB substrate. This was ready to use and 100 µl was added directly to each well.
10) This substrate incubation took place at 25°C with the excluson of light. The incubation time for the substrate was 15 min.
11) The reaction was stopped by the addition of 50µl of stopping reagent per well.
12) Results were read immediately on a plate reader measured with a dual wavelength of 450nm and 630nm.

2.4.5 Inactivation of Goat Serum

This procedure was carried out to inactivate all proteins in the goat serum prior to use in the ELISA conjugate diluent.

1) The waterbath was switched on and allowed to heat until it reached a temperature of 56 ±1°C.
2) The temperature was recorded with a calibrated thermometer.
3) The container containing the goat serum was placed in the waterbath when it reached the desired temperature.
4) The goat serum was stirred occasionally while heating.
5) The temperature of the serum was monitored carefully until it reached a temperature of 56±1°C.
6) The temperature was taken only after mixing ensuring uniformity.
7) Once the serum had reached the desired temperature it was maintained for a period of 30 min.
8) Readings were taken every 5 min. during the 30 min. period.
9) When the incubaton time had elapsed the serum was removed from the waterbath and allowed to cool to room temperature.
10) The serum was then 0.2µm filtered.
11) It was then aliquotted into glass universals and stored at -20°C.

2.5 Methods Used in Rapid Test

2.5.1 Procedure for Conjugation of Peptide to Protein

This procedure was carried out prior to the spotting of peptide to the membrane. Due to the porous nature of the membrane the smaller weight peptide was conjugated to a carrier protein of large molecular weight, BSA.

1) The quantity of peptide to be conjugated was carefully weighed (*x*mgs).
2) Dialysed BSA (6.5mg/ml) was added to this peptide at a fixed ratio. For every mg of peptide weighed, 0.33ml of BSA was added. The BSA and peptide solution was allowed to stir for 15 min. during which time light was excluded.
3) The solution was then dispensed in 1ml volumes into glass tubes containing magnetic stirring bars.
4) A 1% (v/v) glutaraldehyde solution was prepared by adding 40µl of 25% (v/v) glutaraldehyde to 960µl of column elution buffer, previously removed which did not contain azide.
5) To each 1ml volume of the peptide/BSA soluton was slowly added 50µl of 1% (v/v) glutaraldehyde solution. Each tube was covered with parafilm and allowed to stir, again excluding light.
6) Towards the end of the designated conjugation time a 20mg/ml sodium cyanoborohydride solution was prepared. This was done by adding 2ml of deionised water to 40mgs of sodium cyanoborohydride. This serves to stop the conjugation reaction.
7) 25µl of above stopping solution was added per 1ml of conjugated peptide/BSA solution. Each tube was allowed to stir for 15 min., again ensuring exclusion of light.
8) All the conjugated peptide/BSA tubes was pooled into a glass container and was purified either by dialysis or by column purification.

2.5.2 Purification of Conjugated Peptide by Dialysis

Following conjugation of peptide to BSA, it was purified, as described below, to remove any unbound or free peptide or BSA.

1) Column elution buffer, prepared during the conjugation, of peptide is used as dialysis buffer.
2) Into the dialysis buffer was added a piece of dialysis/visking tubing of sufficient length to hold the conjugated peptide. This served to soften the tubing.
3) Having sealed one end of the tubing with a clamp or a double knot the peptide was carefully pipetted into the tubing and the outer end sealed.
4) The sealed tubing was placed into a large beaker containing approximately 2 litres of buffer. The beaker was covered with parafilm and placed at 4°C, stirring constantly.
5) Over a period of 2 days the dialysis buffer was replaced with 2 litres of fresh buffer to give a total of 4 changes using approximately 10 litres of dialysis buffer.
6) On completion of dialysis the conjugated peptide was removed from the tubing and stored in a glass container at 4°C.

2.5.3 Purification of Conjugated Peptide by Column Chromatography

As an alternative method to dialysis, the conjugated material could also be purified by column chromatography. This also served to separate the unbound material from the conjugated mixture.

1) A Sephacryl S-200 column was equilibrated with column elution buffer.
2) A number of tubes were labelled and the chromatography syytem was prepared to allow for the collection of 2ml fractions from the column.
3) Allowing the gel bed to run until almost dry the conjugated peptide was loaded onto the top of the column and the fractions were collected immediately. When the sample had eluted into the gel the buffer was used to top up the column and the fractions were collected either by gravity or by using the pump connected to the conjugation system.
4) Using a visual check along with absorbance readings obtained from the spectrophotometer set at 280nm, fractions were identified which were believed to contain purified conjugated peptide. When using absorbance readings the spectrophotometer was blanked with column elution buffer before the fractions were analysed.

2.5.4 Determination of Peptide Fractions-Neutralisation Assay

After the purification of peptide by chromatography, the fractions containing conjugated peptide were identified using the neutralisation assay. A visual check and the specrophotometer readings served as an aid to identify these fractions.

1) The fractions obtained from the absorbance readings were identified. Five additional fractions either side of the identified fractions were also chosen.
2) 1ml of peptide-specific positive serum was dispensed into a sufficient number of labelled tubes. This serum served as the neutralisation reagent.
3) 10µl of each identified fraction was added into its corresponding tube and the mixture was inverted gently.
4) The tubes were incubated at room temperature for approximately 30 min. This allowed time for any peptide in the fraction to bind to specific antibody in the neutralisation reagent, therefore, neutralising the serum.
5) Using these samples as test specimen, the rapid assay was carried out as per usual. From the results it was possible to determine which fractions contained peptide. A positive result indicated neutralisation did not occur and therefore no peptide was present in that corresponding fraction.
6) All peptide-containing fractions were pooled and stored at 2-8°C.

2.5.5 Concentration of Conjugated Peptide

In certain cases, the volume of conjugated peptide purified by chromatography was large due to a large quantity of peptide fractions. Reduction of volume was carried out using concentration units or by osmosis.

1) The concentration units involved loading the sample onto Amicon B15 units using a clean glass pasteur pipette.
2) After a certain length of time depending on volume the sample reached a set mark, for example 1ml, and was carefully removed.
3) The other method involved the use of osmosis. The sample was placed in dialysis tubing and left in a bed of sucrose.
4) When the sample reduced to a final set volume it was removed.
5) The reduced sample in either case was placed in a glass container and stored at 2-8°C until required for card spotting.

2.5.6 Spotting of Peptide on Solid Phase Membrane

Following purification of peptide either by dialysis or chromatography, it was evaluated for use on the membrane solid phase. This involved the spotting or adhesion of peptide as described.

1) The peptide was spotted on the card membrane following conjugation.
2) A stock spotting solution was made prior to spotting. A 1/10 stock was made by diluting 50µl of conjugated peptide in 450µl of 2X PBS. A series of working dilutions were then made.
3) Cards were then spotted using a multipipette by pipetting 5µl of spotting solution onto the top right port of the card.
4) 6µl of 2X PBS were pipetted onto the top left port and 10µl of control dilution buffer onto the bottom right port.
5) Cards were covered and dried overnight at a temperature of 18-25°C for 18-24 hours.
6) Following drying, the cards were blocked by pipetting 50µl of blocking buffer to each of the two top ports.
7) They were then dried overnight in a vacuum drying chamber.
8) Following removal from the vacuum drier they were pouched in foil pouches containing an indicating desiccant.

2.5.7 Assay Protocol for Rapid Test

This procedure outlines the protocol followed for the testing of samples on the rapid test card.

1) One drop of wash reagent was added to both bottom ports to wet the membrane.
2) Approximately 50µl of sample was added to both bottom ports and allowed to absorb into the membrane.
3) Sample was incubated for 30 seconds.
4) One drop of wash reagent was added to the sample ports to wash the sample towards the reactive sites (top ports).
5) After two min., one drop of wash buffer was added to both top ports.
6) This was followed by one drop of enzyme conjugate reagent to both ports.

7) Upon absorption the enzyme was incubated for two minutes.
8) The unbound conjugate was washed by adding one drop of wash reagent to each of the top ports and absorbed.
9) This wash procedure was repeated until washed four times each time ensuring the drop had absorbed before adding a subsequent drop.
10) The final step was the addition of two drops of substrate reagent to the top ports. This was followed by a five minute incubation.
11) Results were read immediately after the five min. had elapsed.

2.5.8 Preparation of Dialysed BSA

Dialysed BSA at a concentration of 6.5mg/ml was used as the carrier protein in the conjugation of peptide as described in section 2.5.1. It serves as the larger molecule, which binds with the smaller weight peptide allowing spotting of the peptide on to the porous membrane.

1) The first step in the preparation of dialysed BSA was the formulation of approximately 12 litres of 1X PBS which was used as dialysis buffer.
2) A quantity of the prepared dialysis buffer equal to half the desired final volume of dialysed BSA was placed in a glass container.
3) For each ml of the desired final volume of dialysed BSA required 6.5mg of BSA was weighed and added to the container.
4) The BSA was allowed to dissolve into the PBS buffer without stirring.
5) A length of dialysis tubing capable of holding the solution was softened by placing in the dialysis buffer.
6) Once dissolved the hydrated BSA was pipetted into the dialysis tubing and both ends of tubing were sealed.
7) The tubing was placed into a beaker containing approximately 2 litres of dialysis buffer.
8) A magnetic stirring bar was placed in the beaker and the solution was stirred at a temperature of 2-8°C.
9) The entire volume of dialysis buffer was changed three times per day over a period of two days.
10) At the end of dialysis the contents of the tubing was placed in a graduated glass cylinder.

11) The solution was made up to the final volume with dialysis buffer.

12) The top of the cylinder was covered with parafilm and the dialysed BSA was mixed by inverting five times.

13) The solution was aliquoted into volumes of approximately 1ml and stored at a temperature of -20°C.

Sections 3 and 4-

Results

3.1 Evaluation of Synthetic Peptides

Sections 3.1 to 3.6 describe the procedures involved in the process of development of the microtitre plate assay. Various assay parameters were investigated to reach the optimised test. Commencing with the solid phase peptides, various diluents and dilutions for the enzyme conjugate were investigated along with the sample diluent and sample dilution.

At various stages of development, all parameters outlined above were tested with clinical samples to ascertain specificity and sensitivity of the assay. These clinical samples were obtained from three suppliers, International Enzymes, Inc. (IEI), Cork Blood Bank and Boston Biomedica, Inc. It is not known whether the samples were positive by PCR. Interpretation was carried out by the supplier based on results of the Ortho 2.0 Anti-HCV EIA Test and Abbott 2.0 Anti-HCV EIA Test. Specimens had not been inactivated and were treated as potentially infectious material, following good laboratory safety practice. All samples had been tested and found negative for anti-HIV antibody and Hepatitis B surface antigen.

During the evaluation of assay parameters, the method employed by Trinity Biotech for the assay of samples was followed. Samples were assayed in duplicate, the mean of these determinations taken as the absorbance value. For the purpose of the initial evaluation steps, a value of 0.25 absorbance units was assigned as the cut-off value above which a sample was considered positive and below which the interpretation was negative. For subsequent steps, which involved large volume testing, a cut-off value was determined. The value was calculated as the mean negative value ± two standard deviation units ($x \pm 2$ SD). Throughout development, result interpretation involved no further statistical analysis.

3.1.1 Suitability of Peptides for Microtitre Plate Coating

A total of 15 peptides were available for evaluation in this study. Initial experiments were carried out to predict the best peptide for coating, as there was no prior recommendation. The peptides available for evaluation along with the viral region specific to each peptide is outlined in table 3.1.1a. Prior to the adhesion onto the microtitre wells, each peptide was conjugated to the carrier protein BSA, according to

Section 2.5.1. This method of conjugation of peptide to protein is well established within Trinity Biotech and has been used efficiently and successfully to conjugate HIV peptides. Each complex was stored at 4°C prior to use.

Peptide No.	Peptide ID	Region
1	HCV NS3-1 peptide	NS3
2	E47 (Envelope) peptide	Envelope
3	HCV 52 peptide	NS4
4	HCV Core peptide	Core
5	HCV NS5 peptide	NS5
6	HCV 15 peptide	Core
7	E51 (Envelope) peptide	Envelope
8	MS94-23 (Core) peptide	Core
9	PS9401-1 (NS3) peptide	NS3
10	PS9401-2 (NS3) peptide	NS3
11	PS9401-12 (NS3) peptide	NS3
12	PS9307-8 (NS5) peptide	NS5
13	PS9307-9 (NS5) peptide	NS5
14	NS4a peptide	NS4
15	NS3 Recombinant Protein	NS3

Table 3.1.1a Specific viral regions of peptides evaluated for suitability for microtitre plate coating.

Each conjugated peptide was then coated at coarse coating dilutions between 1/10 and 1/5,000. Coating solutions were made up in carbonate coating buffer and coated following procedure in Section 2.4.1.

For the purpose of this initial testing, the following protocol was used.

Sample Dilution	1 in 20
Incubation Times	30 min.
Incubation Temperature	37°C
Plate Washing	300µl x 5 washes
Conjugate Dilution	1/70,000 (as per manufacturer's instructions)

Following the procedure described in Section 2.4.4, each peptide dilution was tested. For the purpose of this investigation only one known HCV positive serum sample and one known HCV negative sample was tested. The data, which accompanied the positive sample, confirmed that it represented all regions of the HCV genome.

Results are expressed as a positive/negative (P/N) ratio of absorbance values read at 450nm with a reference filter of 630nm. (Figure 3.1.1a). Only readings of peptide coated at 1/5,000 are shown because at stronger dilutions the absorbance of the positive serum gave a value beyond the range of the plate reader.

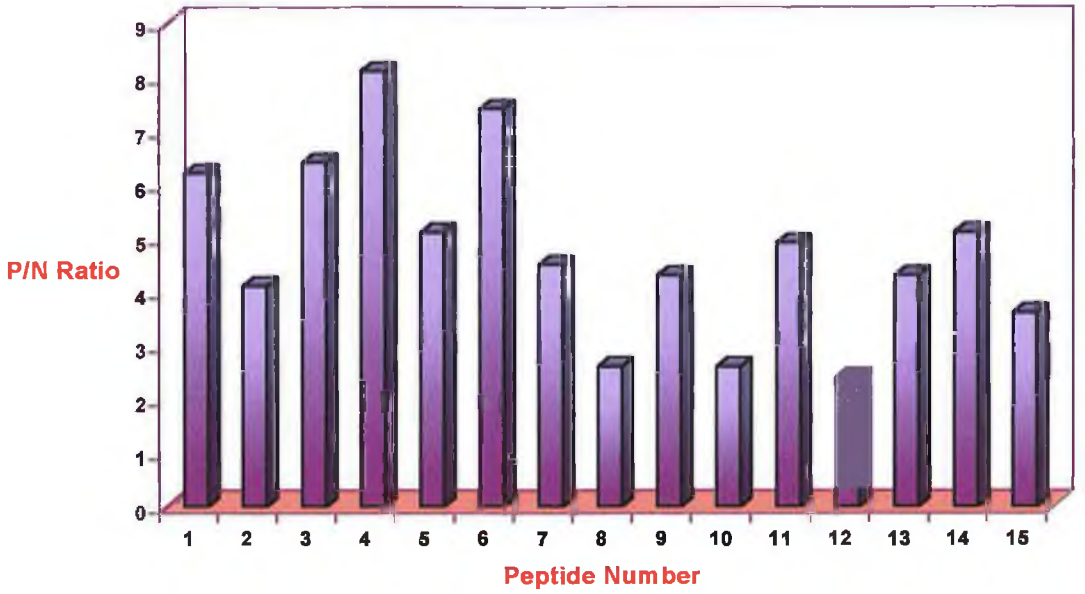


Figure 3.1.1a Positive-negative ratios obtained for initial evaluation of peptides tested on microtitre plate ELISA with known positive and negative sample.

Based on the results from Figure 3.1.1a, four peptides were chosen for further analysis. These peptides were selected as they gave the highest P/N ratios as shown in Table 3.1.1b.

Peptide Number	Name	Viral Region	P/N Ratio
1	HCV NS3-1	NS3	6.2
3	HCV 52	NS4	6.4
4	HCV Core	Core	8.1
6	HCV 15	Core	7.4

Table 3.1.1b P/N ratios of chosen peptides from initial evaluation.

Two of the four peptides selected were specific to the HCV Core region (HCV 15, HCV Core); one was specific to the NS4 region (HCV 52) and one to the NS3 region (HCV NS3-1).

3.1.2 Additional Titration of Peptides

These four peptides were further evaluated in an attempt to improve assay sensitivity. It was decided to coat these peptides individually at coating dilutions between 1/5,000, which gave the positive/negative ratios in Table 3.1.1b, and also at weaker coating dilutions up to 1/15,000.

To evaluate the effect of combining peptides, two additional coating solutions were investigated. The core peptides HCV 15 and HCV Core were pooled and also a full combination of the four peptides was examined. Table 3.1.2a shows the final dilutions and the combinations used.

Each dilution of each peptide or combination was tested with one positive sample and one negative sample in order to choose a coating dilution. Again this positive sample represented all regions of the HCV genome.

Figure 3.1.2a represents the positive/negative ratio achieved. An optimum coating dilution was then chosen to test the peptides with characterised sera.

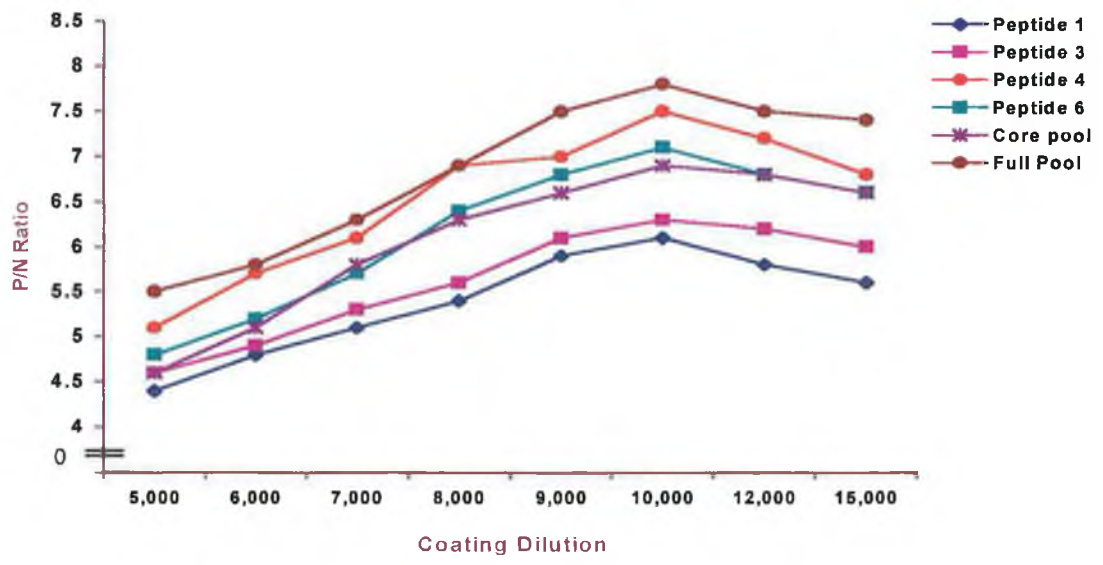


Figure 3.1.2a Further titration of peptides for ELISA solid phase: results are expressed as P/N ratio for each coating dilution.

Peptide/Combination	Viral Region	Coating Dilution
Peptide 1	HCV NS3-1	All peptides at final
Peptide 3	HCV 52	coating dilutions of
Peptide 4	HCV Core	1/5,000, 1/6,000,
Peptide 6	HCV 15	1/7,000, 1/8,000,
Core Pool	HCV 15, HCV Core	1/9,000, 1/10,000,
Full Pool	HCV NS3-1, HCV 52,HCV Core, HCV 15	1/12,000 and 1/15,000.

Table 3.1.2a Dilutions and viral regions of peptides and combinations coated in titration of peptides for ELISA solid phase.

As can be seen from Figure 3.1.2a, the optimum coating dilution for all peptides and peptide combinations was at 1/10,000.

3.1.3 Testing with Characterised Sera

With a coating dilution of 1/10,000, the peptides above were tested with characterised HCV sera to determine which solid phase gave the best sensitivity and specificity.

A total of nine samples (ID 1-9) were used in this evaluation including three negative serum samples. The assay protocol in section 3.1.1 was followed.

The samples supplied had been characterised using of the Ortho RIBA 3.0 Assay. The viral regions specific to the samples are shown in Table 3.1.3a, along with the corresponding coated peptides specific to these viral regions.

Sample ID	Viral Region	Specific Peptide
1	NS3, Core	1,4,6
2	NS4, Core	3,4,6
3	Core	4,6
4	NS3, NS4	1,3
5	Core	4,6
6	NS4, Core	3,4,6
7	Neg.	None
8	Neg.	None
9	Neg.	None

Table 3.1.3a Nine samples characterised by Ortho RIBA 3.0 assay showing the viral region specific to samples and the coated peptide specific to the viral regions.

Each sample was tested on the four peptides individually and also on the core pool and the full pool combinations.

The absorbance reading of each sample was read at 450nm with a reference filter of 630nm. The readings obtained with each sample are represented in Figure 3.1.3a.

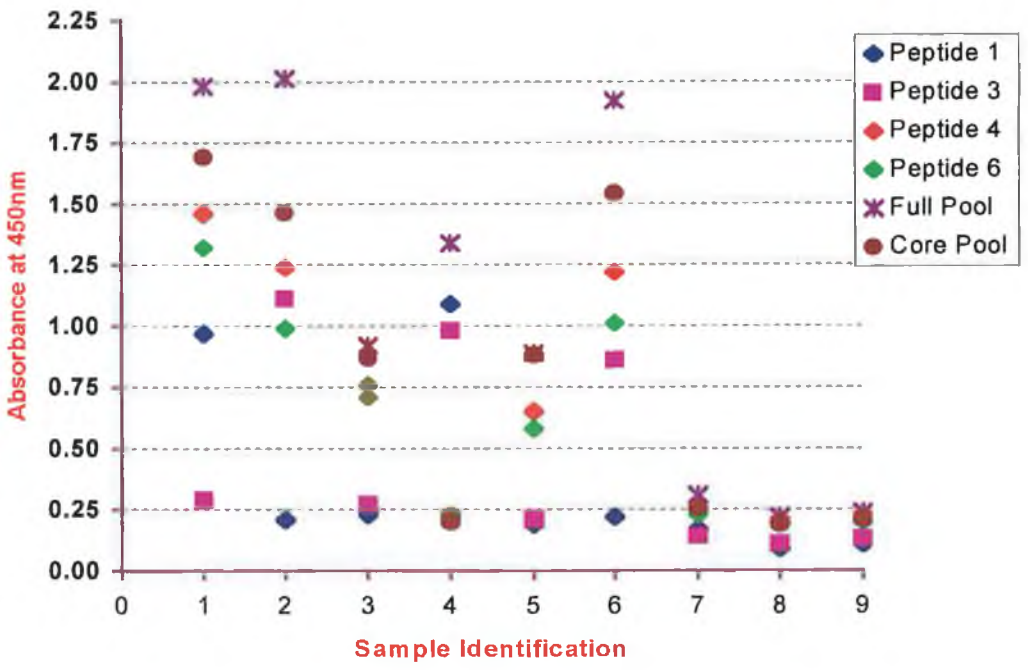


Figure 3.1.3a Absorbance readings of nine characterised serum samples on wells with peptide coated at a dilution of 1/10,000.

To represent these results and determine which peptide gave the best specificity and sensitivity, an initial coarse value of >0.25 absorbance units was interpreted as a positive reading and <0.25 absorbance units as a negative reading.
These values served as a guideline only, the main area of interest lay in achieving a peptide or combination, which gave the highest positive value with a corresponding low negative reading. Based on this criteria, Table 3.1.3b shows the sensitivity and specificity achieved.

As can be seen from the table, the full pool was the only combination in picking up all characterised samples 1 to 6. The negative samples were close to the cut-off value of 0.25 absorbance units, samples 7, 8 and 9 giving readings of 0.21, 0.22 and 0.24 absorbance units, respectively. The individual peptides and the core pool were unable to detect all the samples.

Sample	Viral Region	P1 (NS3)	P3 (NS4)	P4 (Core)	P6 (Core)	Core Pool	Full Pool
1	NS3, Core	+	-	+	+	+	+
2	NS4, Core	-	+	+	+	+	+
3	Core	-	-	+	+	+	+
4	NS3, NS4	+	+	-	-	-	+
5	Core	-	-	+	+	+	+
6	NS4, Core	-	+	+	+	+	+
7	Neg.	-	-	+	-	+	-
8	Neg.	-	-	-	-	-	-
9	Neg.	-	-	-	-	-	-
Sensitivity		33.3%	50%	83.3%	83.3%	83.3%	100%
Specificity		100%	100%	66.6%	100%	66.6%	100%

Table 3.1.3b Initial sensitivity and specificity of peptide combinations;
+ represents a positive absorbance reading;
- represents a negative absorbance reading.

The full pool solid phase, with all peptides coated at a dilution of 1/10,000, was chosen for further evaluation tests.

3.2 Improvements to Assay Sensitivity and Specificity

In an attempt to improve the differentiation between positive and negative readings, certain components and parameters were investigated.

Up to this point, the coating solution was the only parameter to be evaluated and all work was carried out without examining other areas.
These areas were evaluated and included

- Sample Diluent
- Conjugate Titration
- Conjugate Diluent
- Sample Dilution

3.2.1 Evaluation of Sample Diluent

The sample diluent was the first of these to be evaluated. All the solid phase evaluation tests were carried out with the samples diluted in control dilution buffer. The addition of certain components to this buffer was investigated to determine which, if any, would give any improvement to test sensitivity and specificity. It was decided to add protein and serum to the diluent in the hope that it would reduce the negative readings by binding non-specific proteins in the sample.

The proteins chosen to add to control dilution buffer were Bovine Serum Albumin (BSA), Human Serum Albumin (HSA), Rabbit Serum (RS) and Goat Serum (GS). Each component was added to a final concentration of 1% (v/v), 2% (v/v), 5% (v/v) and 10% (v/v).

As some precipitate was present in the diluents following addition of protein, each diluent was filtered with a 0.2µm filter containing a 0.45µm pre-filter prior to use.

To evaluate the sample diluent, one positive serum sample and three negative serum samples were diluted in each test diluent and tested as normal. Based on the results of

Section 3.1, the solid phase used for this evaluation was the full pool combination coated at a dilution of 1/10,000.

Table 3.2.1a gives the absorbance values achieved for each sample in each diluent. Figure 3.2.1a shows these results expressed as a positive/negative ratio for each component added. As three negative samples were tested, the mean absorbance of these readings was calculated and used to obtain the positive/negative ratio. Again, the samples were assayed in duplicate.

Component added, % (v/v)		Positive Reading	Mean Negative Absorbance	P/N Ratio
BSA	***1%***	1.346	0.237	5.7
	2%	1.142	0.224	5.1
	5%	1.366	0.305	4.5
	10%	1.371	0.298	4.6
HSA	***1%***	1.117	0.228	4.9
	2%	1.012	0.199	5.1
	5%	1.126	0.209	5.4
	10%	1.236	0.221	5.6
RS	***1%***	1.194	0.230	5.2
	2%	1.281	0.268	4.8
	5%	1.198	0.292	4.1
	10%	1.267	0.325	3.9
GS	***1%***	1.221	0.207	5.9
	2%	1.186	0.189	6.3
	5%	1.202	0.156	7.7
	10%	1.179	0.178	6.6

Table 3.2.1a. Absorbance and P/N values of positive and negative samples diluted in sample diluents for evaluation.

Table 3.2.1a and Figure 3.2.1a indicate that the addition of goat serum gave the highest positive/negative ratios. As can be seen from Table 3.2.1a, 5% (v/v) goat serum gave the lowest absorbance reading for the negative samples and also gave the best positive to negative ratio.

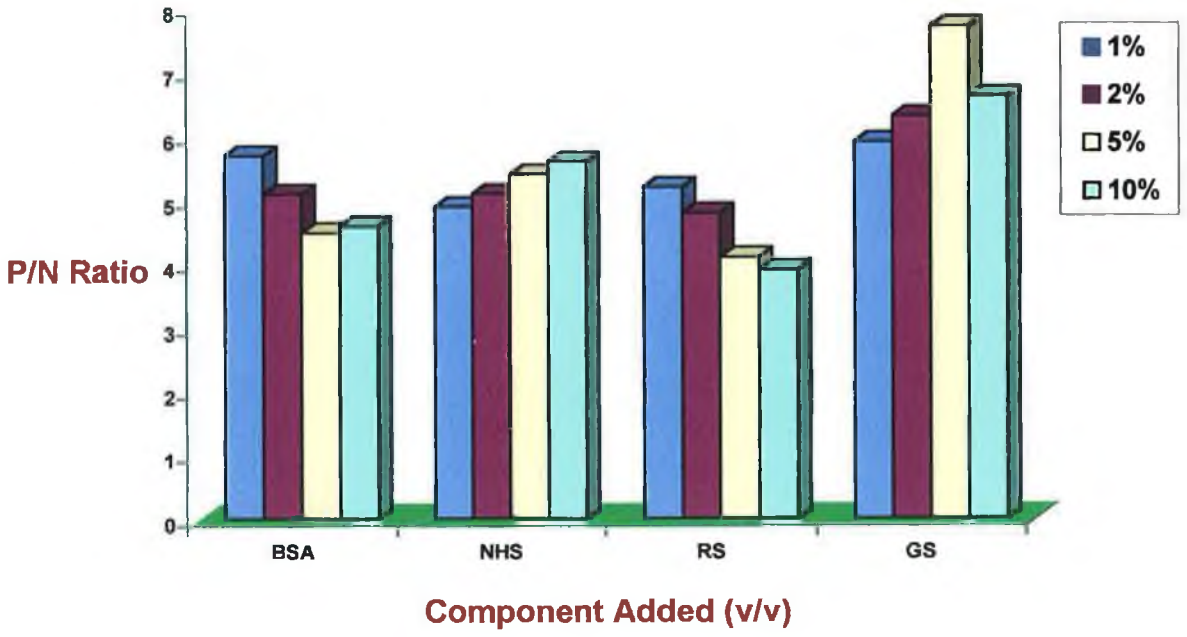


Figure 3.2.1a. P/N ratio achieved with sample added to sample diluent, containing bovine serum albumin, normal human serum, rabbit serum and goat serum.

All further evaluations used 5% (v/v) goat serum in sample diluent.

3.2.2 Evaluation of Enzyme Conjugate

Following the evaluation of sample diluent was that of the enzyme conjugate. The HCV indirect assay utilised anti-human IgG labelled to peroxidase as the enzyme.

A total of two goat anti-human IgG peroxidase-labelled conjugates were purchased for evaluation, one lot from Kirkegarrd and Perry Laboratories (KPL) and the other from Genzyme. All previous work had utilised the KPL conjugate at 1/70,000. This working dilution was recommended by the manufacturer and was used as a coarse titre. The KPL conjugate was titred between 1/10,000 and 1/120,000 and Genzyme between 1/1,000 and 1/12,000. These values were above and below their recommended working dilutions for use in EIA's.

For this initial evaluation all dilutions were made in a cytochrome C-based diluent supplied by Trinity Biotech, plc., and used in other EIA procedures.

One positive sample and one negative sample were used in this initial evaluation of both conjugates.

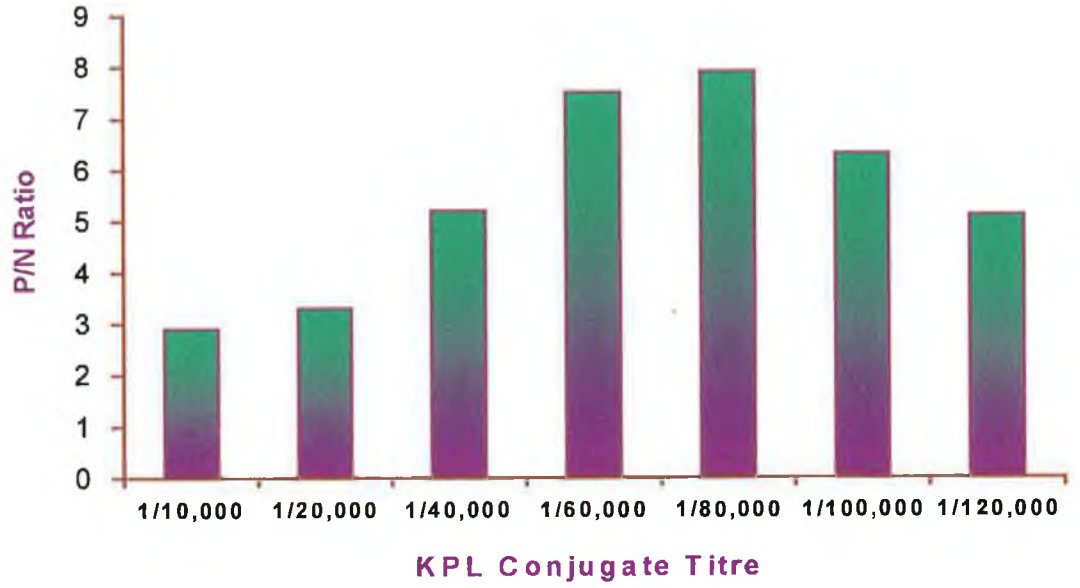


Figure 3.2.2a. P/N ratios achieved in titre of KPL anti-human IgG-peroxidase conjugated.

Figure 3.2.2a represents the positive to negative ratio achieved with the KPL conjugate and Figure 3.2.2b shows that of the Genzyme conjugate.

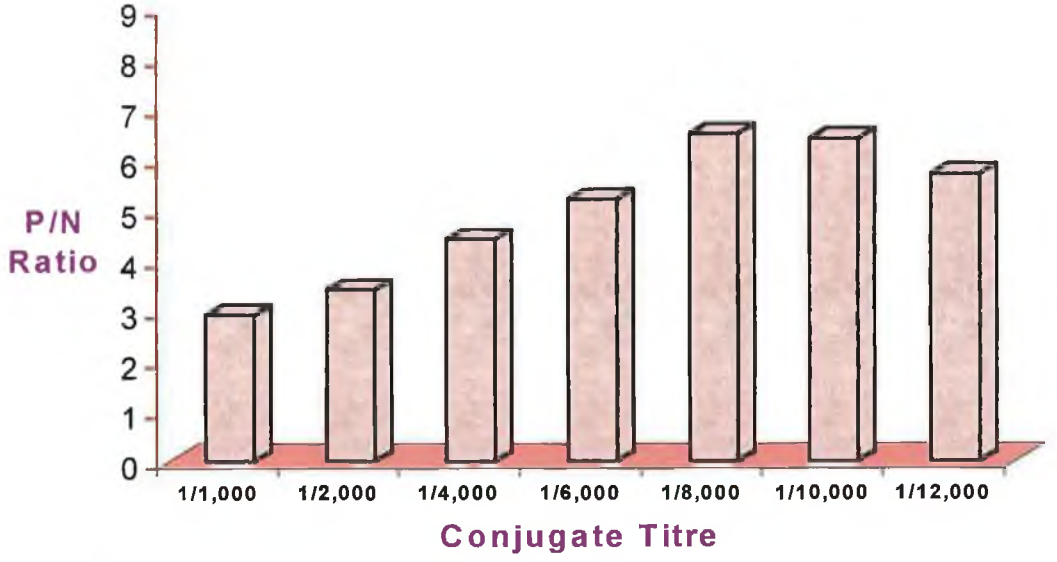


Figure 3.2.2b. P/N ratios achieved in titre of Genzyme anti-human IgG-peroxidase conjugated.

As can be seen from Figure 3.2.2a the KPL conjugate titred well between 1/10,000 and 1/120,000. The best positive to negative ratio was obtained at a dilution of 1/80,000. In the case of the Genzyme conjugate the best ratio was obtained at a dilution of 1/8,000 although the absorbance value for the positive sample was lower than that achieved with the KPL conjugate.

These absorbance readings and P/N ratios for both conjugates are shown in Table 3.2.2a and 3.2.2b.

Dilution	Positive reading	Negative reading	P/N ratio
1/10,000	1.988	0.686	2.9
1/20,000	1.699	0.514	3.3
1/40,000	1.654	0.318	5.2
1/60,000	1.587	0.212	7.5
1/80,000	1.458	0.184	7.9
1/100,000	1.127	0.179	6.3
1/120,000	0.893	0.175	5.1

Table 3.2.2a. Absorbance and P/N values of KPL conjugate evaluation.

Dilution	Positive reading	Negative reading	P/N Ratio
1/1,000	1.669	0.541	2.9
1/2,000	1.521	0.447	3.4
1/4,000	1.395	0.317	4.4
1/6,000	1.288	0.247	5.2
1/8,000	1.257	0.193	6.5
1/10,000	1.199	0.187	6.4
1/12,000	1.022	0.179	5.7

Table 3.2.2b. Absorbance and P/N values of Genzyme conjugate evaluation.

The absorbance values and the positive to negative ratios of both conjugate evaluations indicate that the best differentiation was observed with the KPL conjugate at a dilution of 1/80,000.

3.2.3 Evaluation of Enzyme Conjugate Diluent

As stated in Section 3.2.2 the KPL and Genzyme goat anti-human peroxidase conjugates were diluted in a cytochrome C-based diluent. This diluent did not contain any serum or detergent or additional components, which are known to reduce negative readings. The addition of such components to the KPL enzyme conjugate diluent was the next parameter to be investigated. The KPL conjugate at 1/80,000 was chosen.

For this purpose, two detergents, Tween 20 and Triton X-100 were evaluated. They were added separately to the cytochrome C diluent at a concentration of 0.1% (v/v), 0.05% (v/v), 0.02% (v/v) and 0.01% (v/v). Goat serum was also added, at a concentration of 5%. Cytochrome C conjugate diluent without added components was used as a control diluent.

For this investigation, one positive serum sample and three negative serum samples were assayed. Table 3.2.3a shows the absorbance readings obtained on the assay.

Component added, (v/v)		Positive	Neg. 1	Neg. 2	Neg. 3	Mean Neg.
Tween 20	**0.1%**	1.906	0.241	0.247	0.266	0.251
	0.05%	1.727	0.283	0.262	0.251	0.265
	0.02%	2.069	0.299	0.276	0.254	0.276
	0.01%	1.998	0.287	0.269	0.277	0.277
Triton X-100	**0.1%**	1.708	0.228	0.191	0.208	0.209
	0.05%	1.659	0.158	0.175	0.186	0.173
	0.02%	1.565	0.161	0.185	0.201	0.182
	0.01%	1.762	0.187	0.202	0.225	0.204
Goat Serum	**5%**	0.563	0.151	0.198	0.246	0.198
Reference		1.693	0.221	0.213	0.196	0.211

Table 3.2.3a. Absorbance readings obtained upon addition of components to the diluent for KPL anti-human IgG-peroxidase conjugated, in ELISA test.

The positive/negative ratios are given in Table 3.2.3b. The mean of the three negative readings is used to calculate the P/N ratio. These positive to negative ratios are represented diagrammatically in Figure 3.2.3a.

Component (v/v)		*P/N Ratio*
Tween 20	0.1%	7.59
	0.05%	6.51
	0.02%	7.49
	0.01%	7.21
Triton X-100	0.1%	8.17
	0.05%	9.58
	0.02%	8.59
	0.01%	8.63
Goat Serum	5%	2.84
Reference		8.04

Table 3.2.3b. P/N ratios achieved with addition of components to the diluent for KPL anti-human IgG-peroxidase conjugated, in ELISA test.

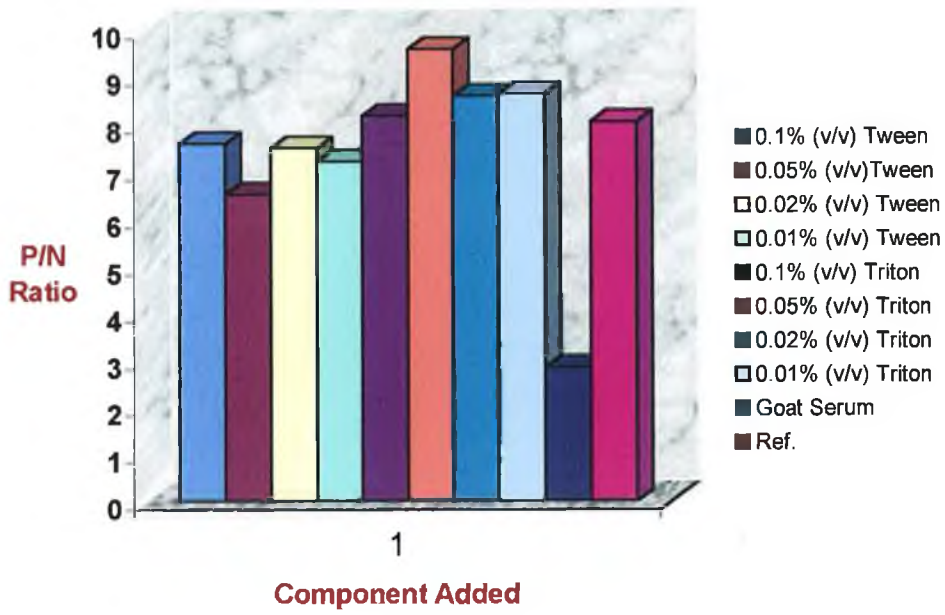


Figure 3.2.3a. P/N ratios achieved with above components added to KPL conjugate diluent, in ELISA test.

From the data shown it was clear that Triton X-100 was the best diluent. Table 3.2.3a shows that it gave cleaner negative readings with high positive values. It was selected based on its specificity and positive/negative ratio. The best Triton X-100 dilution was 0.05%.

3.2.4 Investigation of Optimum Sample Dilution

Up to this stage, all investigative work was carried out by premixing serum samples in tubes, at an approximate dilution of 1 part sample in 19 parts control dilution buffer i.e. a 1 in 20 dilution. The next step was to investigate the effect of mixing the samples in the microwell, which eliminated the premixing step in the tube. This was done by adding the required quantity of sample to the well followed by the addition of diluent, which served to mix the sample.

Each sample was mixed to give a final dilution of 1 in 5, 1 in 10, 1 in 20 and 1 in 40 as shown in Table 3.2.4a. The final 'in-well' volume was 100µl. Samples applied undiluted to the well were also examined.

	Undiluted	1/5	1/10	1/20	1/40
Volume Sample	100µl	20µl	10µl	5µl	2.5µl
Volume Diluent	None	80µl	90µl	95µl	97.5µl

Table 3.2.4a. Sample dilutions (µl) investigated for ELISA evaluation.

For the purpose of this investigation, four positive samples, numbered 1 to 4 and four negative samples, numbered 5 to 8 were diluted as described above.

Two of the positive samples, 1 and 4 were chosen for this evaluation, as they were weakly positive samples, whereas samples 2 and 3 were known stronger samples.

Table 3.2.4b gives the absorbance values obtained for each of the eight samples at each dilution and these figures are represented in Figure 3.2.4a.

Dilution	Pos. 1	Pos. 2	Pos. 3	Pos. 4	Neg. 1	Neg. 2	Neg. 3	Neg. 4
Undiluted	0.966	2.500	1.549	0.837	0.306	0.154	0.199	0.189
1 in 5	0.869	1.977	1.498	0.654	0.256	0.126	0.165	0.163
1 in 10	0.759	1.935	1.256	0.625	0.198	0.092	0.132	0.124
1 in 20	0.578	1.382	0.925	0.412	0.167	0.089	0.101	0.105
1 in 40	0.457	1.251	0.797	0.367	0.135	0.087	0.087	0.093

Table 3.2.4b. Absorbance readings obtained in ELISA sample dilution evaluation.

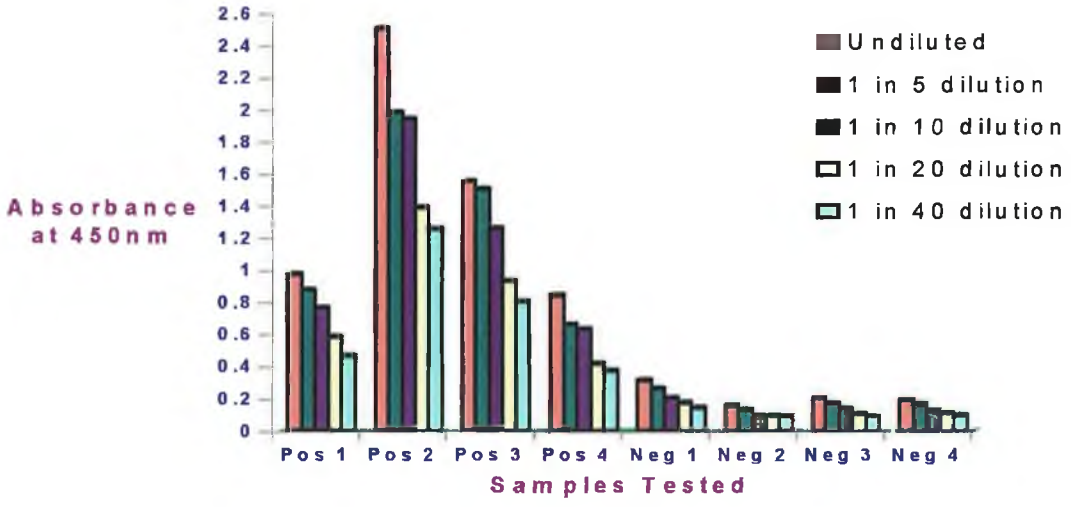


Figure 3.2.4a. Sample absorbance readings obtained with samples tested on ELISA plate test, for evaluation of various sample dilutions.

The positive/negative ratio for each positive sample was calculated for all sample dilutions. The negative reading was taken as the mean of the four negative sample readings. This is shown in Table 3.2.4c.

	Undiluted	1 in 5	1 in 10	1 in 20	1 in 40
Mean Negative absorbance ⇨	0.212	0.178	0.137	0.116	0.101
Positive 1	4.96	4.88	5.54	4.98	4.52
Positive 2	11.79	11.16	14.12	11.91	12.38
Positive 3	7.30	8.41	9.16	7.97	7.89
Positive 4	3.94	3.67	4.56	3.55	3.63

Table 3.2.4c. P/N ratios of samples diluted 'in well' as shown in Table 3.2.4a.

The ratios for the positive samples are represented in Figure 3.2.4b. The results indicated that the best in-well dilution was at 1 in 10 of sample in control dilution buffer as it gave the best positive/negative ratios for all four positive samples tested.

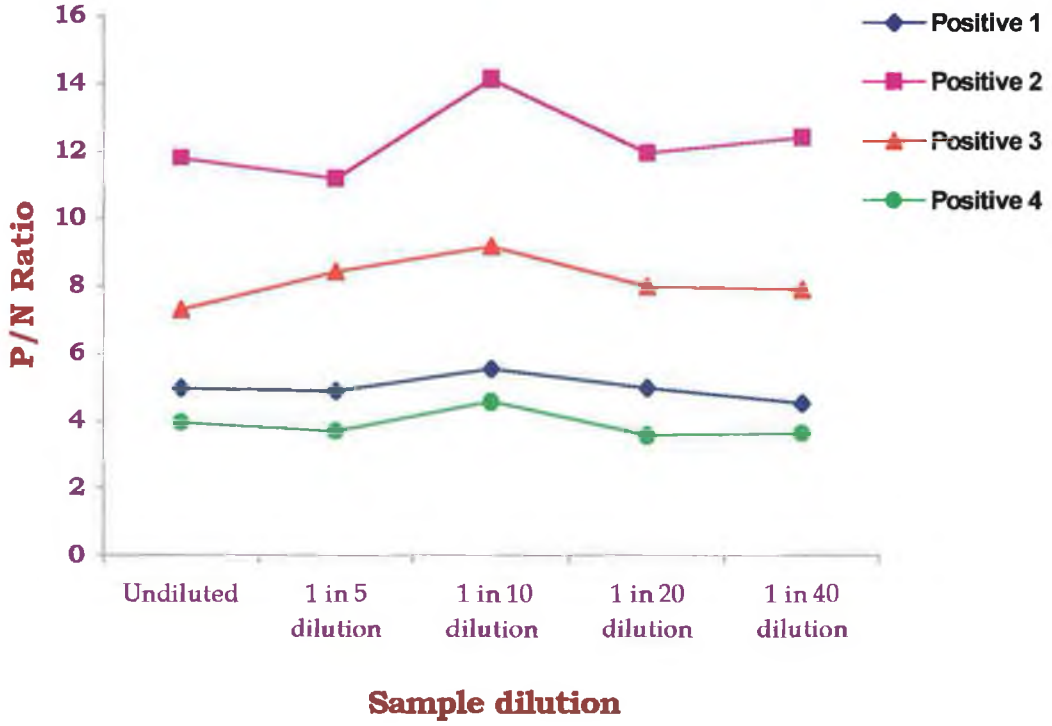


Figure 3.2.4b. P/N ratios of positive samples obtained with samples tested on ELISA plate test for evaluation of various sample dilutions.

3.2.5 Final Assay Parameters

As a result of the investigations described in Section 3.1 and 3.2, the following assay parameters were used in the analysis of serum samples in the proceeding sections. The assay protocol is outlined below.

Plate:	Full Pool of peptides coated at 1/10,000 HCV NS3-1, HCV 52, HCV Core and HCV 15
Sample:	1/10 in-well dilution in control dilution buffer with 5% (v/v) goat serum (10µl sample + 90µl diluent)
Conjugate:	1/80,000 KPL goat anti-human IgG-peroxidase conjugated, in cytochrome C diluent with 0.05% (v/v) Triton X-100 added.

Assay Protocol:

100µl sample
30 min. @ 37°C
Wash x 5 times
100µl conjugate
30 min. @ 37°C
Wash x 5 times
100µl substrate
15 min. @ 25°C
Read at 450nm with a reference filter of 630nm

Sensitivity and Specificity

The following formulae were used to calculate the sensitivity and specificity for all evaluations carried out (Hart, 1980).

Sensitivity	Specificity
$\frac{\text{True positives-false negatives}}{\text{True positives}}$	$\frac{\text{True negatives-false positives}}{\text{True negatives}}$

3.3 Initial Clinical Trial-Analysis of Clinical Sera

At this point, using the chosen parameters outlined in Section 3.2.5, a number of HCV positive and negative serum or plasma samples were evaluated.

The samples, which were received from International Enzymes Incorporated (IEI) were known to be specific for certain viral regions as recommended by the supplier.

3.3.1. Interpretation of Analysis Data

A total of 88 samples were received from IEI. These samples had been previously characterised by Ortho™ Recombinant ImmunoBlot Assay (RIBA).

These characteristics were represented as RIBA bands, each band representing a specific viral region of the HCV genome, which is shown in Table 3.3.1a.

RIBA Band	Viral Region
C100	NS4
C33	NS3
C22	Core
5-1-1	NS5

Table 3.3.1a. Ortho™ RIBA banding of IEI samples and corresponding viral region.

A total of two or more bands must be present if the sample to be termed a positive sample. The presence of one band only indicates a negative or indeterminate (Ind.) sample. It is not known whether or not indeterminate samples are positive by PCR. They were termed negative or indeterminate as a result of Ortho Anti-HCV RIBA 2.0 testing carried out by the supplier.

All IEI samples, along with 45 negative samples received from Cork Blood Bank were tested as described previously. Table 3.3.1b shows the samples available for analysis and the RIBA banding. The absorbance values obtained on testing are included.

Sample	IEI Lot Number	RIBA Band	Viral Region(s)	Interpretation	Absorbance
1	473	C100, C33	NS4, NS3	Pos.	0.520
2	678	C33, C22	NS3, Core	Pos.	0.292
3	682	C33, C22	NS3, Core	Pos.	0.641
4	687	C33, C22	NS3, Core	Pos.	1.348
5	694	C33, C22	NS3, Core	Pos.	0.613
6	696	C33, C22	NS3, Core	Pos.	0.852
7	699	C33, C22	NS3, Core	Pos.	0.819
8	700	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	1.198
9	701	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.482
10	702	C22	Core only	Neg./Ind.	0.138
11	703	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	1.237
12	706	C33, C22	NS3, Core	Pos.	0.646
13	708	C33, C22	NS3, Core	Pos.	0.173
14	709	C100, C33, C22	NS4, NS3, Core	Pos.	1.339
15	710	C33, C22	NS3, Core	Pos.	0.564
16	711	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	1.290
17	712	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.835
18	713	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.203
19	714	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.408
20	717	C100, C33, C22	NS4, NS3, Core	Pos.	0.822
21	718	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.613
22	719	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	1.683
23	722	C22	Core only	Neg./Ind	0.169
24	725	C100, C33, C22	NS4, NS3, Core	Pos.	0.081
25	727	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	1.125
26	728	C33, C22	NS3, Core	Pos.	0.417
27	729	C100, C33, C22	NS4, NS3, Core	Pos.	0.900
28	731	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.092

Sample	IEI Lot Number	RIBA Band	Viral Region(s)	Interpretation	Absorbance
29	736	C33, C22	NS3, Core	Pos.	0.956
30	738	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.373
31	739	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.430
32	741	C33, C22	NS3, Core	Pos.	0.225
33	742	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.121
34	743	C33, C22	NS3, Core	Pos.	0.214
35	746	C33, C22	NS3, Core	Pos.	0.815
36	748	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.286
37	750	C33, C22	NS3, Core	Pos.	0.171
38	753	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.405
39	754	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	0.211
40	762	C22	Core only	Neg./Ind.	0.121
41	767	C22	Core only	Neg./Ind.	0.112
42	779	C100, C33, C22	NS4, NS3, Core	Pos.	0.670
43	859	C100, C33, C22	NS4, NS3, Core	Pos.	0.316
44	865	C33, C22	NS3, Core	Pos.	1.468
45	894	C22	Core only	Neg./Ind.	0.122
46	903	C22	Core only	Neg./Ind.	0.123
47	904	C22	Core only	Neg./Ind.	0.065
48	913	C22	Core only	Neg./Ind.	0.076
49	935	C22	Core only	Neg./Ind.	1.103
50	937	C22	Core only	Neg./Ind.	0.078
51	943	C100, C33	NS4, NS3	Pos.	0.214
52	944	C22	Core only	Neg./Ind.	0.087
53	945	C33, C22	NS3, Core	Pos.	0.303
54	946	C100, C33, C22	NS4, NS3, Core	Pos.	0.845
55	947	C22	Core only	Neg./Ind.	0.882
56	950	C33, C22	NS3, Core	Pos.	0.894
57	951	C33, C22	NS3, Core	Pos.	0.348
58	953	C22	Core only	Neg./Ind.	0.102

Sample	IEI Lot Number	RIBA Band	Viral Region(s)	Interpretation	Absorbance
59	958	C33, C22	NS3, Core	Pos.	0.082
60	959	C22	Core only	Neg./Ind.	0.060
61	961	C33, C22	NS3, Core	Pos.	1.589
62	963	C33, C22, 5-1-1	NS3, Core, NS5	Pos.	0.748
63	981	C33, C22	NS3, Core	Pos.	0.190
64	985	C22	Core only	Neg./Ind.	0.095
65	991	C22	Core only	Neg./Ind.	0.133
66	992	C22	Core only	Neg./Ind.	0.083
67	993	C22	Core only	Neg./Ind.	0.164
68	995	C33, C22	NS3, Core	Pos.	0.916
69	996	C100, C33, C22	NS4, NS3, Core	Pos.	0.112
70	997	C22	Core only	Neg./Ind.	0.158
71	998	C22	Core only	Neg./Ind.	0.197
72	999	C22	Core only	Neg./Ind.	0.091
73	1013	C22	Core only	Neg./Ind.	0.114
74	1025	C22	Core only	Neg./Ind.	0.134
75	1039	C22	Core only	Neg./Ind.	0.194
76	1053	C100, C33, C22, 5-1-1	NS4, NS3, Core, NS5	Pos.	0.619
77	1055	C22	Core only	Neg./Ind.	0.090
78	1059	C22	Core only	Neg./Ind.	0.129
79	1070	C33	NS3 only	Neg./Ind.	0.085
80	1072	C22	Core only	Neg./Ind.	0.282
81	1076	C22	Core only	Neg./Ind.	0.074
82	1077	C33	NS3 only	Neg./Ind.	0.074
83	1081	C22	Core only	Neg./Ind.	0.220
84	1086	C22	Core only	Neg./Ind.	0.116
85	1110	C22	Core only	Neg./Ind.	0.287
86	1112	C22	Core only	Neg./Ind.	0.113
87	1115	C33, C22	NS3, Core	Pos.	0.108
88	1265	C33	NS3 only	Neg./Ind.	0.263

Table 3.3.1b. IEI samples with RIBA banding and absorbance readings with ELISA parameters outlined in section 3.2.5.

The absorbance values obtained for the 45 Cork blood bank negative samples are shown in Figure 3.3.1a. See Appendix 1 for raw data.

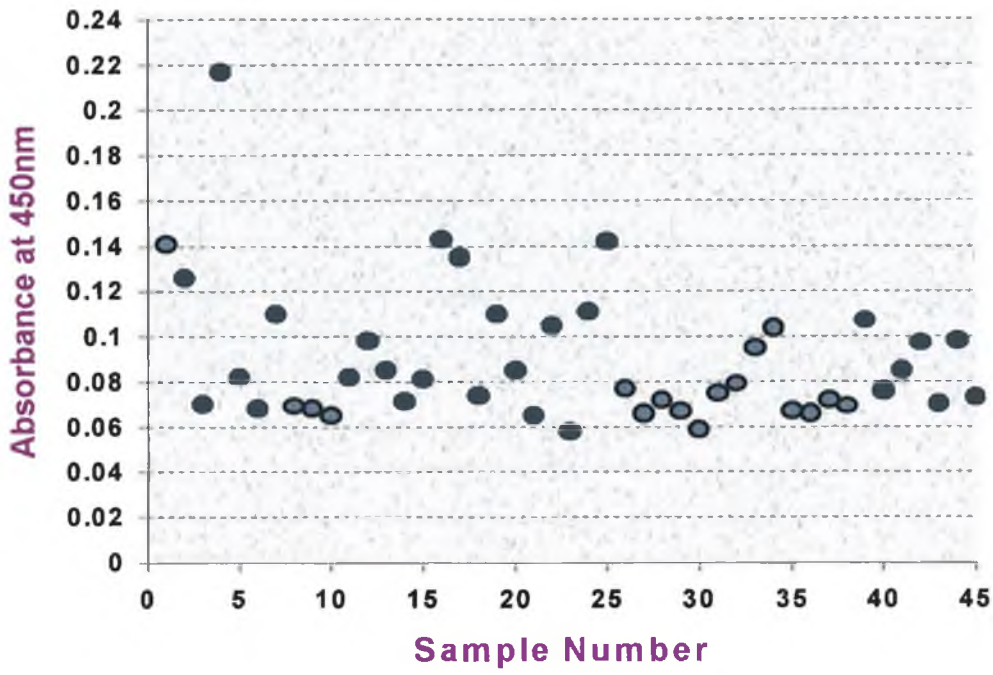


Figure 3.3.1a. Absorbance values of 45 negative samples in ELISA test with parameters outlined in section 3.2.5.

3.3.2 Discrepant Samples

From the 45 negative samples, a cut-off value was determined. This value was calculated by adding two standard deviations to the mean negative value (x + 2 SD) and was given a value of 0.145 absorbance units.

3.3.2.1 False Negative Samples

Sample	Absorbance	RIBA Banding	Expected Result
725	0.081	C100, C33, C22	Positive
731	0.092	5-1-1, C100, C33, C22	Positive
742	0.121	5-1-1, C100, C33, C22	Positive
958	0.082	C33, C22	Positive
996	0.112	C100, C33, C22	Positive
1115	0.108	C33, C22	Positive

Table 3.3.2a. False negative IEI samples from initial evaluation of ELISA test, using parameters outlined in section 3.2.5.

3.3.2.2 False Positive Samples

Sample	Absorbnace	RIBA Banding	Expected Result
722	0.169	C22	Neg./Ind.
935	1.103	C22	Neg./Ind.
947	0.882	C22	Neg./Ind.
993	0.164	C22	Neg./Ind.
997	0.158	C22	Neg./Ind.
998	0.197	C22	Neg./Ind.
1039	0.194	C22	Neg./Ind.
1072	0.282	C22	Neg./Ind.
1081	0.220	C22	Neg./Ind.
1110	0.287	C22	Neg./Ind.
1265	0.263	C33	Neg./Ind.
C4	0.217	Not available	Neg.

Table 3.3.2b. False positive samples from initial evaluation of ELISA test. A negative (Neg.) or indeterminate (Ind.) reading is based on RIBA bands as outlined in section 3.3.1.

3.3.3 Sensitivity and Specificity from Initial Evaluation

A total of 88 IEI samples were assayed. The analysis data shows that 35 of these samples contained only one RIBA band and were therefore treated as negative samples and included in the specificity results along with the 45 Cork Blood Bank negative samples. This gave a total of 80 negatives. The remaining 53 positive samples contained two or more RIBA bands.

Table 3.3.3a shows the initial specificity and sensitivity results based on the cut-off value of 0.145 absorbance units.

Sensitivity	Specificity
53 True Pos.-6 False Neg. 53 True Pos.	80 True Neg.-12 False Pos. 80 True Neg.
47/53 x 100	68/80 x 100
88.6%	85%

Table 3.3.3a. Initial Sensitivity and Specificity on negatives and IEI positives, using parameters outlined in section 3.2.5.

3.4 Further Attempts to Improve Assay Sensitivity

Based on the sensitivity and specificity results obtained in Section 3.3, the solid phase was further evaluated. The peptides contained in the full pool were coated as shown in Table 3.4a.

Peptide	Viral Region	RIBA Band	Coating Concentration
HCV NS3-1	NS3	C33	1/10,000
HCV 52	NS4	C100	1/10,000
HCV Core	Core	C22	1/10,000
HCV 15	Core	C22	1/10,000

Table 3.4a. Peptides and coating concentrations used in ELISA full pool solid phase.

3.4.1 Additional Evaluation of Peptide Titration

Each of the peptides contained in the full pool was titred individually at dilutions greater than the full pool dilution of 1/10,000. A set of combinations were coated and designated A to G. These combinations contained peptide with coating combinations as shown in Table 3.4.1a. The full pool was included as a reference solid phase.

	HCV NS3-1	HCV 52	HCV Core	HCV 15
A	-	-	-	1/800
B	-	-	1/3,000	-
C	-	1/800	-	-
D	1/500	-	-	-
E	1/600	-	-	-
F	-	1/600	1/2,500	-
G	-	1/800	-	1/800
Full Pool	1/10,000	1/10,000	1/10,000	1/10,000

Table 3.4.1a. Peptides and coating concentrations of combinations in re-titre of full pool peptides, designated A to G.

To determine which solid phase gave the best sensitivity and specificity, the combinations were tested with 18 of the IEI samples, the discrepant samples from the full pool peptide coated at 1/10,000, and 10 of the Cork blood bank negatives.

Sample Lot	A	B	C	D	E	F	G	FP
473(+)	0.206	0.220	0.417	0.242	0.244	0.380	0.368	0.420
687(+)	2.223	1.272	0.139	0.122	0.128	1.225	2.001	1.248
694(+)	Over	1.757	0.113	0.150	0.125	1.658	2.036	0.513
700(+)	1.195	2.110	Over	0.203	0.124	1.816	1.604	1.098
706(+)	2.165	1.796	0.175	0.168	0.137	1.709	2.016	0.545
709(+)	Over	2.308	0.249	0.228	0.199	0.543	2.196	1.139
727(+)	Over	2.020	1.474	0.306	0.343	2.264	2.427	1.025
728(+)	0.377	0.338	0.146	0.147	0.144	0.340	0.362	0.317
741(+)	1.877	1.792	0.559	0.922	1.037	1.813	1.827	0.127
743(+)	0.584	1.561	0.127	0.125	0.116	1.630	0.638	0.119
748(+)	0.127	1.273	0.666	0.214	0.138	1.465	0.592	0.183
779(+)	0.284	0.287	0.698	0.091	0.077	0.720	0.742	0.470
937(Ind)	0.168	0.235	0.130	0.140	0.156	0.251	0.148	0.068
944(Ind)	0.103	0.081	0.098	0.088	0.104	0.086	0.094	0.077
959(Ind)	0.099	0.107	0.103	0.085	0.099	0.089	0.097	0.050
985(Ind)	0.175	0.112	0.123	0.208	0.099	0.108	0.176	0.085
991(Ind)	0.175	0.112	0.119	0.137	0.136	0.112	0.165	0.103
1053(+)	Over	1.916	0.725	0.146	0.156	1.996	Over	0.519
C1	0.146	0.131	0.167	0.081	0.082	0.081	0.190	0.098
C2	0.109	0.125	0.109	0.124	0.103	0.121	0.191	0.107
C3	0.097	0.102	0.126	0.067	0.068	0.084	0.103	0.087
C4	0.154	0.147	0.135	0.124	0.188	0.154	0.161	0.165
C5	0.130	0.136	0.155	0.082	0.073	0.136	0.126	0.108
C6	0.095	0.084	0.073	0.058	0.075	0.069	0.078	0.056
C7	0.085	0.076	0.095	0.088	0.091	0.072	0.086	0.091
C8	0.056	0.065	0.078	0.095	0.101	0.087	0.069	0.102
C9	0.087	0.102	0.098	0.087	0.100	0.084	0.095	0.099
C10	0.125	0.112	0.135	0.123	0.115	0.124	0.111	0.135

Table 3.4.1b. Absorbance readings of selected samples on new combinations of full pool peptides, designated A to G. The readings of the full pool of peptides all at 1/10,000 are shaded, for comparison. Samples are characterised based on RIBA banding as shown in Table 3.3.1a. These results are represented in Figure 3.4.1a.

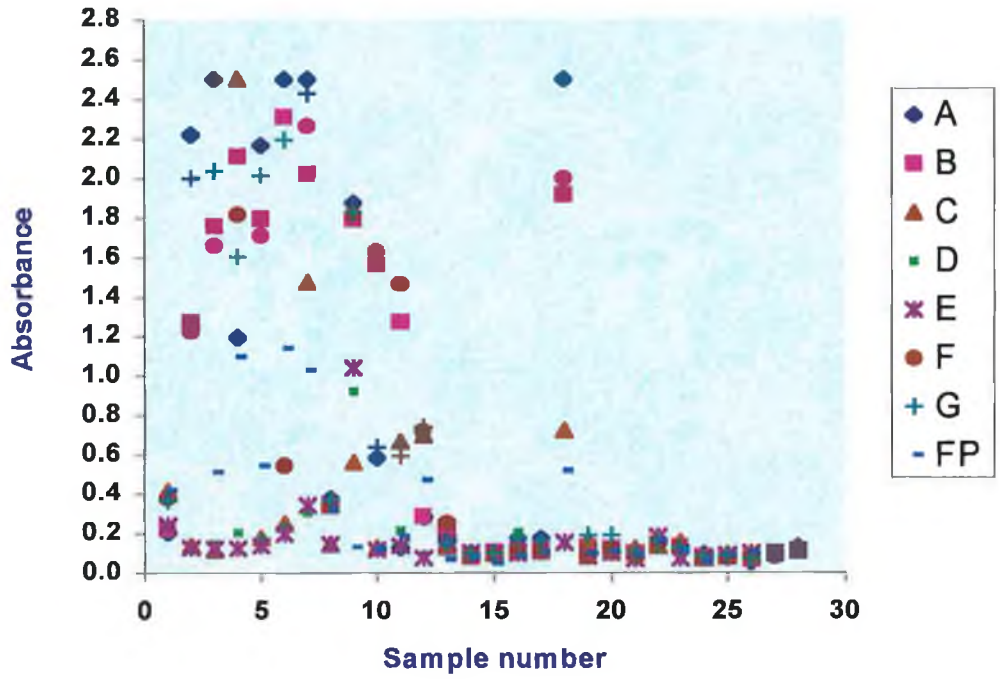


Figure 3.4.1a. Absorbance readings of selected samples on re-titre of full pool peptide combinations, A to G, and also full pool peptides, coated at 1/10,000.

Table 3.4.1c compares the absorbance of samples, which gave false positive results on full peptide pool coated at 1/10,000 (Table 3.3.2b), with the value obtained with the new combination of full pool peptides, designated A to G.

Sample Lot	A	B	C	D	E	F	G	FP
722	0.107	0.124	0.154	0.145	0.121	0.103	0.114	0.109
935	0.124	0.157	0.236	0.154	0.099	0.137	0.189	0.254
947	0.112	0.138	0.248	0.141	0.151	0.125	0.252	0.322
993	0.145	0.178	0.166	0.157	0.136	0.153	0.179	0.168
997	0.083	0.101	0.135	0.099	0.087	0.098	0.112	0.154
998	0.147	0.158	0.175	0.151	0.135	0.154	0.165	0.177
1039	0.092	0.121	0.201	0.132	0.127	0.174	0.121	0.154
1072	0.071	0.124	0.187	0.112	0.212	0.142	0.128	0.181
1081	0.189	0.211	0.274	0.201	0.164	0.196	0.107	0.250
1110	0.084	0.179	0.212	0.154	0.167	0.145	0.128	0.187
1265	0.143	0.169	0.176	0.122	0.163	0.149	0.198	0.263

Table 3.4.1c. Comparison of absorbance values of samples, which gave false positive readings on full pool peptide coated at 1/10,000, with combinations designated A to G.

Table 3.4.1d compares the absorbance values of the false negative samples, on full pool peptides coated at 1/10,000 from Table 3.3.2a, with the readings achieved on combinations A to G.

Sample Lot	A	B	C	D	E	F	G	FP
725	0.234	0.204	0.422	0.315	0.550	0.429	0.495	0.132
731	2.099	1.648	0.687	0.144	0.139	1.670	1.943	0.231
742	2.419	1.974	Over	0.152	0.126	Over	Over	0.265
958	Over	2.280	1.338	0.199	0.172	2.284	Over	0.326
996	0.525	0.369	0.208	0.149	0.126	0.467	0.366	0.135
1115	0.625	0.456	0.136	0.123	0.156	0.599	0.561	0.108

Table 3.4.1d. Comparison of absorbance values of samples, which gave false negative readings on full pool of peptides coated at 1/10,000, now tested with combinations designated A to G.

3.4.2 Testing of Chosen Combination

As can be seen from Table 3.4.1c, there was an improvement in sensitivity with an increase in the absorbance readings of the false negative samples previously not detected with the full peptide pool, HCV 15, HCV Core, HCV 52 and HCV NS3-1, coated at 1/10,000. Specificity was also improved, as the absorbance values of the false positive samples were lower as shown in Table 3.4.1d.

Based on this analysis, Table 3.4.2a shows a chosen solid phase combination.

HCV 15	*HCV Core*	*HCV 52*	*HCV NS3-1*
1/800	1/2,500	1/600	1/500

Table 3.4.2a. New peptide coating dilutions chosen after testing of samples giving false positive and negative results on full pool peptides coated at 1/10,000.

On the above peptide combination, all available HCV positive and negative material was assayed. This consisted of 45 negative sample from cork blood bank and the 88 samples from IEI. Along with these samples a HCV seroconversion panel PHV 901 supplied by Boston Biomedica, Inc. was also assayed. The specific region represented

by the members of this panel is shown in Table 3.4.2b. The data was obtained using the Ortho™ Anti-HCV RIBA 2.0 Assay.

Panel ID	Specific Region	Expected Result	Absorbance
PHV901-01	-	Negative	0.187
PHV901-02	-	Negative	0.176
PHV901-03	NS3	Indeterminate	0.354
PHV901-04	NS3	Indeterminate	0.234
PHV901-05	NS3	Indeterminate	0.203
PHV901-06	NS3	Indeterminate	0.232
PHV901-07	NS3, NS4	Positive	1.051
PHV901-08	NS3, NS4	Positive	0.829
PHV901-09	NS3, NS4	Positive	1.100
PHV901-10	NS3, NS4	Positive	1.029
PHV901-11	NS3, NS4	Positive	1.562

Table 3.4.2b. Viral region and absorbance readings of HCV seroconversion panel; panel tested on the new full pool peptide combination shown in Table 3.4.2a.

Figure 3.4.2a compares the absorbance reading of the 88 IEI samples tested with new combination in Table 3.4.2a with that obtained with the full pool of peptides, all coated at 1/10,000. See Appendix 2 for raw data.

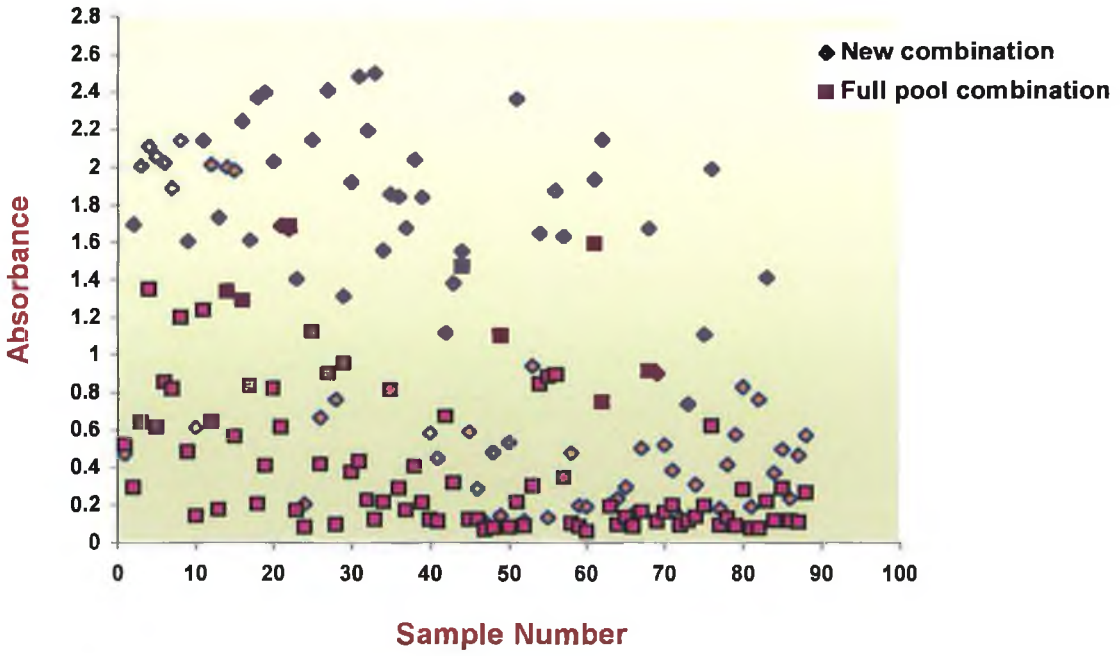


Figure 3.4.2a. Comparison of IEI samples tested on new combination of full pool peptides as shown in Table 3.4.2a, and also on full pool peptides, HCV 15, HCV Core, HCV 52 and HCV NS3-1 all coated at 1/10,000.

The absorbance readings obtained for the negative samples from Cork blood bank are shown in Table 3.4.2d.

Sample	New Combination	Full Pool	Sample	New Combination	Full Pool
C1	0.151	0.141	C24	0.153	0.111
C2	0.174	0.126	C25	0.184	0.142
C3	0.153	0.070	C26	0.143	0.077
C4	0.256	0.217	C27	0.165	0.066
C5	0.190	0.082	C28	0.163	0.072
C6	0.103	0.068	C29	0.116	0067
C7	0.147	0.110	C30	0.172	0.059
C8	0.148	0.069	C31	0.187	0.075
C9	0.133	0.068	C32	0.132	0.079
C10	0.127	0.065	C33	0.198	0.095
C11	0.099	0.082	C34	0.212	0.104
C12	0.139	0.098	C35	0.102	0.067
C13	0.160	0.085	C36	0.155	0.066
C14	0.182	0.071	C37	0.111	0.072
C15	0.150	0.081	C38	0.141	0.069
C16	0.232	0.143	C39	0.165	0.107
C17	0.167	0.135	C40	0.125	0.076
C18	0.136	0.074	C41	0.194	0.085
C19	0.149	0.110	C42	0.191	0.097
C20	0.127	0.085	C43	0.185	0.070
C21	0.113	0.065	C44	0.197	0.098
C22	0.122	0.105	C45	0.181	0.073
C23	0.143	0.058			

Table 3.4.2d. Absorbance readings of negative samples tested on new full pool combination and the full pool with all peptides, HCV 15, HCV Core, HCV 52 and HCV NS3-1, coated at 1/10,000.

The increase in solid phase coating dilutions resulted in an increase in the absorbance values of the positive readings as is seen from Table 3.4.2b. However Table 3.4.2d shows that, although sensitivity had improved, there was also an increase in the absorbance values of some of the negative readings.

3.4.3 Sensitivity and Specificity on Improved Solid Phase

To calculate the sensitivity and specificity of the new solid phase, a cut-off value was determined. The cut-off value was calculated as the mean of the negative readings ± 2 standard deviation units (x ±2 SD).

Using a cut-off value of 0.206 the sensitivity and specificity was calculated as shown in Table 3.4.3a.

The specificity figures were calculated without the inclusion of the 35 indeterminate samples.

Specificity	*Sensitivity*
45 True Neg. – 3 False Pos. 45 True Neg.	53 True Pos.- 3 False Neg. 53 True Pos.
42/45 x 100	50/53 x 100
93.3%	94.3%

Table 3.4.3a. Sensitivity and specificity based on samples tested on new combination of peptides. HCV 15 at 1/800, HCV core at 1/2,500, HCV 52 at 1/600 and HCV NS3-1 at 1/500.

3.5 Evaluation of Direct Coating of Peptide

In an attempt to reduce the negative values and also to improve sensitivity, it was decided to coat the HCV peptides directly on to the microwells omitting the conjugation of peptide to BSA.

This procedure involved dissolving a known quantity of the peptide raw material in a coating buffer and then coating as per Section 2.3.1.

3.5.1 Comparison of CCB and PBS as Direct Coating Buffers

A stock of 1mg/ml of the coating peptide was made. An accurate quantity of approximately 1mg of peptide was weighed and the corresponding volume of buffer was added to give a 1mg/ml solution.

e.g. 1mg/ml solution contains
1,100µg peptide + 1.1ml buffer
700µg peptide + 700µl buffer

Two coating buffers were evaluated for this purpose, carbonate coating buffer, pH 9.6 (CCB), used previously, and 0.1M phosphate buffered saline, pH 7.4 (PBS).

To evaluate both buffers, HCV 15 peptide was chosen and dilutions made as shown in Table 3.5.1a. A sub-stock of 10µg/ml was made from 1mg/ml (1000µg/ml) stock.

	4µg/ml	2µg/ml	1µg/ml	0.5µg/ml
Volume 10µg/ml stock	4.8ml	6ml of 4µg/ml	6ml of 2µg/ml	6ml of 1µg/ml
Volume CCB/PBS	5.2ml	6ml	6ml	6ml

Table 3.5.1a. Dilutions of HCV 15 peptide coated for evaluation of direct coating of peptide.

The wells were tested with two positive samples, 741, which gave high absorbance values previously, 700 which gave a medium to low positive reading, and two negative samples, C11 and C12.

Figure 3.5.1a represents the absorbance readings of the samples assayed and compares both coating buffers.

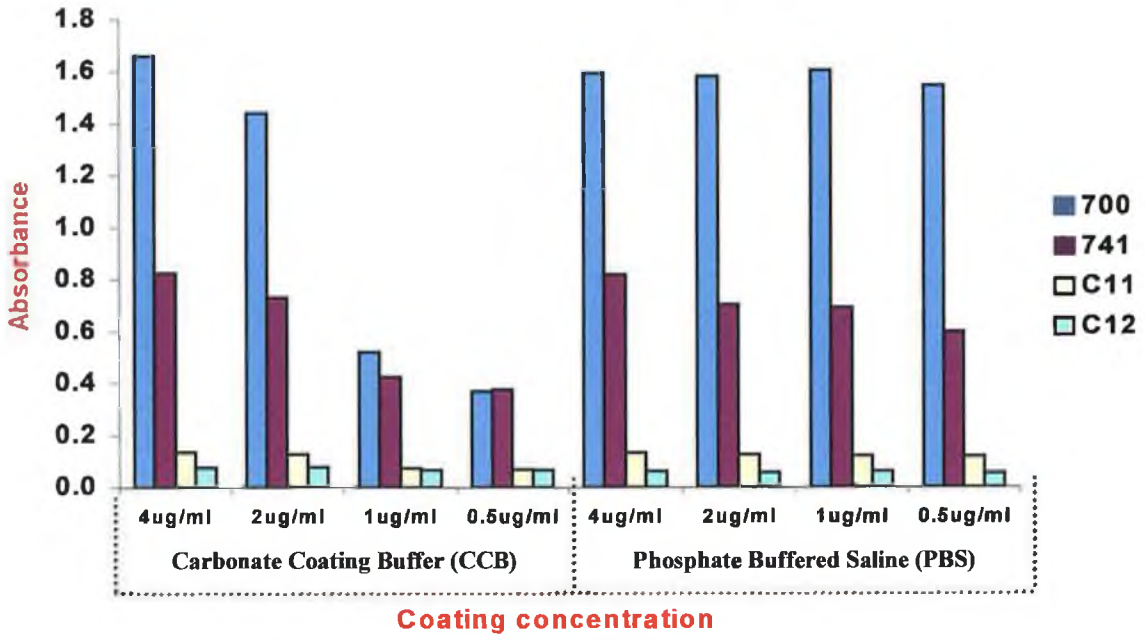


Figure 3.5.1a. Absorbance readings of samples in comparison of carbonate coating buffer and phosphate buffered saline as buffers for direct coating of peptide.

As can be seen phosphate buffered saline was the preferred buffer because at lower coating concentrations at 1µg/ml and 0.5µg/ml of HCV 15, the absorbance values did not decrease. This was not observed in the case of carbonate coating buffer.

3.5.2 Titration of Peptides Coated Directly

Based on results obtained from directly coating the HCV 15 peptide, the remaining peptides were also coated in 0.1M PBS, pH 7.4.

A set of peptide combinations at varying concentrations was made and designated 1 to 6, as shown in Table 3.5.2a.

Combination	HCV NS3-1 (µg/ml)	HCV 52 (µg/ml)	HCV Core (µg/ml)	HCV 15 (µg/ml)
1	0.25	0.5	0.5	0.5
2	0.25	0.5	0.25	0.25
3	0.25	1.0	1.0	1.0
4	0.25	1.0	0.5	0.5
5	0.25	1.0	0.5	1.0
6	0.25	0.25	0.25	0.25

Table 3.5.2a. Peptide combinations, designated 1 to 6, to evaluate direct coating of unconjugated peptide to the plate.

Each of the above combinations 1 to 6, was evaluated using 54 of the IEI positive samples and 20 Cork blood bank negative samples.

The readings obtained are represented in Figure 3.5.2a. See Appendix 3 for raw data.

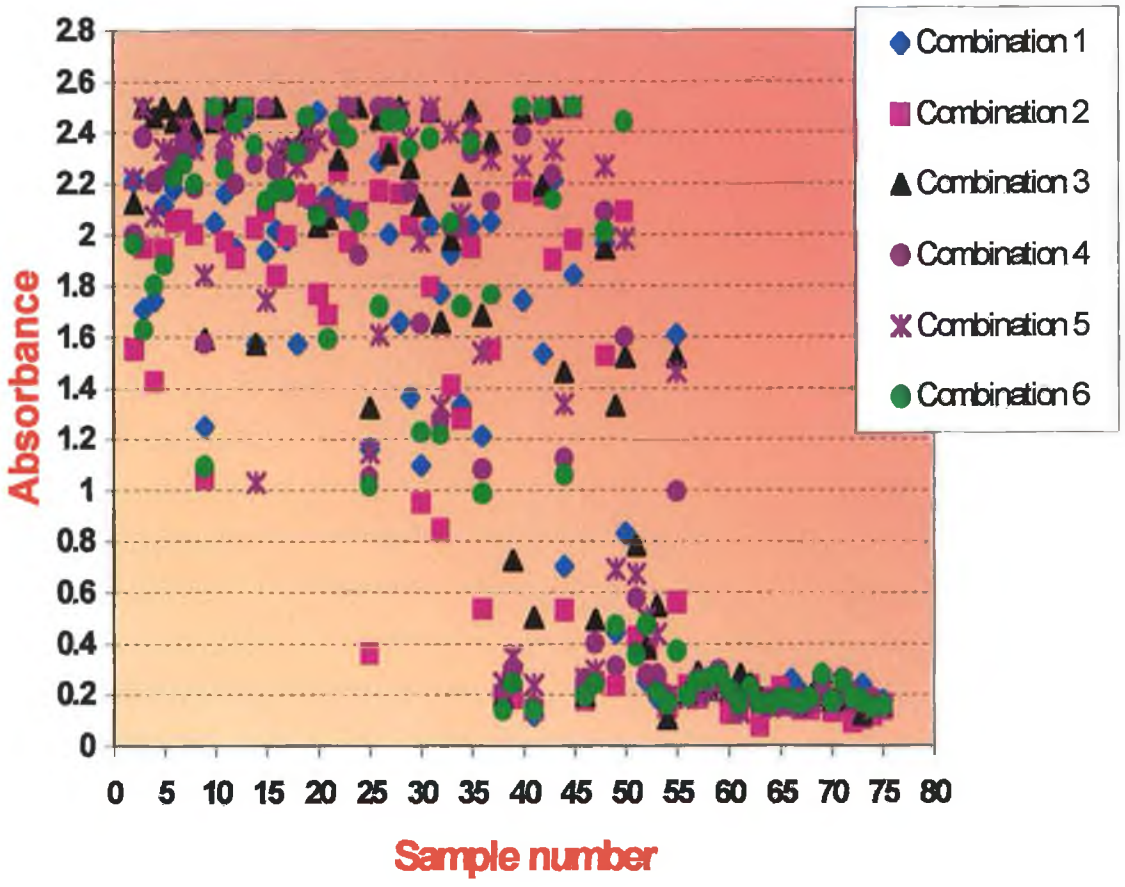


Figure 3.5.2a. Absorbance values of IEI positive and negative samples on wells coated with directly coated unconjugated peptide combinations, designated 1 to 6.

Analysis of results indicated that the best combination of peptide and coating concentration was achieved with combination 5. This combination succeeded in picking up all 45 positive samples with higher absorbance reading than the other combinations, although negative samples were slightly higher than had been previously achieved where the solid phase was coated with conjugated peptide.

Combination 5 contained	HCV 15	1.0µg/ml
	HCV 52	1.0µg/ml
	HCV Core	0.5µg/ml
	HCV NS3	0.25µg/ml

Figure 3.5.2b compares the absorbance readings of the negative samples C1 to C20, tested on combination 5, coated with unconjugated peptide in the direct coating method, and the new full pool solid phase coated with conjugated peptide.

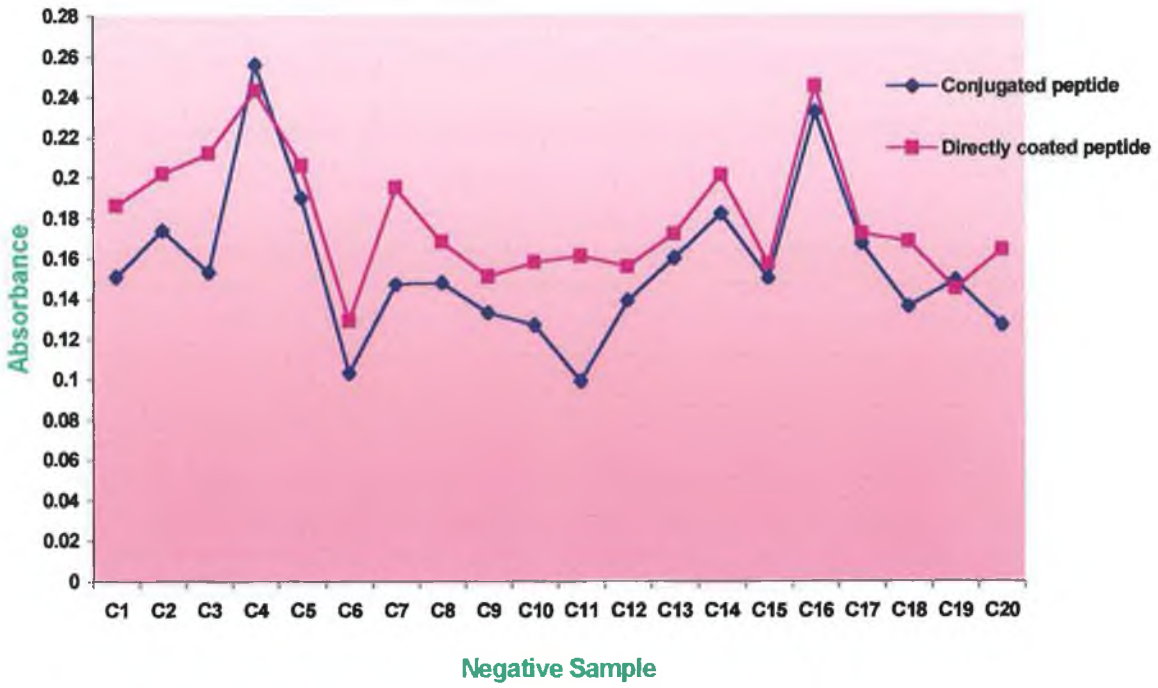


Figure 3.5.2b. Comparison of negative samples assayed on ELISA wells coated with the full pool of conjugated peptides, with directly coated unconjugated peptide.

Although there was an improvement in sensitivity with the direct coating method the readings for the negative samples are slightly higher. In order to reduce the slight background observed with this method, the enzyme conjugate was re-evaluated.

3.5.3 Approaches to Improve Specificity with Direct Coating Method

Based on previous evaluation, the KPL conjugate was at a dilution of 1/80,000 (Figure 3.2.2a) in Cytochrome C diluent with 0.05% Triton X-100 (Figure 3.2.3a).

3.5.3.1 Titre of the KPL Conjugate

The conjugate was further titred between 1/80,000 and 1/120,000. The titred conjugate was tested with negative samples C1 to C12 and also with one positive sample, 709, and one indeterminate sample, 996, on solid phase combination 5, directly coated.

Table 3.5.3a shows the absorbance values obtained with all samples. The absorbance readings of the 12 negative readings are represented in Figure 3.5.3a.

Sample	1/80,000	1/90,000	1/100,000	1/120,000
C1	0.193	0.164	0.145	0.132
C2	0.212	0.198	0.175	0.163
C3	0.213	0.185	0.162	0.144
C4	0.225	0.201	0.157	0.132
C5	0.210	0.187	0.169	0.155
C6	0.132	0.121	0.101	0.087
C7	0.199	0.163	0.142	0.129
C8	0.175	0.156	0.136	0.122
C9	0.163	0.145	0.132	0.119
C10	0.175	0.135	0.121	0.109
C11	0.189	0.165	0.143	0.125
C12	0.162	0.139	0.120	0.106
709	2.500	2.213	2.196	1.845
996	0.573	0.544	0.491	0.387

Table 3.5.3a. Absorbance readings of samples in further titre of KPL anti-human IgG-peroxidase conjugated, to improve specificity with direct coating of unconjugated peptide.

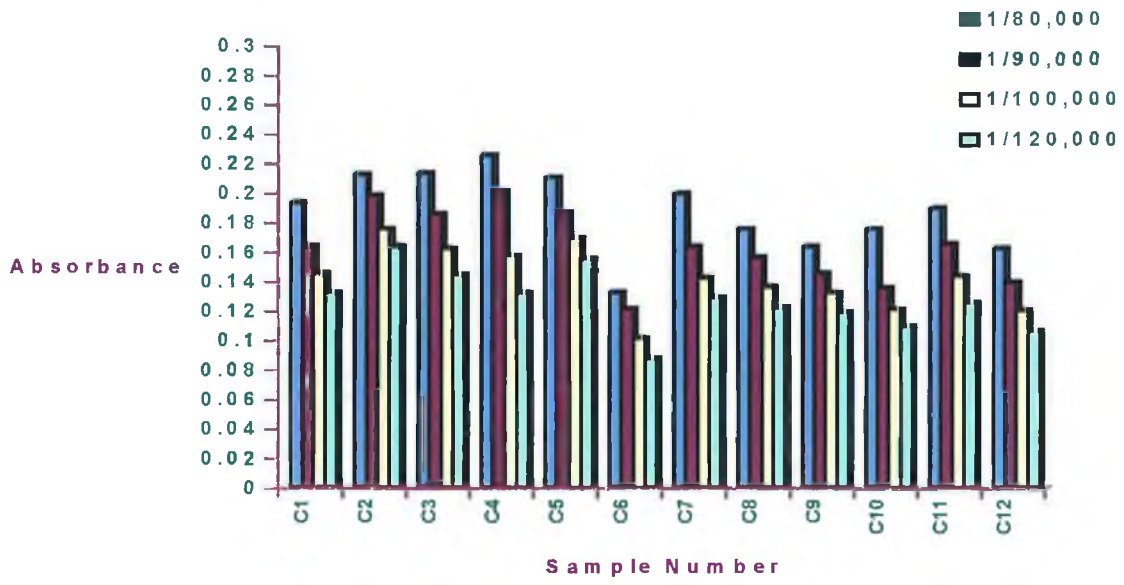


Figure 3.5.3a. Absorbance readings of negative samples in evaluation of KPL anti-human IgG-peroxidase conjugated, to improve specificity tested on directly coated peptide.

The conjugate titre chosen was at 1/100,000. As can be seen from the above data, the absorbance values of the negative readings decreased at this dilution while not compromising on sensitivity, as the readings for samples 709 and 996 show.

3.5.3.2 Addition of Serum to the Conjugate Diluent

To further improve specificity, the conjugate diluent was further evaluated. Various quantities of Foetal Bovine Serum (FBS) and Goat Serum (GS) were added to the Cytochrome C diluent. The components were added at 5% (v/v), 10% (v/v), 20% (v/v). With the conjugate diluted at 1/100,000, the same samples were assayed. Cytochrome C diluent with no additives was used as a control conjugate.

	20% (v/v) FBS	10% (v/v) FBS	5% (v/v) FBS	20% (v/v) GS	10% (v/v) GS	5% (v/v) GS	Control
C1	0.071	0.079	0.073	0.065	0.072	0.081	0.145
C2	0.098	0.111	0.102	0.053	0.065	0.078	0.175
C3	0.074	0.081	0.086	0.048	0.052	0.065	0.162
C4	0.070	0.069	0.072	0.041	0.045	0.057	0.157
C5	0.089	0.096	0.095	0.082	0.078	0.096	0.169
C6	0.065	0.061	0.069	0.046	0.048	0.052	0.101
C7	0.062	0.073	0.071	0.047	0.053	0.066	0.142
C8	0.075	0.081	0.086	0.049	0.042	0.054	0.136
C9	0.078	0.084	0.082	0.045	0.054	0.059	0.132
C10	0.075	0.072	0.074	0.041	0.047	0.045	0.121
C11	0.099	0.123	0.112	0.050	0.055	0.062	0.143
C12	0.081	0.089	0.088	0.051	0.046	0.054	0.120
709	1.872	1.925	2.139	0.596	0.621	0.832	2.196
996	0.379	0.422	0.478	0.133	0.195	0.265	0.491

Table 3.5.3b. Absorbance readings of samples tested with addition of serum to conjugate diluent to improve specificity.

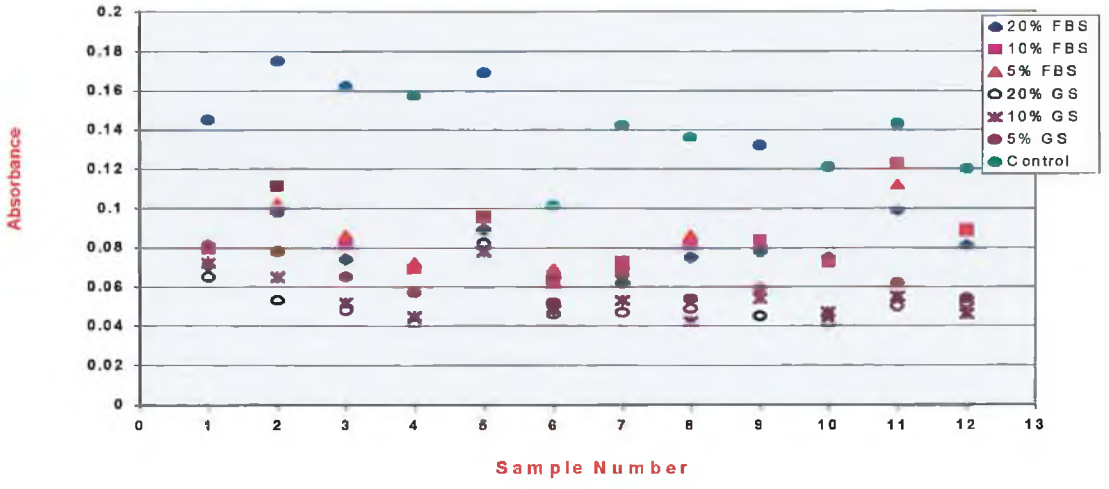


Figure 3.5.3b. Absorbance readings of negative samples tested, with addition of serum to conjugate diluent, to improve specificity on directly coated peptide.

Figure 3.5.3b shows the absorbance values of the negative samples only. The addition of goat serum gave the lowest negative readings but sensitivity was reduced significantly as can be seen in Table 3.5.3b.

The diluent, which gave the best specificity and sensitivity based on both negative and positive readings, was cytochrome C diluent with the addition of 5% (v/v) FBS.

3.5.4 Improved Assay Parameters

As a result of the investigation of directly coating the peptide as described in Section 3.5, the following assay parameters were used in the final evaluation of samples. The assay protocol is outlined below.

Plate: Combination 5 Coated Directly

HCV 15	1.0µg/ml
HCV 52	1.0µg/ml
HCV Core	0.5µg/ml
HCV NS3	0.25µg/ml

Sample: 1 in 10 in-well dilution in control dilution buffer with 5% (v/v) goat serum (10µl sample + 90µl diluent)

Conjugate: 1/100,000 KPL conjugate in cytochrome C diluent with 0.05% (v/v) Triton X-100 and 5% (v/v) Foetal Bovine Serum

Assay Protocol:

100µl sample
30 min. at 37°C
Wash x 5 times
100µl conjugate
30 min. at 37°C
Wash x 5 times
100µl substrate
15 min. at 25°C
Read at 450nm with a reference filter of 630nm.

3.6 Final Evaluation of HCV ELISA

For the final evaluation of the ELISA assay, a total of 93 negative samples and 88 IEI samples were evaluated along with the 11 members of the seroconversion panel.
These 192 samples were tested with the parameters outlined in Section 3.5.4 and compared with the previous parameters as outlined in Section 3.2.5.
Table 3.6a shows the peptide quantity contained in both methods.

Peptide	HCV 15	HCV Core	HCV 52	HCV NS3-1
Direct Coat	1.0µg/ml	0.5µg/ml	1.0µg/ml	0.25µg/ml
Conjugated	1/800	1/2,500	1/600	1/500

Table 3.6a. Peptides combinations contained in directly coated and conjugated coating methods.

3.6.1 Comparison of Absorbance Readings with both methods

3.6.1.1 Assay of IEI Samples

Figure 3.6.1a compares the absorbance readings obtained for the IEI samples using both solid phase coating methods. See Appendix 4 for raw data.

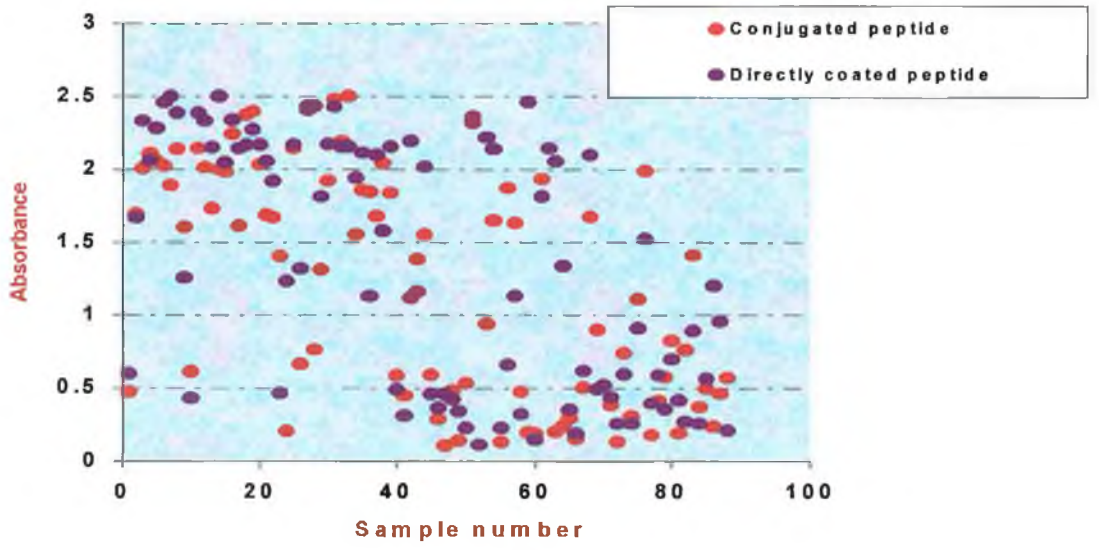


Figure 3.6.1a. Absorbance of IEI samples on conjugated and directly coated peptide for final evaluation of HCV ELISA.

3.6.1.2 Assay of Negative Samples

Figure 3.6.1b compares the absorbance readings obtained for the negative samples using both methods. See Appendix 5 for raw data.

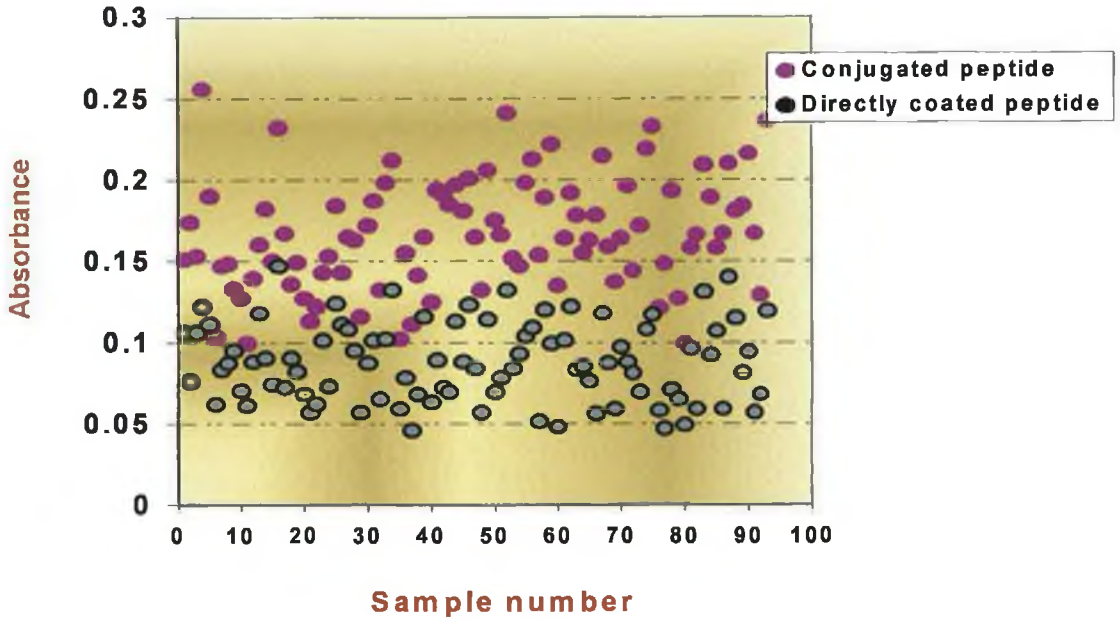


Figure 3.6.1b. Absorbance of negative samples assayed on directly coated and conjugated peptide for final evaluation of HCV ELISA.

3.6.1.3 Assay of Seroconversion Panel

Panel ID	Specific Region	Expected Result	Conjugated	Directly
PHV901-01	-	Negative	0.187	0.098
PHV901-02	-	Negative	0.176	0.084
PHV901-03	NS3	Indeterminate	0.354	0.365
PHV901-04	NS3	Indeterminate	0.234	0.451
PHV901-05	NS3	Indeterminate	0.203	0.318
PHV901-06	NS3	Indeterminate	0.232	0.209
PHV901-07	NS3, NS4	Positive	1.051	1.265
PHV901-08	NS3, NS4	Positive	0.829	1.023
PHV901-09	NS3, NS4	Positive	1.100	0.897
PHV901-10	NS3, NS4	Positive	1.029	1.255
PHV901-11	NS3, NS4	Positive	1.562	2.112

Table 3.6.1a. Absorbance readings of HCV seroconversion panel on solid phase peptide, both coated directly and conjugated to carrier protein.

3.6.2 Sensitivity and Specificity of 'In-house' Evaluation of ELISA

A cut-off value was determined using the method applied previously. This value was used to calculate the sensitivity and specificity achieved with both coating methods.

The Ortho™ RIBA banding indicated that 53 IEI samples were positive and these results were used to calculate the sensitivity results. As previously done, the specificity results were calculated with and without the inclusion of the indeterminate samples.
The calculations for both coating methods are shown in Table 3.6.2a and 3.6.2b.

Specificity	*Sensitivity*
93 True Neg. – 6 False Pos. 93 True Neg.	53 True Pos.- 3 False Neg. 53 True Pos.
87/93 x 100	50/53 x 100
93.5%	94.3%

Table 3.6.2a. Sensitivity and specificity with solid phase coated with conjugated peptide for 'in-house' evaluation.

Specificity	*Sensitivity*
93 True Neg. – 1 False Pos. 93 True Neg.	53 True Pos.- 0 False Neg. 53 True Pos.
92/93 x 100	53/53 x 100
98.9%	100%

Table 3.6.2b. Sensitivity and specificity with solid phase coated with directly coated peptide for 'in-house' evaluation.

A comparison of the sensitivity and specificity results as shown above, is represented in Figure 3.6.2a.

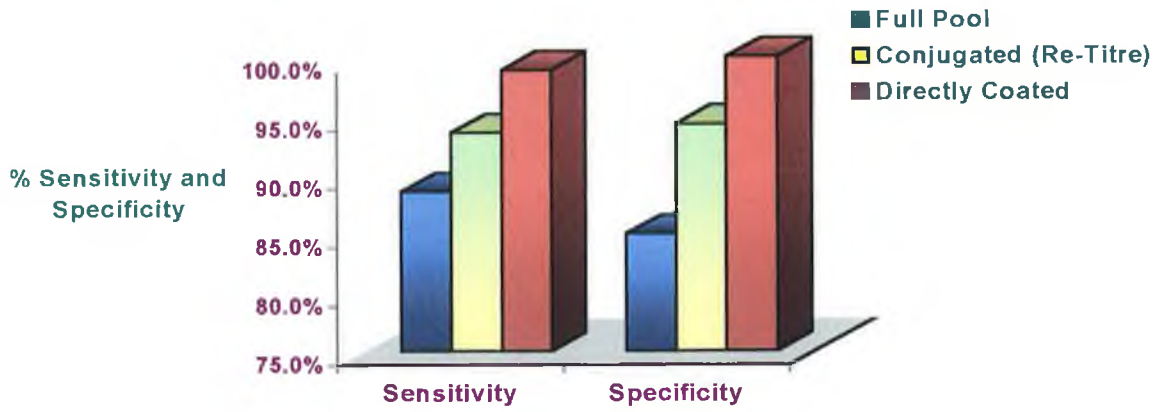


Figure 3.6.2a. Sensitivity and specificity achieved during various stages of development of HCV ELISA.

The peptide combinations and coating concentrations used in each method is shown in Table 3.6.2c.

Solid Phase	HCV 15	HCV Core	HCV 52	HCV NS3-1
Full pool conjugated peptide	All Coated at 1/10,000			
Improved conjugated peptide	1/800	1/2,500	1/600	1/500
Directly coated peptide	1.0µg/ml	0.5µg/ml	1.0µg/ml	0.25µg/ml

Table 3.6.2c. Peptide combinations and dilutions of peptide evaluated during development of HCV ELISA assay.

Based on the data shown in Figure 3.6.2a, all clinical evaluations were carried out on plates coated with peptide directly coated on to the well.

3.6.3 Performance Evaluation at an External Site

The performance of the HCV ELISA test was evaluated at an external trial site. The off-site location chosen was Public Health Laboratory Service (PHLS), Birmingham. Based on the internal evaluation results as outlined in the previous section, the evaluation at the PHLS was carried out with plates on which the peptide was directly coated. The assay parameters were as shown in Section 3.5.4.

3.6.3.1 HCV ELISA Evaluation Results

A total of 530 serum or plasma specimens were tested at the Public Health Laboratory, Heartlands Hospital, Birmingham. All specimens had been previously tested as part of routine testing at the Serology Laboratory, PHLS, and had been confirmed by RIBA testing. Analysis of these samples showed that 281 samples were found to be anti-HCV negative and 249 were anti-HCV positive by RIBA banding. All specimens had been stored at -20°C prior to thawing for testing by the ELISA test. The cut-off value was determined as before by calculating the mean negative control absorbance reading + 2 standard deviation units.

Of the 249 confirmed positive samples assayed, a total of 233 samples gave a positive reading on the HCV ELISA test. A total of 275 samples of the 281 known negatives gave a negative reading on the ELISA. These results are summarised in Table 3.6.3a.

Specificity	Sensitivity
281 True Neg. – 6 False Pos.	249 True Pos.- 16 False Neg.
281 True Neg.	249 True Pos.
275/281 x 100	233/249 x 100
97.9%	93.6%

Table 3.6.3a. ELISA performance evaluation results from PHLS, Birmingham.

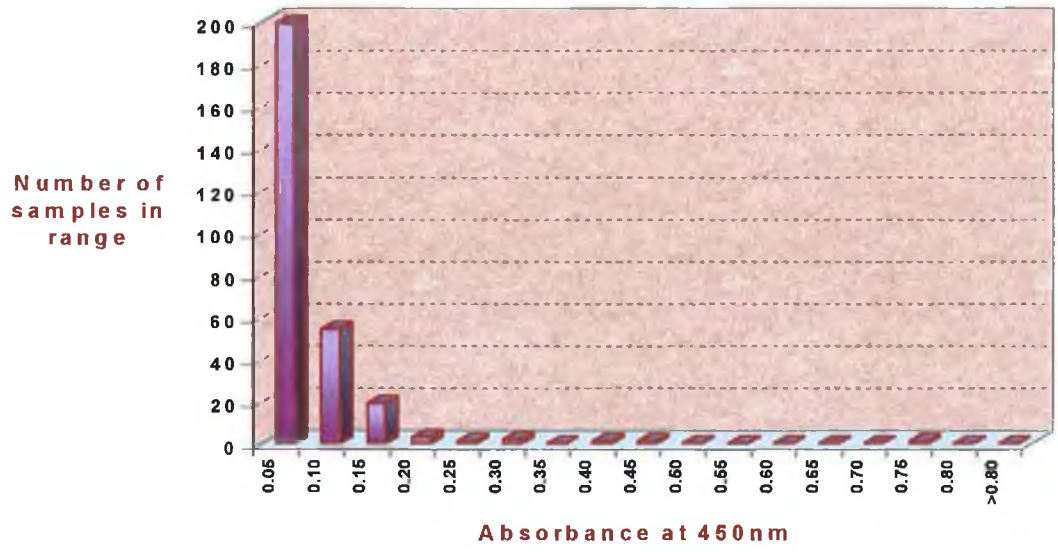


Figure 3.6.3a. Distribution of negative sample absorbance readings at PHLS HCV ELISA evaluation.

The absorbance readings obtained at the performance evaluation are represented in the histograms in Figures 3.6.3a and 3.6.3b. The distribution of the readings for the negative samples tested is shown in Figure 3.6.3a and that of the positive readings in Figure 3.6.3b. The absorbance readings are divided into different histogram ranges and the amount of samples, which gave a reading within a particular range, are shown. As can be seen from Figure 3.6.3a, 199 of the 281 negative samples tested gave a reading within a range of 0 to 0.05 absorbance units.

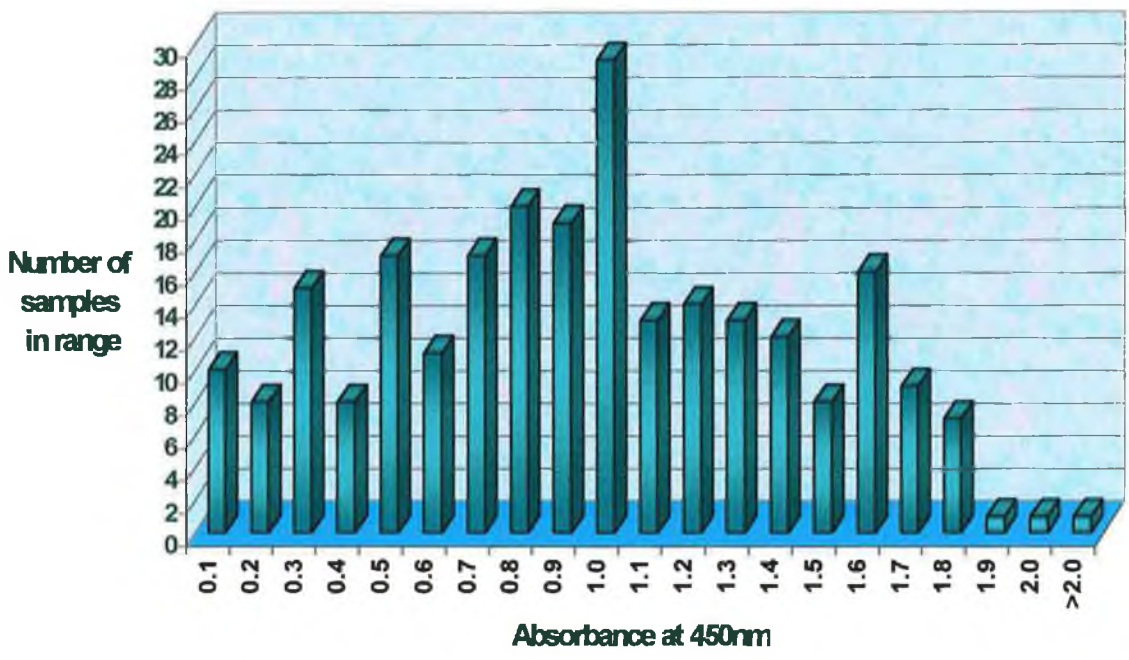


Figure 3.6.3b. Distribution of positive sample absorbance readings at PHLS HCV ELISA evaluation.

4.1 Evaluation of Peptides on the Test Card Membrane

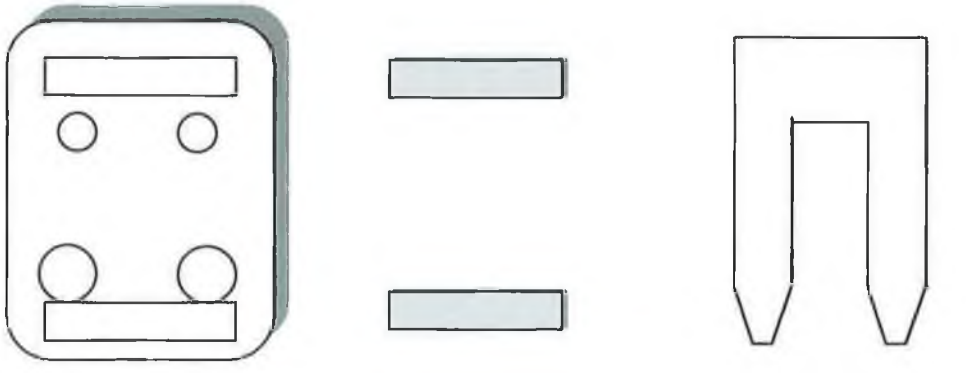
The development of the rapid assay followed a similar format to that used in the development of the ELISA test. Both assays are examples of an indirect ELISA as outlined in section 1.7.4.2. Due to the success achieved in the ELISA format with the immobilised HCV peptides, the same peptides were analysed for use on the test card format. Many tedious steps are omitted in the development of the rapid test with the availability of reagents and buffers for evaluation. The first step involved the assembly of the test card device.

4.1.1 Assembly of Test Cards

The assembly of each rapid card device consisted of four raw material components.

- A moulded 2-port test card,
- An absorbent pad,
- A glass cut membrane and
- A white backing label.

The test card components are shown in Figure 4.1.1a and the assembled test card (front view and back view) shown in Figure 4.1.1b. The diagrams are shown without the addition of the backing label.



Test Card Mould **Absorbent Pads** **Glass Cut Membrane**

Figure 4.1.1a Components used to assemble SeroCard test device.

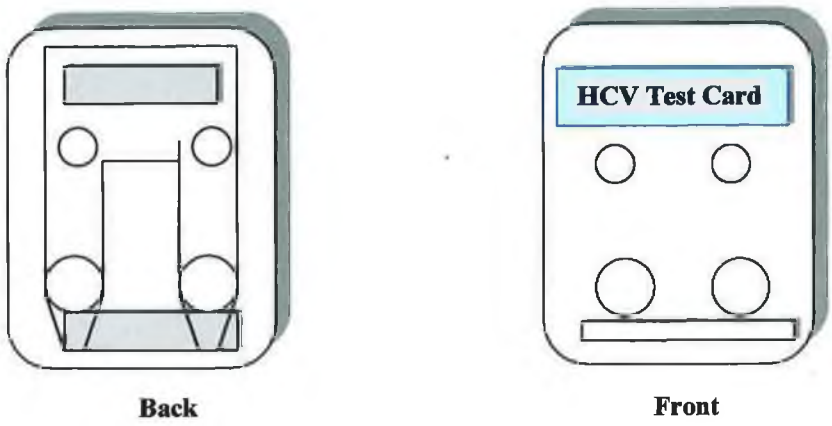


Figure 4.1.1b Front and back view of assembled SeroCard device without backing label.

The cards were assembled manually. As is seen from the diagram, the absorbent pads were placed in grooves at either end of the test card. The membrane was then placed in position over these pads stretching the length of the card. Finally, the backing label was applied to ensure the components remained in position.

4.1.2 Adhesion of Peptides to Card Membrane

In the same manner as peptides were coated to microtitre plate wells, the peptides were evaluated for adhesion to the card membrane. To do this, a quantity of peptide was spotted or dispensed on to the top ports of the test card membrane. The spotting sites are shown in Figure 4.1.2a.

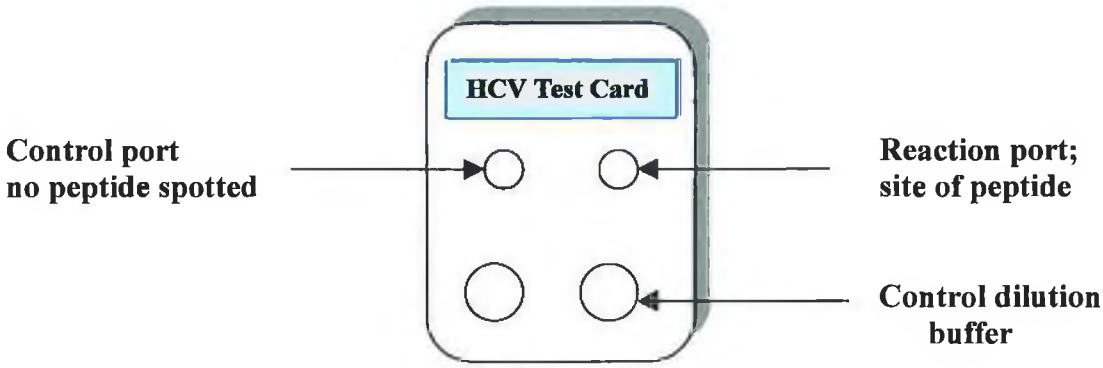


Figure 4.1.2a Site of spotted peptide and control spots on spotted SeroCard test device

Due to the high specificity and sensitivity obtained with the peptide combination used in the microtitre plate assay, the same combination of peptide was evaluated for use on the test card membrane.

In the case of the microtitre plate assay, the peptides were coated both as conjugated and as unconjugated peptide. Due to the porous nature of the test card membrane the peptides were conjugated to the carrier protein, BSA, prior to spotting. This conjugation procedure is shown in section 2.1.1 and the peptides spotted as in section 2.1.7.

4.1.2.1 Determination of Spotting Dilution

The coating volume used in the microtitre plate test was 100µl per well. The proposed spotting volume for the rapid method was supplied by Trinity Biotech plc., and was 5µl of spotting solution per port.

This card spotting volume was twenty times smaller than the corresponding plate coating volume of 100µl. Due to this volume decrease, the concentrations of the peptides were increased accordingly, also by a factor of twenty.

The peptides and the coating concentrations used in the plate assay (section 3.4.2) are shown in Table 4.1.2a. Based on these coating dilutions at 100µl, the corresponding spotting dilutions at 5µl are also shown.

Peptide	Volume Applied		Coating Concentration	
	Plate	Test Card	Plate	Test Card
HCV 15	100µl	5µl	1/800	1/40
HCV Core	100µl	5µl	1/2,500	1/125
HCV 52	100µl	5µl	1/600	1/30
HCV NS3-1	100µl	5µl	1/500	1/25

Table 4.1.2a Coating dilutions of peptides used in ELISA test and the corresponding spotting dilution on test card membrane.

Using the dilutions outlined in Table 4.1.2a, spotting solutions were formulated for each of the four peptides. In addition, the four peptides were combined in one solution

giving the above final spotting dilutions. All dilutions were made in 2x phosphate buffered saline (2x PBS).

4.1.2.2 Spotting of Cards

The procedure used to spot, block and dry the cards followed a method supplied by Trinity Biotech plc. To spot, 5µl of the four individual peptide spotting solutions, along with the full combination, were applied to the top right port as shown in Figure 4.1.2a. As a control, 5µl of the diluent, 2x PBS was spotted on to the top left port. On the bottom right port under the peptide spot 10µl of control dilution buffer was spotted.

The cards were incubated, blocked and dried as per the spotting procedure described in section 2.5.5.

4.1.3 Test Format and Interpretation of Results

The wash reagent, enzyme conjugate reagent, substrate reagent and stopping reagent, along with the initial test format, were all supplied by Trinity Biotech plc. They were supplied in bottles containing a dropper tip to dispense the reagent. One drop of reagent corresponded to a volume of approximately 50µl. Samples were applied using a dropper pipette, which dispensed approximately 40µl of liquid.

4.1.3.1 Test Format for HCV Rapid Assay

Step	Reagent	Volume Applied	Location	Incubation time
1	Wash reagent	1 drop	Bottom ports	Until absorption
2	Test specimen	1 drop	Bottom ports	30 seconds
3	Wash reagent	1 drop	Bottom ports	30 seconds
4	Wash reagent	1 drop	Top ports	Until absorption
5	Enzyme reagent	1 drop	Top ports	1 min.
6	Wash reagent	4 x 1 drop	Top ports	Until absorption
7	Substrate reagent	2 drops	Top ports	5 min.
8	Stopping reagent	1 drop	Top ports	Until absorption

Table 4.1.3a Test protocol for assay of HCV SeroCard.

4.1.3.2 Interpretation of Results

Results were interpreted based on the formation of colour on the reactive top right port of the test card after the substrate incubation time had elapsed. In the case of a positive reading, a blue colour formed at the reaction site following the addition of substrate. The intensity of colour was directly proportional to the amount of HCV antibody present in the sample. To indicate a negative reading, no colour appeared at the reactive port. In the case of a colour of equal strong intensity on both top ports, the result was termed invalid. Figure 4.1.3a highlights these interpertations.

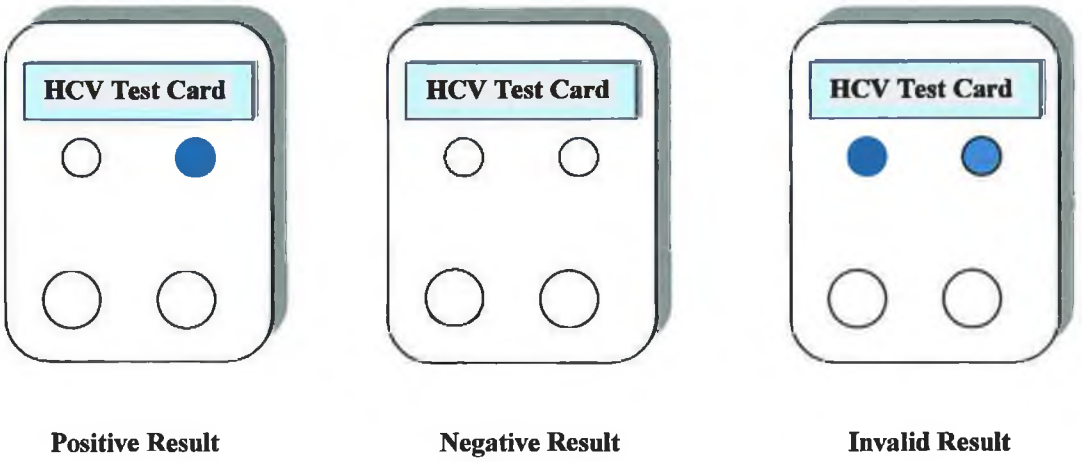


Figure 4.1.3a Colour interpretation of SeroCard assay results showing a positive, negative and invalid test result.

To enable the recording of results, during the development of the assay, the colour intensity was given a numerical value 0 to 10. A result was designated two numbers, for example, (0,7), which reads the colour intensity of both the left and the right ports. The stronger the colour intensity the higher the number. Figure 4.1.3b shows the intensity and the corresponding numerical value used.

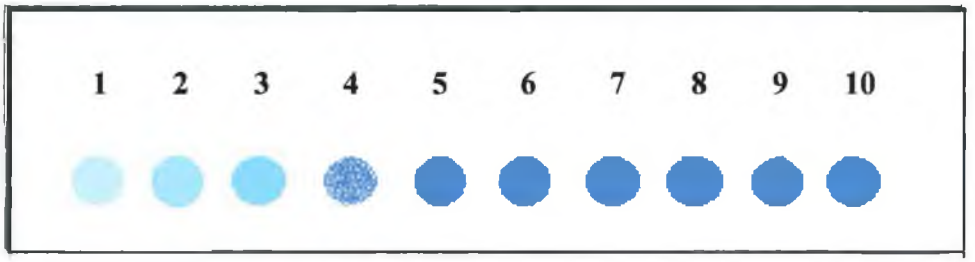


Figure 4.1.3b Representation of colour intensity with the corresponding numerical value for interpretating SeroCard test result.

4.1.4 Testing with Characterised Sera

Using the cards spotted with the dilutions shown in Table 4.1.2a and with the format outlined in Table 4.1.3a, a total of nine samples were tested. The samples were those used in the initial evaluation of the solid phase in section 3.1.3 of the ELISA test development and were characterised using the Ortho™ RIBA 3.0 Assay. The characterisation is shown in Table 4.1.4a

Sample ID	Viral Region	Specific Peptide
1	NS3, Core	NS3-1, HCV Core, HCV 15
2	NS4, Core	HCV 52, HCV Core, HCV 15
3	Core	HCV Core, HCV 15
4	NS3, NS4	HCV NS3-1, HCV 52
5	Core	HCV Core, HCV 15
6	NS4, Core	HCV 52, HCV Core, HCV 15
7	Neg.	None
8	Neg.	None
9	Neg.	None

Table 4.1.4a Characterisation of nine samples by RIBA banding for evaluation of SeroCard test device.

The results achieved for the above samples on the individually coated cards along with the combination cards are shown in Table 4.1.4b.

Sample ID	HCV 15 at 1/40	HCV Core at 1/125	HCV 52 at 1/30	HCV NS3-1 at 1/25	Combination of full pool
1	0,2	0,3	0,0	0,1	0,4
2	0,3	0,4	0,2	0,0	0,5
3	0,2	0,2	1,1	0,0	0,2
4	0,0	0,0	0,1	0,1	0,2
5	0,3	0,2	0,0	0,0	0,3
6	0,4	0,3	0,3	0,0	0,5
7	0,0	0,0	0,1	0,0	1,1
8	0,0	1,1	0,0	0,0	0,0
9	0,0	0,0	0,0	0,0	0,0

Table 4.1.4b Results of initial testing of nine characterised samples on combinations of peptides spotted on SeroCard membrane as shown in Table 4.1.2a.

Table 4.1.4b is represented graphically in Figure 4.1.4a. Each result was assigned a value, called a numerical difference, derived from subtracting the lower control port

value from the reactive port value. If the result '0,5' was achieved then the numerical difference was '5' and in the case of '1,1' the value was '0'. The highest specificity was achieved for negative sample giving a value of '0', while the opposite was true in the case of positive samples, where a higher numerical difference was preferable.

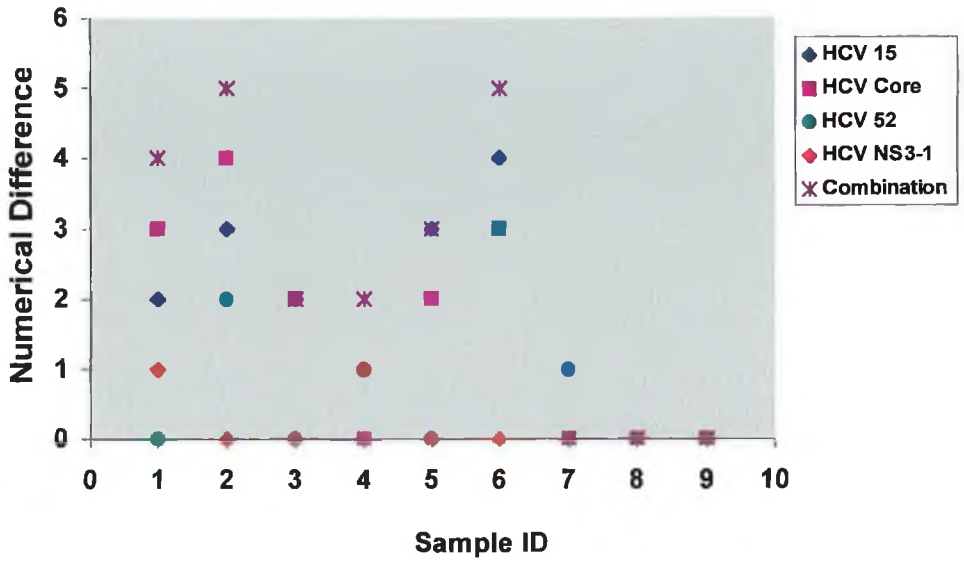


Figure 4.1.4a Numerical difference values achieved for each peptide combination in initial testing of characterised samples.

As can be seen from Figure 4.1.4a, the cards spotted with a combination of all four peptides gave the highest numerical difference for the six positive samples while a numerical value of '0' was obtained with the negative samples. Closer examination of the graph shows that 100% specificity was achieved with all peptides coated, with the exception of HCV 52 which showed one false positive (sample 7 gave a reading of 0,1).

4.1.4.1 Initial Sensitivity and Specificity

Based on the results achieved in the previous section, the following specificity and sensitivity was obtained as shown in Table 4.1.4c.

	HCV 15	HCV Core	HCV 52	HCV NS3-1	Combination of full pool
Sensitivity	5/6 83.3%	5/6 83.3%	3/6 50%	2/6 33.3%	6/6 100%
Specificity	3/3 100%	3/3 100%	2/3 66.6%	3/3 100%	3/3 100%

Table 4.1.4c Sensitivity and specificity results achieved for initial SeroCard testing.

4.2 Attempts to Improve Rapid Assay Sensitivity and Specificity

Following the sensitivity and specificity results achieved in table 4.1.4c, the cards spotted with a combination of all four peptides were chosen for further evaluation work. Although the sensitivity and specificity obtained with the combination of peptides was excellent, some improvements were needed to improve the numerical difference achieved with the positive samples tested. The intensity of the colour also needed improvement.

The parameters to be examined to improve these areas are

- Spotting dilution
- Incubation times

4.2.1 Investigation of Optimum Spotting Dilution

In an attempt to increase the colour intensity of the positive samples, variations in the spotting dilutions of the peptides on the cards were investigated. As Table 4.1.4c indicates, the peptide HCV 52 gave the poorest specificity result at 66.6%. Therefore, the spotting dilution of this peptide was not increased. A number of spotting solutions was formulated with peptide dilutions as shown in Table 4.2.1a. Combination 'A' was the combination used in Section 4.1.

Spotting Combination	HCV 15	HCV Core	HCV 52	HCV NS3-1
A	1/40	1/125	1/30	1/25
B	1/35	1/110	1/30	1/20
C	1/30	1/100	1/40	1/20

Table 4.2.1a. SeroCard spotting combinations used to optimise spotting dilution in an attempt to improve test performance.

The peptides were spotted, blocked and dried using the same procedure as previously used. A total of 50 cards were spotted with each combination. They were pouched along with a desiccant and stored at 2-8°C for use.

4.2.1.1 Testing of Combinations A to C.

A total of 25 samples were tested on the above combination of cards. These comprised of 13 positive samples and 12 negatives. They were characterised by Recombinant ImmunoBlot Assay (RIBA) and were some of the samples used in Section 3.3.1 of the ELISA assay development.

RIBA Band	Viral Region	Specific Peptide
C100	NS4	HCV 52
C33	NS3	HCV NS3-1
C22	Core	HCV Core/HCV15
5-1-1	NS5	None

Table 4.2.1b. RIBA banding and corresponding viral region by Ortho™ assay.

A list of the positive samples tested, along with the corresponding RIBA banding are shown in Table 4.2.1c.

Sample	IEI Lot Number	RIBA Banding	Viral Region(s)	Interpretation
1	711	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.
2	712	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.
3	713	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.
4	714	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.
5	717	C100, C33, C22	NS4, NS3, Core	Pos.
6	718	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.
7	753	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.
8	754	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.
9	859	C100, C33, C22	NS4, NS3, Core	Pos.
10	865	C33, C22	NS3, Core	Pos.
11	937	C22	Core only	Neg./Ind.
12	995	C33, C22	NS3, Core	Pos.
13	997	C22	Core only	Neg./Ind.

Table 4.2.1c. Positive samples and RIBA banding of samples tested on SeroCard spotting combinations A to C.

The results of the positive and negative samples tested are shown in Table 4.2.1d. The negative samples, C1 to C12 were obtained from Cork Blood Bank. The samples were tested in duplicate and both readings are shown.

Sample	Combination A		Combination B		Combination C	
1. 711	1,5	0,5	1,6	0,5	0,6	0,5
2. 712	0,3	1,3	0,5	1,5	1,6	0,6
3. 713	1,6	0,6	0,6	1,7	0,6	0,7
4. 714	1,6	1,6	0,6	1,7	0,7	0,6
5. 715	0,7	0,6	1,8	0,7	0,7	0,7
6. 718	0,6	0,5	0,6	0,6	0,7	0,7
7. 753	1,3	1,4	1,4	1,5	0,5	0,4
8. 754	1,6	1,7	0,6	1,7	0,6	0,6
9. 859	0,3	0,4	0,4	0,4	0,4	1,5
10. 865	0,2	0,3	0,3	0,3	0,4	0,3
11. 937	1,1	1,0	1,1	1,1	1,1	0,1
12. 995	0,5	1,6	0,6	1,5	1,6	0,6
13. 997	2,2	2,2	1,2	1,1	1,2	1,2
14. C1	0,0	0,0	0,0	0,0	0,0	0,0
15. C2	1,1	1,1	0,0	1,1	1,1	0,0
16. C3	0,0	0,0	0,0	0,0	0,0	0,0
17. C4	1,1	1,1	0,0	1,1	0,0	0,0
18. C5	2,2	2,1	1,1	1,1	1,1	1,0
19. C6	0,0	0,0	0,0	0,0	0,0	0,0
20. C7	0,1	1,1	1,1	0,0	0,0	0,0
21. C8	0,0	0,0	1,0	0,0	1,1	0,0
22. C9	0,0	0,0	1,0	0,0	0,0	0,0
23. C10	2,2	2,2	2,1	1,1	1,1	0,0
24. C11	0,0	0,0	1,0	0,0	1,1	0,0
25. C12	0,0	0,0	0,0	0,0	0,0	0,0

Table 4.2.1d. Results of samples from Table 4.2.1c tested with SeroCard spotting combinations, designated A to C (Table 4.2.1a).

Taking the best duplicate result for each combination, the numerical difference was calculated for each sample and was plotted as shown in Figure 4.2.1a.

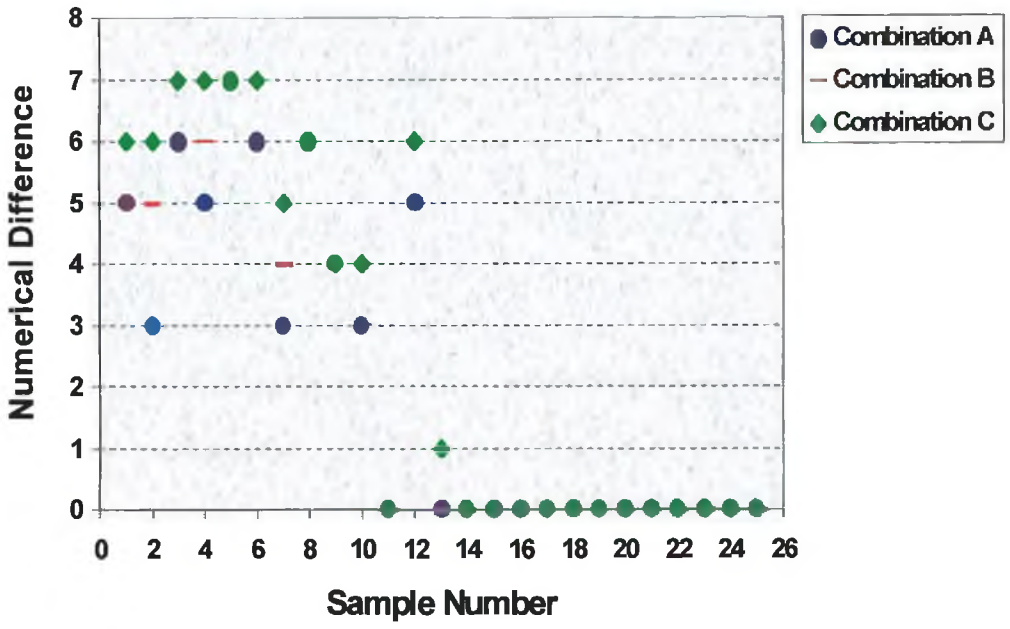


Figure 4.2.1a. Numerical difference values for 25 samples tested on three peptide spotting combinations on SeroCard.

The above representation of results indicates that combination 'C' gave the best readings. It gave the highest numerical difference values for the positive samples 1 to 13 and was the only combination, which succeeded in picking up sample 13. For the negative samples, all combinations gave the desired numerical value, however, in the case of combination 'C', the lowest actual readings were observed.

All future investigative studies were carried out on cards spotted using the peptide dilutions in combination 'C', HCV 15 spotted at 1/30, HCV Core at 1/100, HCV 52 at 1/40 and HCV NS3-1 at 1/20.

4.2.2 Investigation of Assay Incubation Times

The next study involved varying the incubation times of the assay to attempt to improve the colour intensity while keeping the negative sample readings as low as possible. The times used up to this point are shown in Table 4.1.3a, and are summarised as follows.

Sample Incubation	30 seconds
Post-sample Wash Incubation	30 seconds
Enzyme Incubation	60 seconds
Substrate Incubation	5 min.

The incubation times of three stages of the assay were varied. These variations are reflected in Table 4.2.2a.

Incubation	Time 1	Time 2	Time 3
Sample Time (S_1, S_2, S_3)	120 seconds	60 seconds	30 seconds
Post-sample Wash Time (W_1, W_2, W_3)	30 seconds	60 seconds	120 seconds
Enzyme Time (E_1, E_2, E_3)	60 seconds	90 seconds	120 seconds

Table 4.2.2a. Proposed variations in assay incubation times of SeroCard assay to improve colour intensity.

4.2.2.1 Sample Incubation Time

A total of nine samples were tested with sample incubated as shown above. Six of these samples were positive, three of them represented by three RIBA bands and three by two RIBA bands. The other three samples were negative. The results are shown in Table 4.2.2b.

Sample tested		S_1 (120s)	S_2 (60s)	S_3 (30s)
Three Band	***701***	0,2	0,2	0,3
	727	0,4	0,5	0,7
	742	0,3	0,4	0,6
Two Band	***696***	0,3	0,5	0,6
	678	0,1	0,2	0,3
	736	0,0	0,0	0,1
Negative	***C13***	0,0	0,0	0,0
	C15	0,0	0,0	0,0
	C16	0,0	0,0	0,0

Table 4.2.2b. Result of nine samples used in study of variation of sample incubation time in SeroCard assay. S_1, S_2 and S_3 represent three different sample incubation times investigated.

The highest positive readings were achieved with incubation S_3, 30 seconds. Due to the clean negative readings observed with this sample incubation, this was chosen for the next stage.

4.2.2.2 Post-Sample Wash Incubation Time

Using the same samples as were used previously, and with a sample incubation of 30 seconds, the incubation of the post-sample wash was examined as in Table 4.2.2c.

Sample tested		W_1 (30s)	W_2 (60s)	W_3 (120s)
Three Band	*701*	0,3	0,2	0,3
	727	0,6	0,6	0,8
	742	0,6	0,6	0,7
Two Band	*696*	0,6	0,5	0,7
	678	0,3	0,4	0,5
	736	0,1	0,2	0,3
Negative	*C13*	0,0	0,0	0,0
	C15	0,0	0,0	0,0
	C16	0,0	0,0	0,0

Table 4.2.2c Result of nine samples used in study of variation of post-sample wash incubation time in SeroCard assay. W_1, W_2 and W_3 represent three different post-sample wash incubation times investigated.

A post-sample wash time of 120 seconds (W_3) gave the highest positive readings with low negatives. This was chosen for the evaluation of enzyme incubation time.

4.2.2.3 Enzyme Incubation Time

With a sample incubation time of 30 seconds (S_1), and wash time of 120 seconds (W_3), the enzyme conjugate times were investigated. The results are recorded in Table 4.2.2d.

Sample		E_1 (60s)	E_2 (90s)	E_3 (120s)
Three Band	*701*	0,2	0,3	0,4
	727	0,7	0,7	0,8
	742	0,7	0,8	0,8
Two Band	*696*	0,7	0,7	0,8
	678	0,5	0,5	0,6
	736	0,3	0,4	0,5
Negative	*C13*	0,0	0,0	0,0
	C15	0,0	0,0	0,0
	C16	0,0	0,0	0,0

Table 4.2.2d. Result of nine samples used in study of variation of enzyme conjugate incubation time in SeroCard assay. E_1, E_2 and E_3 represent three different sample times investigated.

At the longer incubation time of 120 seconds (E_3), the negative sample readings remained low. This proved to be the best enzyme incubation time with an increase in the positive sample readings. In summary, a sample time of 30 seconds, a post-sample wash time of 120 seconds and an enzyme time of 120 seconds were chosen as assay times.

4.3 Performance Evaluation-Analysis of Characterised Sera

Upon completion of the studies to optimise the assay incubation times and parameters, a number of HCV positive and negative serum and plasma samples were evaluated for performance.

The assay parameters, which were chosen for the evaluations, were as shown below.

Card Spotting: 5µl peptide spotted on top right port as follows

HCV 15 at 1/30

HCV Core at 1/100

HCV 52 at 1/40

HCV NS3-1 at 1/20

5µl 2X PBS spotted on top left port

10µl control dilution buffer on bottom right port

Cards were dried, blocked and pouched according to procedure in Section 2.4.7

Assay Protocol: As per Section 2.4.6., with the following incubation times

Sample incubation	30 seconds at 18-25°C
Post-Sample Wash Incubation Time	120 seconds at 18-25°C
Enzyme Incubation Time	120 seconds at 18-25°C
Substrate Incubation Time	5 minutes at 18-25°

4.3.1 In-house Clinical Trial

A total of 76 positive and 20 negative samples were used to evaluate the HCV cards. The positive material consisted of 53 samples supplied by International Enzymes Inc. (IEI), and 21 samples from Boston Biomedica Inc. (BBI). The negative samples were supplied by Cork Blood Bank. The IEI samples were characterised according to Table 4.2.1b.

The test card results for each of the IEI samples analysed are shown in Table 4.3.1a along with the RIBA banding. The ELISA reading obtained for the chosen samples are also included in this table.

Sample Lot No	RIBA Banding	Viral Region(s)	Interpretation	ELISA Result	SeroCard Result
678	C33, C22	NS3, Core	Pos.	1.669	0,6
682	C33, C22	NS3, Core	Pos.	2.332	1,8
687	C33, C22	NS3, Core	Pos.	2.057	1,6
694	C33, C22	NS3, Core	Pos.	2.282	0,8
696	C33, C22	NS3, Core	Pos.	2.459	0,7
699	C33, C22	NS3, Core	Pos.	2.499	1,5
700	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.387	0,5
701	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	1.257	0,5
703	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.387	0,8
706	C33, C22	NS3, Core	Pos.	2.331	2,6
708	C33, C22	NS3, Core	Pos.	2.150	0,5
709	C100, C33, C22	NS4, NS3, Core	Pos.	Over	0,10
710	C33, C22	NS3, Core	Pos.	2.046	0,7
711	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.338	0,5
712	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.145	0,3
713	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.168	0,6
714	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.274	0,6
717	C100, C33, C22	NS4, NS3, Core	Pos.	2.168	0,8
718	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.055	0,4
719	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	1.917	0,4
727	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.168	0,9
729	C100, C33, C22	NS4, NS3, Core	Pos.	2.435	1,6
731	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.435	1,8
736	C33, C22	NS3, Core	Pos.	1.810	0,5
738	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.170	0,6
739	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.428	0,9
741	C33, C22	NS3, Core	Pos.	2.155	0,7

Lot No	RIBA Banding	Viral Region(s)	Interpretation	ELISA Result	SeroCard Result
742	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.155	0,10
743	C33, C22	NS3, Core	Pos.	1.942	0,6
746	C33, C22	NS3, Core	Pos.	2.114	0,5
748	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	1.129	0,3
750	C33, C22	NS3, Core	Pos.	2.101	1,8
753	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	1.579	0,3
754	5-1-1, C100, C33, C22	NS5, NS4, NS3, Core	Pos.	2.155	0,6
865	C33, C22	NS3, Core	Pos.	2.016	0,4
935	C22	Core only	Neg./Ind.	0.341	1,1
937	C22	Core only	Neg./Ind.	0.231	1,1
943	C100, C33	NS4, NS3	Pos.	2.317	1,8
944	C22	Core only	Neg./Ind.	0.114	0,1
945	C33, C22	NS3, Core	Pos.	2.220	0,8
950	C33, C22	NS3, Core	Pos.	0.661	0,7
951	C33, C22	NS3, Core	Pos.	1.134	1,3
958	C33, C22	NS3, Core	Pos.	2.462	0,10
959	C22	Core only	Neg./Ind.	0.147	0,1
961	C33, C22	NS3, Core	Pos.	1.814	0,5
981	C33, C22	NS3, Core	Pos.	2.056	0,4
993	C22	Core only	Neg./Ind.	0.620	0,2
995	C33, C22	NS3, Core	Pos.	2.100	0,5
996	C100, C33, C22	NS4, NS3, Core	Pos.	0.494	0,5
997	C22	Core only	Neg./Ind.	0.522	1,1
998	C22	Core only	Neg./Ind.	0.440	2,2
999	C22	Core only	Neg./Ind.	0.256	1,1
1112	C22	Core only	Neg./Ind.	1.203	0,0

Table 4.3.1a. Result of IEI samples tested on SeroCard and ELISA test achieved during in-house performance evaluation.

The results of the BBI and the negative samples are shown in Table 4.3.1b. There was no RIBA banding available for the BBI positive samples.

Sample	Interpretation	Card Result	Sample	Interpretation	Card Result
3304-0424	Positive	0,3	C25	Negative	0,0
1305-1	Positive	0,0	C23	Negative	0,0
1305-4	Positive	0,1	C22	Negative	1,1
3305-0070	Positive	0,0	C31	Negative	0,0
3304-0437	Positive	0,1	C21	Negative	0,0
1305-14	Positive	0,4	C26	Negative	0,0
3305-0076	Positive	0,4	C11	Negative	1,1
3305-0077	Positive	0,3	C24	Negative	0,0
3312-0028	Positive	0,0	C13	Negative	0,0
3305-0060	Positive	0,1	C20	Negative	0,0
3305-0101	Positive	0,1	C14	Negative	1,1
3311-0063	Positive	0,2	C8	Negative	0,0
3311-0312	Positive	0,2	C19	Negative	0,0
3308-0197	Positive	1,8	C18	Negative	1,1
3304-0302	Positive	0,10	C27	Negative	0,0
3311-0341	Positive	1,9	C10	Negative	0,0
3311-0403	Positive	0,10	C16	Negative	0,0
3304-0262	Positive	0,9	C17	Negative	0,0
3311-0315	Positive	0,9	C9	Negative	0,0
3311-0167	Positive	0,8	C28	Negative	1,1
3308-0154	Positive	0,4			

Table 4.3.1b. Result of BBI and negative samples tested on SeroCard assay achieved during in-house performance evaluation.

4.3.2 Trial Sensitivity and Specificity

Based on the above results, the sensitivity and specificity were calculated. As before, the formulae in section 3.2.5 was used to calculate test sensitivity and specificity.

A total of 74 positive samples and 20 negative samples were analysed. Data analysis of the IEI samples show that 9 of them contained only one RIBA band and were, therefore, indeterminate samples. They were not included in the calculation of the specificity

value. Sensitivity calculations were based on 65 positive samples. Results were interpreted as outlined in Section 4.1.3.2.

Specificity	*Sensitivity*
20 True Neg.-0 False Pos. 20 True Neg.	65 True Pos.-3 False Neg. 65 True Pos.
20/20 x 100	62/65 x 100
100%	95.4%

Table 4.3.2a. Sensitivity and specificity achieved during in-house performance evaluation of HCV SeroCard assay.

4.3.3 External Performance Evaluation

The performance of the HCV SeroCard was evaluated at two external trial site. As with the ELISA test, one location chosen was Public Health Laboratory Service (PHLS), Birmingham and samples results were compared with those achieved with RIBA banding. The other site chosen was the Virus Reference Laboratory, University College Dublin (UCD). All specimens had been stored at -20°C prior to thawing for testing by the rapid test.

4.3.3.1 Evaluation Results-PHLS, Birmingham

A total of 509 serum or plasma specimens were tested at the Public Health Laboratory, Heartlands Hospital, Birmingham. All specimens had been previously tested as part of routine testing at the Serology Laboratory, PHLS, and had been confirmed by RIBA testing. Analysis of these samples showed that 244 samples were found to be anti-HCV negative and 265 were anti-HCV positive by RIBA banding.

Of those tested, 480 specimens agreed with the RIBA result after initial testing. The 29 discordant samples were repeat tested. After re-testing, a total of ten discordant samples remained, consisting of 6 indeterminate samples, 2 false positive and 2 false negative samples. For the purpose of sensitivity and specificity calculations, the 6 indeterminate sera were omitted from the final calculations. Therefore, a total of 259 negative and 244 positive specimens were included in the calculations as shown in Table 4.3.3a.

Specificity	Sensitivity
259 True Neg. – 2 False Pos. 259 True Neg.	244 True Pos.- 2 False Neg. 244 True Pos.
257/259 x 100	242/244 x 100
99.2%	99.1%

Table 4.3.3a. SeroCard performance evaluation results at trial carried out at PHLS.

4.3.3.2 Evaluation Results- University College, Dublin

The reference test used to confirm the status of the samples was also the RIBA test. A total of 150 samples and 98 negative samples were positive by RIBA banding. The results obtained are shown in Table 4.3.3b.

Specificity	Sensitivity
98 True Neg. – 0 False Pos. 98 True Neg.	150 True Pos.- 2 False Neg. 150 True Pos.
98/98 x 100	148/150 x 100
100%	98.6%

Table 4.3.3b. SeroCard performance evaluation results at trial carried out at UCD.

The combined SeroCard performance results from both evaluation sites are combined in Table 4.3.3c. This gives a total of 394 positive and 357 negative samples.

Specificity	Sensitivity
357 True Neg. – 2 False Pos. 357 True Neg.	394 True Pos.- 4 False Neg. 394 True Pos.
355/357 x 100	390/394 x 100
99.4%	98.9%

Table 4.3.3c. Combined SeroCard performance evaluation results from sites at PHLS, Birmingham and at University College, Dublin.

Section 5-

Discussion

Discussion

5.1 Importance of Diagnosis of HCV Infection

The hepatitis C virus (HCV), described in 1989, is an RNA virus, of which there are at least nine genotypes. Infection with HCV is the leading cause of chronic liver disease. Detecting the presence of antibodies to the virus initially identifies the presence of HCV infection. The enzyme- linked immunosorbent assay (ELISA) is a serological diagnostic test used to confirm the presence of anti-HCV antibodies in serum. The Recombinant Immunoblot assay (RIBA), also a serological antibody test, specifically identifies antibodies to hepatitis C viral proteins represented as RIBA bands. A positive RIBA result is usually followed by the PCR test. The RNA structure of the Hepatitis C virus is examined by a quantitative PCR test, which confirms whether the virus itself is present and replicating in the blood. This is known as the viral load. Liver function tests or a liver biopsy is carried out if the previous tests point toward viral presence.

5.1.1 Routine Screening of Serum Samples for HCV Infection

The importance of polymerase chain reaction (PCR) in the diagnosis of HCV infection has established it as the gold standard of HCV-RNA detection (described in section 1.6.1). Viral presence and replication are critical to HCV diagnosis and to monitor treatment of HCV infection. PCR, discovered in 1983 by Kary Mullis (Hengen, 1999), works in a specific manner, amplifying a desired sequence of DNA or RNA, regardless of whether the sequence is known. It is a primer extension reaction for amplifying specific nucleic acids, allowing a short stretch of RNA to be amplified to such an extent that the size and nucleotide sequence can be determined.

In many cases, testing for HCV infection involves multiple screening of samples. Such wide-scale testing is not feasible by PCR, in most laboratories, as it is a time-consuming, high-cost method, requiring extensive specialised equipment, specific sample handling procedures and operator training. In developed countries, automated systems are widely used to cater for multiple screening. Serological methods of diagnosis are required, where testing can be carried out at low cost using standard laboratory equipment, operational with minimum training. The developed anti-HCV

ELISA plate fills these prerequisites and may be used where initial multiple screening of serum samples is necessary. Table 1b highlights the high-risk and low-risk categories where multiple sample screening is required.

While the majority of screening of serum samples is carried out in the comfort of a modern laboratory, this is not always the case. In poorly developed countries, the requirement for sample screening is often paramount but not always feasible. Temporary mobile clinics replace the standard laboratory and sensitive screening tests must be as simple and user-friendly as possible. The development of the rapid SeroCard test provides a means of carrying out testing of serum or plasma samples with minimal training and no laboratory equipment. Multiple testing using the SeroCard test is not feasible, as a limited number of samples may be assayed simultaneously. However, the advantage is that test results can be obtained within minutes and that testing can be conducted at point-of-care (POC). This means that, if a positive result is achieved on a rapid test performed in the presence of a patient, a fresh blood sample can be taken for confirmatory PCR testing at that moment. This would result in considerable savings in the money and time spent on recalling patients to give a second sample, if a positive reading is achieved at off-site laboratory testing.

5.1.2 Limitations of the Assays developed during this Research

It must be pointed out that an important limitation of the anti-HCV antibody ELISA and SeroCard rapid test, developed in this study, is also encountered in other serological assays such as Murex Anti-HCV (Version III) ELISA® and the Ortho HCV 3.0 ELISA®. They do not indicate the presence of current or recent viral infection and is only suitable for anti-HCV antibody screening; a positive result must be followed up by the steps outlined above.

In daily laboratory routine, reliable diagnosis with any of the tests described is not always possible since it is well known that EIA's produce a number of false-positive results. A study carried out in Germany indicates that the rate of false-positive HCV results by enzyme immunoassays was determined to be at least 10% among 1,814 reactive serum samples (Schroter *et al*, 1999). The results were based on negative results achieved in an independent confirmation assay, negative PCR results along with the lack of development of clinical or biochemical signs of infection during a 1-year

follow-up. This is a serious drawback of serological assays and although much can be done to improve specificity the total alleviation of false-positive results cannot be guaranteed.

The development of a different class antibody assay such as an IgM-specific assay would possibly have not improved potential false-positive readings. The presence of rheumatoid factor in sera from anti-HCV positive blood donors has been known to interfere with the detection of HCV-specific IgM resulting in false positive reactivity (Stevenson *et al*, 1996). Rheumatoid factor is an immune complex found primarily, but not limited to serum and synovial fluid of patients with rheumatoid arthritis. This complex is composed of immunoglobulin autoantibodies directed against and complexed with normal serum IgG. They react specifically with antigenic determinants on the constant regions of IgG heavy chains. IgG rheumatoid factors form aggregates, which appear to be self-associated complexes (Williams *et al.,* 1977). The mechanism responsible for rheumatoid factor formation is not known, however, the serum of rheumatoid arthritis patients is said to contain IgG molecules, which are conformationally altered toward the hinge region. Removal of IgG from samples prior to IgM testing by Stevenson *et al*, resulted in a significant loss of reactivity in the IgM anti-HCV assay in 30/41 sera that were positive when tested untreated; 70% of anti-HCV-positive sera were also positive for rheumatoid factor.

The sensitivity of the HCV ELISA plate and SeroCard assay could have been investigated by the introduction of the avidin–biotin amplification system. Avidin is a glycoprotein found in the egg white and tissues of birds, reptiles and amphibia. It contains four identical subunits having a combined molecular mass of 67-68kDa. Biotin, a 244 dalton vitamin found in tissue and blood, binds with high affinity to both avidin and streptavidin, a 60kDa protein that is isolated from *Streptomyces avidinii.* A format was considered which involved the use of commercially available streptavidin-HRP conjugate coupled to biotinylated specific antibody and antigen complex. It was believed that despite signal amplification, the use of this tri-complex would be of little advantage as numerous titration experiments would be required. The use of a streptavidin-coated plate was also eliminated as it proved to be a high-cost material. A system involving the biotinylation of anti-human IgG was also considered but not practised.

5.1.3 HCV-Infected Victims on our Doorstep

The need for multiple screening of serum samples for antibodies to HCV was highlighted in recent years in Ireland as a result of the contamination of blood products with the virus. An estimated 62,667 Irish women were screened for the presence of anti-HCV antibodies between 1994 and 1997 (Government Publications, 1997). At the time of initial screening, the availability of sensitive second and third generation assays was relatively new; the virus itself identified only five years previously.

Extensive research by the Blood Transfusion Service Board (BTSB) revealed that a plasma pool containing anti-D immunoglobulin (anti-D), a manufactured blood product, was the source of HCV contamination. Anti-D has been manufactured by the BTSB since 1970 and was intended for injection intravenously, rather than the more common method of intramuscularly, to prevent Rhesus (Rh) haemolytic disease. This disease may occur when a woman with Rh-negative blood produces a newborn Rh-positive. When administered within 48 hours of pregnancy, anti-D prevents the disease, which has caused over 100 Irish stillbirths, by blocking the maternal production of antibodies to the foetal red blood cells. The invasion of Rh-positive antibodies, in subsequent pregnancies, damages the foetus and can lead to severe anaemia and brain damage (Government Publications, 1997).

A therapeutic course of plasma exchange is necessary in cases where the prevention of harmful antibodies has not been successful. In September 1976, the BTSB used the plasma removed from an Rh-positive female patient, during exchange treatment, to manufacture anti-D for use in other patients. A month later, the patient became jaundiced and was later diagnosed as having developed hepatitis C. Five batches of anti-D immunoglobulin, with an average of 250-400 doses per batch had been manufactured and distributed. Table 5a highlights the number of infections, which occurred as a result of the receipt of transfusion, highlighted in Table 1b as a high-risk mode of HCV transmission. The statistics presented in Table 5a leave no doubt as to the absolute importance of the availability of a sensitive anti-HCV test, not only as a diagnostic tool but in the conduction of multiple screening of human samples in the hope of preventing the reoccurrence of such a catastrophe

Recipients	Number of People	Details
Recipients screened for HCV	62,667	56,151 in 1994, 2,636 in 1995, 3,696 in 1996 and 184 in 1997
Identified recipients of infected anti-D	1,147	294 type 1b infections
Identified recipients who became blood donors	103	60% positive by PCR
Blood donations given	504	606 labile components issued
Infections as a result of donations	61	49% viral presence by PCR
Positive diagnosis from direct or indirect anti-D infection	849	55% show continuing viral presence by PCR
Compensation claims paid	1,066	A cost of £150,393,732

Table 5a. Details of the effects of HCV contamination in Ireland in the mid 90's (Statistics received from Government Publications, 1998)

5.2 Development and Optimisation of HCV Assays

One of the first questions posed at the onset of test development was the basic issue of assay technique. Along with the short-lived options outlined in section 5.1.2, was the choice of developing a test using the direct or indirect test protocol. The direct method originated in the 1940's using fluorescent-labelled antibodies to mark tissue antigens (Pierce, 1997). In this method, a primary antibody is directly labelled with a fluorescent probe or enzyme such as HRP or AP, which then reacts with bound antigen. The use of a sole primary antibody has its limitations. Although the cross-reactivity of a secondary antibody with primary antibody is eliminated, the immunoreactivity of the sole antibody may be reduced as a result of labelling, and amplification of signal with this method is limited. Therefore, the indirect method was chosen for both the ELISA and SeroCard tests. It utilises a labelled secondary antibody as represented in Figure 1c, and was first described in 1954. A primary HCV specific antibody binds the HCV antigen followed by the secondary antibody directed against the primary, in this case, goat-anti-human IgG peroxidase or phosphatase labelled. This method offers a more sensitive assay as each primary antibody contains several epitopes for binding to the secondary antibody increasing the binding capacity at the site of antigen. Non-specific binding may occur as a result of cross-reactivity between both antibodies, although one of the advantages of the indirect assay over the direct assay is that the primary antibody is not affected by labelling.

5.2.1 Choosing an Optimum Solid Phase

After deciding on the indirect assay technique, the first part of the study involved choosing a suitable solid phase support on which to immobilise the specific antigen. The C-well microtitre plate, devised as a hybrid of the flat-bottomed and the round-bottomed wells, was used as it carries the advantage of good washing and aspirating steps while retaining the optical advantage of the flat bottom well. The surface chosen to bind the solid phase peptides was polystyrene. Adsorption to the well surface is controlled by the chemical properties of the surface and of the peptide. Polystyrene is a long carbon chain with numerous benzene rings, making it a hydrophobic compound. The adsorption of molecules to polystyrene is due to intermolecular attraction forces such as van der Waals forces and hydrogen bonding. The chemical properties of the four HCV synthetic peptides chosen for immobilisation to polystyrene were examined. The amino acid sequence of each peptide is shown in Figure 5a. All four peptides consist of both hydrophobic and hydrophilic amino acids, which favours the use of the NUNC™ Maxisorp microtitre plate. This surface binds molecules with both hydrophobic and hydrophilic regions, the enhanced binding achieved by a physical method which alters the surface molecules (Rowell, 1997a). The solid phase membrane chosen for the SeroCard was supplied by Trinity Biotech.

HCV NS3-1 (m.w. 2885)

Gly-Arg-Glu-Iso-Leu-Leu-Gly-Pro-Ala-Asp-Gly-Met-Val-Ser-Lys-Gly-Try-Arg-Leu-Leu-Ala-Pro-Thr-Iso-Thr-Ala-Tyr-NH_2

HCV 52 (m.w. 3960.5)

Iso-Cys-Asp-Arg-Glu-Val-Leu-Tyr-Arg-Glu-Phe-Asp-Asn-Glu-Cys-Ser-Gln-His-Leu-Pro-Tyr-Iso-Glu-Gln-Gly-Met-Met-Leu-Ala-Glu-Gln-NH_2

HCV Core (m.w. 3417)

Pro-Gln-Asp-Val-Lys-Phe-Pro-Gly-Gly-Gly-Gln-Iso-Val-Gly-Gly-Val-Tyr-Leu-Leu-Pro-Arg-Arg-Gly-Pro-Arg-Leu-Gly-Val-Arg-Ala-Thr-Arg-NH_2

HCV 15 (m.w. 3143)

Met-Ser-Thr-Asp-Pro-Lys-Pro-Cys-Arg-Lys-Thr-Lys-Arg-Asp-Thr-Asp-Arg-Arg-Pro-Cys-Asp-Val-Lys-Thr-Pro-Gly-Gly-Gly-NH_2

Figure 5a. Amino acid sequences of synthetic peptides used in ELISA and rapid tests. Green represents hydrophobic, red represents hydrophilic and blue represents neutral amino acids

5.2.2 Parameters affecting Coating

Protein binding is influenced by a number of factors such as time, concentration and pH, which were considered to optimise coating of the peptide to the solid support. As recommended by NUNC™, after two hours, maximum coating is achieved but cannot withstand stringent washing. Thus, time effects coating stability and so overnight coating at 4°C was recommended although maximum stability can be been achieved after six hours. Coating at 37°C was avoided as the higher temperature can cause the edge effect (Rowell, 1997a). The binding is also dependent on the pH of the coating buffer along with its hydrophobicity. Section 3.1 describes the adsorption of conjugated HCV peptide-BSA to polystyrene with carbonate buffer, pH 9.6, as the coating buffer. These conditions were suitable for binding all fifteen HCV peptides and four peptide-BSA complexes were then chosen. In section 3.5, carbonate buffer, pH 9.6 was compared with phosphate buffer, pH 7.2 as coating buffer for unconjugated peptide. The phosphate buffer, with the lower pH, was chosen for this direct coating, as can be seen in Figure 3.5.1a. The use of PBS is recommended due to its ability to structure water around the surface-bound molecules. Other diluents such as carbonate buffers, do not have the same stabilising effect as PBS (Gibbs, 1995). The higher binding affinity of the unconjugated material in carbonate buffer suggests that molecular weight of the peptide is an additional factor affecting coating. At 1μg/ml and 0.5μg/ml, the absorbance readings of the positive samples reduced dramatically (Figure 3.5.1a). The same effect was not seen to such an extent in Figure 3.1.2a in the case of the conjugated material. This suggests that in carbonate buffer, pH 9.6, the BSA (m.w. 60,000) has a higher affinity than the lower molecular weight peptides (m.w. 3,000-4,000).

5.2.3 The Sequence of Assay Optimisation

Optimisation of the assays followed a sequence of investigations, which lead to subsequent evaluation steps. The first phase in the development of the microtitre plate assay is described in section 5.2.1. The synthetic peptides were adsorbed to the solid surface and the peptides, which gave the highest positive-negative ratio on testing with characterised sera, were chosen (Table 3.1.1b). These four peptides were further coated at various dilutions along with a full peptide pool and core-specific peptide pool as shown in Table 3.1.2a. Optimum binding was achieved on the solid phase coated with full peptide pool at 1/10,000 as seen by the absorbance readings in Figure 3.1.3a. Although each specific peptide had affinity to the polystyrene when coated alone,

optimum binding of the positive samples occurred in the presence of peptides specific to all viral genomic regions (Figure 3.1.3a). This suggests that specific detection of the HCV antibody required the presence of the full compliment of peptides, covering both the structural and non-structural regions of the HCV genome (see Figure 3.1.2a).

On plates coated with the full peptide pool, the sample diluent was next to be evaluated. This study involved the addition of serum and inert proteins to the control dilution buffer to improve the positive-negative ratios (personal communication, Dr. Edward Balbirnie, Trinity Biotech). The effect of bovine serum albumin, normal human serum, rabbit serum and goat serum was investigated. The concept of positive-negative ratio was used to choose 5% goat serum as a component in the sample diluent, as seen in Figure 3.2.1a. Section 5.2.4 on non-specific binding outlines a possible explanation for the beneficial effect of goat serum on negative samples.

With the sample diluent chosen, two anti-human IgG-HRP conjugates were evaluated (section 3.2.2). The Kirkegaard and Perry Laboratories (KPL) conjugate gave greater definition between positive and negative samples tested, than did the Genzyme conjugate (see Figure 3.2.2a and Figure 3.2.2b). Both were titred based on the manufacturer's recommendations, and 1/80,000 dilution of the KPL conjugate was chosen, as the absorbance reading of the positive sample was 1.458 with a negative reading of 0.184 (Table 3.2.2a). Evaluation of the KPL conjugate diluent involved the addition of surfactant and serum (section 3.2.3). Figure 3.2.3a shows that Triton X-100, Tween 20 and goat serum were examined with 0.05% Triton X-100 chosen as an additive. The effect of detergents is discussed in section 5.2.4. Optimum sample dilution was next to be evaluated with dilutions carried out within the microtitre plate well (section 3.2.4). Premixed samples had been used to evaluate various parameters up to this point, which held the disadvantage of an additional assay step. Table 3.2.4c shows that the 1 in 10 in-well sample dilution was chosen based on positive-negative ratios achieved. This is also represented in Figure 3.2.4b.

With the parameters chosen as outlined above, a number of characterised serum samples were evaluated (section 3.3.1). A sensitivity of 88.6% and specificity of 85% was achieved (Table 3.3.3a). Improvements to these results involved the fine-titre of the solid phase peptides at coating dilutions greater than the initial 1/10,000 dilution, as shown in section 3.4.1. Further testing on clinical samples resulted in an improved sensitivity of 94.3% and specificity of 93.3% (Table 3.4.3a). Further work was carried

out on the solid phase in an attempt to improve this data. It was believed that the large carrier protein, bovine serum albumin, with a molecular weight of 60,000 (Rowell, 1997b), may be blocking the complete adsorption of HCV peptide (each with m.w. 3,000 to 4,000) to the surface, allowing only limited binding. This theory was investigated by coating the peptide directly dissolved in coating buffer (section 3.5.1). This method did improve the positive readings but coating of the conjugated peptide gave lower negative readings (Figure 3.5.2b). For this reason, the KPL conjugate and the conjugate diluent were re-evaluated (section 3.5.3). The conjugate was used at a more dilute 1/100,000 and foetal bovine serum was added to the conjugate diluent. These changes in peptide coating methods, along with those of the conjugate titre and conjugate diluent, succeeded in improving specificity to 98.9%, and sensitivity to 100% as described in section 3.6.2 and shown in Table 3.6.2b.

The sensitivity and specificity achieved with peptides coated on the microtitre plate format led progressively to the adsorption of the same peptides on a membrane for use in the rapid assay (section 4.1). The calculation of the spotting dilutions and spotting volumes is shown in Table 4.1.2a. The wash reagent, enzyme conjugate reagent and substrate reagent were supplied by Trinity Biotech plc. As was done in the initial phase of the microtitre plate assay, the peptides were spotted individually and were also combined for spotting as a full peptide pool (section 4.1.2). Figure 4.1.4a shows that, following testing with characterised sera, optimum binding of the positive samples occurred on the membrane spotted with peptides specific to all viral genomic regions. The need for the full compliment of peptides was also seen in the microtitre plate assay. Although the positive samples were higher on the full peptide pool than on individually spotted peptide (Figure 4.1.4a), the spotting dilutions were increased to improve colour intensity of the positive sample, as described in section 4.2.1. Table 4.2.1d shows the values achieved on the re-evaluated combinations, represented in Figure 4.2.1a.

To further improve the colour intensity of the positive readings while keeping the negative values low, the various assay incubation times were examined (section 4.2.2). The sample incubation time, post-sample wash incubation time and enzyme incubation time were optimised using a number of clinical samples. This resulted in a thirty-second sample incubation, two-minute post sample wash incubation and two-minute enzyme incubation time. In the in-house performance evaluation of the rapid test, the sensitivity achieved was 95.4% with a specificity of 100%, as shown in Table 4.3.2a.

5.2.4 The Problem of Non-Specific Binding

The concept of non-specific binding (NSB) occurs frequently and constantly during the various stages of development and optimisation of an immunoassay. This is particularly true of the enzyme immunoassay due to its increased sensitivity. At any stage of the assay, for example, during blocking, an unspecific reactant may have an affinity to the solid surface and become immobilised. This in turn can lead to the immobilisation of succeeding reactants throughout the assay. During the course of an ELISA or during peptide immobilisation, non-bound reagents are removed from the surface leaving a liquid film behind. After coating and spotting, blocking agents were used in the ELISA and rapid test to block excess solid surface. This was to avoid unspecific immobilisation of reactants to the liquid film and also to protect adsorbed protein from surface denaturation. Low background is usually achieved by blocking with an inert protein. Bovine serum albumin was used in the microtitre plate test, with casein and Triton X-405 detergent used to block non-specific sites on the rapid test membrane. After blocking, plates can be stored at refrigerated temperatures without loss of sensitivity.

Detergents are molecules consisting of a distinct hydrophobic and hydrophilic part. Triton X-100 (m.w. 646) contains branched hydrophobic octyl part and the hydrophilic part of Tween (m.w. 1240) is divided into three separate arms (Rowell, 1997b). They are examples of non-ionic surfactants as opposed to ionic sodium dodecyl sulphate (SDS). Their blocking effect is based on the ability to compete for both hydrophobic and hydrophilic sites. One of the main causes of NSB in microtitre plate assays is improper washing and aspirating, leaving unbound residue on the well surface. Tween 20 is present in the ELISA wash buffer, its washing effect based on the ability to disperse hydrophobic molecules in aqueous medium. In some instances, it is of no benefit to simultaneously use a blocking agent, such as BSA and a detergent such as Triton X-405 or Tween 20, as an immobilised detergent may affect specificities in secondary reactants. Detergent is used at a later stage of the ELISA test. The effect of addition of detergent to the conjugate diluent is shown in Figure 3.2.3a. Triton X-100 was chosen based on the positive-negative ratios achieved. No detergent was used in the secondary reactants of the rapid test.

The effect of the addition of serum during optimisation of the ELISA assay was to reduce the non-specific adsorption of molecules from the liquid phase. The addition of

various sera to the sample diluent was investigated. Results were based on positive-negative ratios and as can be seen in Figure 3.2.1a, the addition of goat serum had the greatest effect with 5% (v/v) giving a negative absorbance of 0.156 with a positive reading of 1.202 (Table 3.2.1a). A possible explanation for the effect of goat serum lies in the fact that the affinity purified antibody used in the conjugate was isolated from a pool of serum from goats immunised with purified human IgG. As a protein stabiliser, goat serum was added to the conjugate by the manufacturer. The further introduction of goat serum in the sample diluent may explain the decrease in negative absorbance readings.

Section 3.5.3 describes an attempt to further improve specificity by the addition of serum to the conjugate diluent. Goat serum and foetal bovine serum were introduced at 5, 10 and 20% (v/v). The addition of goat serum reduced the negative readings but also had the effect of significantly reducing the positive readings. As represented in Figure 3.5.3b, foetal bovine serum did not cause a decrease in positive readings but had the desired effect on negative samples; 5% (v/v) was chosen based on absorbance values.

5.2.5 Variations in ELISA and Rapid Tests

The enzyme conjugates used in the ELISA and the rapid assays employ goat anti-human IgG antibody labelled to horseradish peroxidase and alkaline phosphatase respectively. The alkaline phosphatases are a widely distributed family of isoenzymes found in large numbers of species and tissues. Horseradish peroxidase is the most commonly used immunoassay enzyme, which catalyses an enzymatic reaction with a rapid high turnover rate as opposed to the linear reaction rate of alkaline phosphatase (Pierce, 1997). HRP is an inexpensive material and its complexes usually retain enzymatic activity at 4°C for long periods of time. The main distinguishing feature of both enzymes is the molecular weights; HRP was a molecular weight of 40,000 with AP being a far larger enzyme with a molecular weight of 140,000. The characteristics of both enzymes suggest they are not interchangeable between the ELISA and the rapid assays. The smaller molecular weight of HRP is unsuitable for use on a porous membrane-based solid support. An attempt was made to use the peroxidase-labelled conjugate on the rapid test but it was unsuitable and resulted in immediate strong colour on addition of chromagen. The linear reaction rate of alkaline phosphatase was suitable in the rapid assay, whereas the short linear phase of HRP was used on the plate assay. The activity of HRP is inhibited by azide while phosphate buffers inhibit phosphatase activity. HRP is most

commonly detected by the cheap and ready-to-use chromagen, TMB (tetramethyl benzene). It is a safer alternative to the possible use of o-phenylenediamene (OPD) substrate, which is known to have carcinogenic properties. Another safe and inexpensive chromagen, BCIP, is used to detect alkaline phosphatase in the rapid assay.

Throughout the optimisation of both assays and due to the nature of both tests, result interpretation differed. Customer interpretation of the rapid test is visual only; the presence of blue colour on addition of substrate indicating a positive result. The intensity of the blue colour is directly proportional to the quantity of specific antibody present in the test specimen. The concept of numerical difference as described in section 4.1.4, was limited to use as a guide during development of the assay, to enable documentation of results in tabular and in graphical form. On the other hand, result interpretation in the ELISA assay was based on calculation of a cut-off numerical value based on comparison with kit control specimens.

5.3 The use of Protein Coupling and Purification during Development

The concept of protein coupling and its application in ELISA is described in section 1.7. Evaluation of synthetic peptides formed the basis of the first stage in the development of the assays for the detection of antibodies to hepatitis C virus (HCV). Both the microtitre plate format and the rapid membrane-based format incorporated the use of proteins on the solid phase matrix. The protein was coupled to a carrier substance, in this case, bovine serum albumin (BSA), a commercially available protein with a molecular weight of approximately 60,000. This conjugation is an example of homobifunctional coupling. The first cross-linking reagents used for modification and conjugation of macromolecules consisted of bireactive compounds containing the same functional groups at both ends. Most of these homobifunctional reagents were symmetrical in design with a carbon chain spacer connecting the two identical reactive ends as shown in Figure 5.3a.

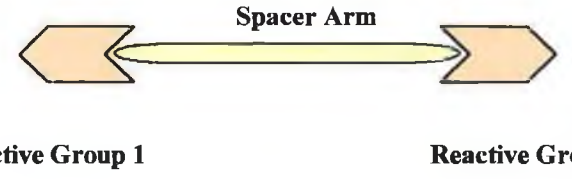


Figure 5.3.a. Representation of a homobifunctional reagent, such as glutaraldehyde used to conjugate HCV peptide to bovine serum albumin

These reagents link one protein to another by covalently reacting the lysine ε-amines or N-terminal amines of one protein to the same functional group on a second protein (Hermanson, 1996). A number of different groups capable of reacting with amines have been described such as aldehydes, succinimide esters, aryl halides and isocyanates.

Glutaraldehyde, also known as glutardialdehyde or pentanedial, is the most commonly used reagent in homobifunctional coupling. The reaction of an aldehyde with an amine to form a Schiff base is shown in Figure 5.3a and is the basis of the conjugation of HCV peptide to BSA, which contains 59 lysine amino groups, 39 of which are free to react.

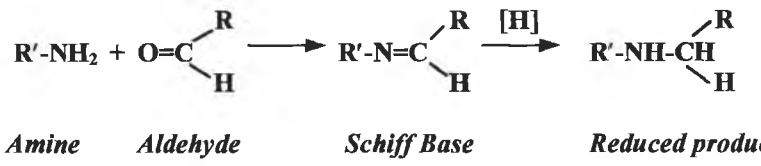


Figure 5.3b. Formation of Schiff base during homobifunctional coupling

The use of glutaraldehyde as a homobifunctional reagent is represented in Figure 5.3c. Quiocho and Richards, in 1966, showed that the chemistry of glutaraldehyde is far more complicated than this reaction suggests (Aslam and Dent, 1998). They observed that the effects of glutaraldehyde could not be reversed by a reagent capable of cleaving imine bonds under many circumstances.

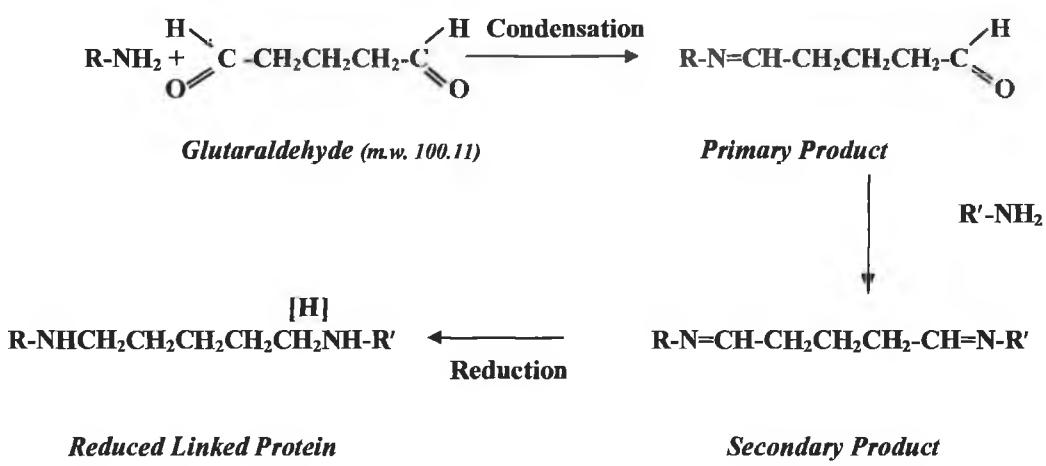


Figure 5.3c. Reaction of glutaraldehyde as a homobifunctional reagent as in the coupling of HCV peptide to protein and its reduction using sodium cyanoborohydride

Richards and Knowles further supported this in 1968 when they carried out NMR on commercial glutaraldehyde solutions. They observed strong evidence for the presence of α, β-unsaturated aldehydes forming conjugates more stable than those expected from simple Schiff base conjugation. In 1974, Whipple and Ruta further evaluated the problem and concluded that commercial 25% (v/v) glutaraldehyde consisted predominantly of *cis* and *trans*-isomers, plus monohydrate, dihydrate and native glutaraldehyde. These studies confirmed that protein cross-linking reactions done using homobifunctional reagents could result in large complexes of multiple sizes and infinite molecular structure including polymerised products that may even precipitate. This inherent potential to polymerise is characteristic of all homobifunctional reagents, even in multistep protocols. The impurity composition varied with temperature and pH. Figure 5.3c represents the reaction of glutaraldehyde with a protein forming an active intermediate, which can cross-link with a second protein.

Reduction of the Schiff base structure was originally carried out using sodium borohydride. However, the products of reduction yielded modifications of the original structure. For this reason the weaker reductant, sodium cyanoborohdride was used in the reduction of the HCV peptide-BSA conjugate as it targeted the Schiff base formation only. Ukeda *et al* carried out an examination of the reaction of BSA with a glutarldehyde-derivatised solid phase (Aslam and Dent, 1998). This revealed complex kinetics and at least two different binding sites. It was discovered that blocking the solid phase with ethanolamine reduced the amount of BSA bound by only 40% with that figure increased to 70% using borohydride as the reductant. Purification of the reduced structure was then carried out, as now described.

Although the protein used in the assay, development in this study, was in solution when ready for purification, many proteins are present in cellular structures that render them insoluble in water. Proteins may frequently be dissolved by extraction with water or with salt solutions of low ionic strength (White *et al*, 1978). Proteins may also be solubilised by the use of weak detergent solutions, such as polyoxyethylenesorbitan monolaurate (Tween 20), by lipid removal from treatment with non-polar solvents, such as acetone and ether, or by treatment with butanol, to disrupt cellular structures.

Advances in chromatography have had the greatest impact on protein purification. Chromatography describes the separation method of a mixture of dissolved substances

through a porous solid support irrespective of the forces that lead to the separation of the mixture (White *et al*, 1978). Purification of the HCV peptide-BSA complex was carried out either by dialysis or by gel filtration chromatography. Gel filtration was chosen because separation of compounds occurred on the basis of molecular size and shape. Purification of the high molecular weight conjugate containing the HCV peptide-BSA complex was carried out by separation from the unconjugated peptide and glutaraldehyde of smaller size. The purification process is described below.

A gel matrix can be prepared by suspending diverse insoluble materials such as synthetic resins, porous glass or polysaccharides such as dextran and agarose in an appropriate solvent. The formation of two solvents occurs, one within the internal space of the gel particles and the other outside the particles. The sephacryl S-200 column of granular matrix acted as a molecular sieve allowing certain molecules to pass through the solid support. Separation of solutes occurred by gel filtration. The smaller solute, the free HCV peptide or glutaraldehyde, could freely enter the internal space within the gel particles. A higher molecular weight molecule, such as unconjugated BSA, could enter the inner space but not as freely as the smaller molecule. The high molecular weight molecule, the HCV peptide-BSA complex could not enter the internal space of the gel particle and passed down the S-200 column outside the particles and was the first molecule to be eluted from the column. Separation occurred as the smaller unconjugated molecules were finally eluted from the column having entered the gel particles of the matrix. The granular matrix can be chemically modified to a fixed porosity and can therefore be used to establish the approximate molecular weight of substances that have the capacity to penetrate the internal phase. The shape of a molecule along with the size determines whether it can enter the gel particle. Spherical molecules of a certain molecular weight may able to enter the internal phase whereas asymmetric molecules of the same molecular weight may not.

Following purification of the peptide-BSA complex by gel filtration chromatography, the eluted material is analysed by spectrophotometry as described in section 2.5.3. The fractions were read at a wavelength of 280nm, where much protein absorption occurs. The protein content of each fraction may be estimated based on the Beer-Lambert law, which states that $A=\varepsilon cl$, where A corresponds to absorbance, ε is the molar extinction coefficient, c is the concentration and l is the light path, usually 1cm (Aslam and Dent, 1998). Measurement of protein content is not essential and serves only to obtain the

yield, if required; the peptide complex is spotted using a spotting dilution, rather than quantitatively. It must be noted that free peptide and free BSA, inevitably present in certain fractions, absorb at 280nm and so the fractions must be further analysed by the neutralisation assay, described in section 2.5.4. This procedure will determine which fractions contain the desired purified conjugated peptide, for immobilisation on the solid phase. A small volume of each fraction to be analysed is added to HCV-positive material, the neutralisation reagent. A positive result, upon testing, indicates that the added fraction did not contain purified HCV peptide; a negative result indicates that any HCV peptide, present in the fraction, binds the HCV antibody in the neutralisation reagent, and so it acts as a negative sample. Whether the conjugated peptide is purified by dialysis or by chromatography, it is titrated to determine the dilution, at which, it will be immobilised on the solid phase. This served as the first evaluation step in assay development and is outlined in section 3.1 and section 4.1, for use in the microtitre plate assay and the membrane-based assay, respectively.

5.4 Comparison of Assays with Current Market Leaders

The world-wide market size for HCV assays was estimated to be worth $900 million in 1998, with a steady growth observed since the mid-nineties. Due to the fact that there are so few rapid HCV tests currently available, this market has not been fully exploited. However, estimates predict that the size of the rapid test market would be 4% of the total available market. The patent related to the marketing of HCV assays is owned by Chiron Corporation and has been granted in over 20 countries. It was not the intention of Trinity Biotech at the time of the development of these kits to market in the United States, therefore, they were not submitted for approval to the Food and Drugs Administration.

Since the discovery of HCV in 1989, various tests have become available for the detection of HCV antibodies. Based on customer requirements, the tests are available in formats such as PCR-based tests, western blot assays, ELISA tests and rapid tests. Roche Diagnostic Systems provide the widely used Amplicor™ HCV detection kit, an easy-to-use PCR assay. Western blot assays include those from ①Ortho Chiron, ② Sanoffi DP and ③ Genelabs Diagnostics. As is the case with PCR-based assays, western blot assays are costly, time-consuming and require complex laboratory equipment.

The ELISA assays offer a cheaper and less complex method of detection of HCV antibodies and are suitable for use with automated systems. Current market leaders in HCV EIA testing include ① Abbott Diagnostics HCV EIA 3.0, ② General Biologicals Corp. NANBASE C-96 anti-HCV EIA, ③ General Biologicals Corp. NANBDINE^{125} C anti-HCV RIA, ④ INNOGENETICS INNOTEST HCV Ab III, ⑤ Murex anti-HCV (Version III), ⑥ Ortho Chiron HCV 3.0 ELISA, ⑦ Sanofi Diagnostics MONOLISA® anti-HCV EIA, ⑧ Syntron Bioresearch Inc., Microwell HCV EIA and ⑨ United Biomedical HCV EIA. A limited number of rapid tests are currently available including ① Bionike Laboratories HCV test, ② Genelabs Diagnostics HCV Spot and ③ SERO-Med® Rapid Test.

There are certain factors to be considered when comparing the assays developed during this research, with those currently available. The performance characteristics of the assay, achieved upon testing of positive and negative clinical specimens, are the most significant factors. Therefore, it is imperative that the test should be both sensitive and specific. The length of time required to achieve a test result, along with the assay temperature, are both influential features of an assay. The incubation times of the commercially available ELISA and rapid tests outlined above, along with the assay incubation temperatures and reagent preparation necessary, are shown in Appendix 6. The clinical trial performance of each these assays were not obtainable and so sensitivity and specificity values were not included (personal communication, Marketing Department, Trinity Biotech).

The HCV ELISA, developed in this study, was compared with two of the market leaders, the Murex anti-HCV Version III EIA and the General Biologicals Corp. NANBASE C-96 EIA. Comparisons were carried out in relation to the aforementioned assay protocols. Excluding washing steps, the Murex assay required a total assay time of two hours and the General Biologicals test required 90 min. The total assay time of the test, developed in this research, was 75 min. In addition, it offered the advantage of ready-to-use conjugate and substrate reagent. The Murex and General Biologicals tests both necessitated two additional steps, the preparation of conjugate reagent and substrate reagent. The incubation temperature of the test, developed in this study, and the Murex test are both at 37°C, whereas that of the General Biologicals test is at 40°C, to be carried out under humid conditions. It also uses o-phenylenediamene, OPD, which is replaced by the safer option, TMB, in the both other tests. In terms of total

assay time, reagent preparation, assay temperature and safety, the assay developed in this research, compared favourably to both the Murex and General Biologicals assays.

The assay protocol of the SeroCard assay was also compared to other membrane–based tests. As stated previously only a limited number of rapid, membrane-based tests are available to detect HCV antibodies. Results can be achieved with the SeroCard assay in 10 min., as is the case with both the Bionike Laboratories HCV test and the Genelabs Diagnostics HCV Spot. The SERO-Med® Rapid Test, a colloidal gold-based assay requires trained staff to carry out the test, which must be performed on fresh whole blood or serum samples.

Along with assay parameters, as described above, the performance of the Murex and General Biologicals assays was evaluated and compared to the both the microtitre plate assay and the rapid SeroCard assay, in a study involving testing of samples of unknown status. A total of 75 samples were tested in this 'blind' study and all procedures were followed according to the manufacturer's instructions. The interpretation of readings is shown only, in Appendix 7, as results were calculated based on the recommended procedure. A comparison of absorbance readings was not made, as different cut-off values were achieved for each assay.

A positive result was achieved on the ELISA test for a total of 47 samples. A total of 43 of these samples gave a positive reading on both the Murex test and the General Biologicals test. A further one of the samples was positive on Murex test only. Three samples positive on the ELISA, developed in this study, were negative on both of the competitor tests. A total of 28 samples gave a negative reading on the ELISA, 23 of which were also negative on both the Murex and the General Biologicals assays, and one of which was positive. Of the remaining four negative samples, two were also negative on the Murex test only and two on the General Biologicals test only. The rapid SeroCard assay also gave good correlation with the competitor assays. Three of the SeroCard readings gave indeterminate results and two negative results were positive on the other tests. All positive readings on the SeroCard assay correlated with positive readings on all three ELISA assays tested.

The favourable comparison of both the microtitre plate assay and the rapid membrane-based assay, developed as a result of this research, indicates that sensitive and specific

assays for the detection of HCV antibodies have been achieved. Both assays incorporate the use of HCV peptides from the Core and non-structural regions of the HCV genome. The microtitre plate assay has a shorter total assay time than current market leaders. It is suitable for use in laboratories, which utilise standard equipment and may be also used where automated systems are preferable. The rapid assay provides a highly sensitive and specific method of HCV antibody detection. This assay may be conducted at point-of-care resulting in a cost-effective test, which could eliminate the time and cost involved in recalling patients to donate a sample for test confirmation, in the event of a positive result on the rapid test. The test does not require sample preparation and may be conducted by untrained personnel. Both the microtitre plate assay and the rapid assay eliminate the preparation steps necessary in the other tests by offering the advantage of safe, ready-to-use assay reagents,.

It is difficult to acknowledge that so much time and effort has been devoted, in recent years, to all aspects of hepatitis C, not least because it is a novel virus, its identification only ten years old. This time has been divided between researching effective treatment options for HCV infection, to HCV genotyping and, in the diagnostic field, developing specific and sensitive tests for antibody detection and HCV-RNA. The current procedures are constantly being improved and updated in an attempt to better the quality of life and the chances of survival of the many people who have fallen victim to this disease. The development of the microtitre plate assay, and the rapid membrane-based assay, has provided an aid to the detection of antibodies to HCV, and is indicative of ongoing research in the hepatitis field.

Section 6-

Conclusion

Conclusion

Hepatitis C virus, HCV, was discovered in 1989 and is the causative agent of the majority of post-transfusion non-A, non-B hepatitis cases (Bradley *et al.*, 1987). The aim of the research undertaken in this study was to develop and evaluate two enzyme immunoassays for the detection of antibodies to HCV in human serum. The first test was in the format of a microtitre plate assay, and was developed for use in clinical laboratories, which utilise either standard laboratory equipment or automated systems. This was followed by the development of a membrane-based assay, which is a simple, and rapid, user-friendly test requiring no laboratory equipment. One of the advantages of the rapid test is that it can be conducted at point-of-care with ready-to-use reagents. Both tests are indirect enzyme immunoassays, incorporating the adhesion of HCV-specific peptide on the solid phase. HCV antibody is detected with anti-human IgG-peroxidase conjugated. The intensity of colour formed, on addition of chromagen, is directly proportional to the quantity of HCV antibody present in the sample.

The evaluation steps, which led to the development of both tests, followed a similar format. A number of peptides, specific for various regions of HCV, was immobilised to the microtitre plate well. They were tested with HCV-positive and negative material, with four peptides subsequently chosen, based on the positive-negative ratio achieved. These four peptides were specific for the core and non-structural regions of the HCV genome. The proceeding steps involved the evaluation of various test parameters such as suitable sample diluent, optimum conjugate titration, suitable conjugate diluent and sample dilution. The optimisation of the rapid test also involved the immobilisation and evaluation of HCV peptides for use on the solid phase membrane. Assay optimisation required less evaluation steps as the reagents were supplied by Trinity Biotech plc. During the course of development of both assays, HCV-positive and HCV-negative samples were used to validate test parameters.

Upon optimisation of both the microtitre plate assay and the rapid assay, the performance of the tests was evaluated. A number of clinical positive and negative samples was tested and the specificity and sensitivity of each assay was obtained. The performance of the microtitre plate assay was determined at Public Health Laboratory

Service (PHLS), Birmingham by analysis of 530 samples. RIBA testing confirmed that 281 samples were found to be anti-HCV negative and 249 were anti-HCV positive. The microtitre plate assay detected 275 negative samples and 233 positive samples, giving a specificity of 97.9% and a sensitivity of 93.6%. The rapid test was evaluated at the Virus Reference Laboratory, University College Dublin (UCD), and also at PHLS, Birmingham. A combined total of 357 negative samples and 394 positive samples were confirmed by RIBA banding. Two false positive and four false negative samples gave an overall specificity and sensitivity of 99.4% and 98.9%, respectively, for the rapid membrane-based assay.

In a separate trial, conducted 'on-site', the plate assay and the rapid assay were compared with two HCV microtitre plate tests from current market leaders, Murex anti-HCV Version III ELISA and General Biologicals Corp. NANBASE C-96 EIA. A total of 75 positive and negative samples, of unknown clinical status, were assayed on all tests. Both the plate assay and the rapid assay correlated well with the results achieved on the competitor assays. Parameters such as total assay time, assay temperature and reagent preparation steps were also evaluated. The microtitre plate assay was shown to have a shorter assay incubation time (75 min.) than both the Murex test (assay time, two hours) and the General Biologicals test (assay time, 90 min.). Also, it was the only assay to supply ready-to-use reagents, requiring no conjugate or substrate preparation. The use of tetramethyl benzidine as a chromagen is favourable over the potentially carcinogenic o-phenylenediamene, used in one of the competitor kits.

In conclusion, the research undertaken in this study resulted in the successful development of a microtitre plate assay and a rapid membrane-based assay for HCV detection. Both tests compare favourably to current market leaders, and whether use is intended for multiple sample screening in the clinical laboratory, or for use at point-of-care, this study provides a sensitive and specific means of detecting antibodies to hepatitis C virus in human serum.

Section 7-

Bibliography

Bibliography

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Section 8-

Appendices

Appendices

Appendix 1.

Absorbance values of 45 Cork blood bank negatives in ELISA test with parameters in section 3.2.5. Represented in Figure 3.3.1a.

Sample	*Absorbance*	*Sample*	*Absorbance*
C1	0.141	**C24**	0.111
C2	0.126	**C25**	0.142
C3	0.070	**C26**	0.077
C4	0.217	**C27**	0.066
C5	0.082	**C28**	0.072
C6	0.068	**C29**	0067
C7	0.110	**C30**	0.059
C8	0.069	**C31**	0.075
C9	0.068	**C32**	0.079
C10	0.065	**C33**	0.095
C11	0.082	**C34**	0.104
C12	0.098	**C35**	0.067
C13	0.085	**C36**	0.066
C14	0.071	**C37**	0.072
C15	0.081	**C38**	0.069
C16	0.143	**C39**	0.107
C17	0.135	**C40**	0.076
C18	0.074	**C41**	0.085
C19	0.110	**C42**	0.097
C20	0.085	**C43**	0.070
C21	0.065	**C44**	0.098
C22	0.105	**C45**	0.073
C23	0.058		

Appendix 2.

Comparison of IEI OD samples tested on new combination and full pool coated at 1/10,000. Represented in Figure 3.4.2a.

Neg.=Negative sample, Ind.=Indeterminate sample

No.	*IEI No.*	*RIBA Band*	*Int.*	*Viral Region(s)*	*New reading*	*Full pool*
1	473	C100, C33	Pos.	NS4, NS3	0.472	0.520
2	678	C33, C22	Pos.	NS3, Core	1.696	0.292
3	682	C33, C22	Pos.	NS3, Core	2.006	0.641
4	687	C33, C22	Pos.	NS3, Core	2.109	1.348
5	694	C33, C22	Pos.	NS3, Core	2.055	0.613
6	696	C33, C22	Pos.	NS3, Core	2.025	0.852
7	699	C33, C22	Pos.	NS3, Core	1.890	0.819
8	700	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	2.141	1.198
9	701	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	1.605	0.482
10	702	C22	Neg/Ind.	Core only	0.612	0.138
11	703	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	2.143	1.237
12	706	C33, C22	Pos.	NS3, Core	2.013	0.646
13	708	C33, C22	Pos.	NS3, Core	1.733	0.173
14	709	C100, C33, C22	Pos.	NS4, NS3, Core	2.001	1.339
15	710	C33, C22	Pos.	NS3, Core	1.983	0.564
16	711	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	2.244	1.290
17	712	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	1.612	0.835
18	713	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	2.372	0.203

No.	IEI No.	RIBA Band	Int.	Viral Region(s)	New reading	Full pool
19	714	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	2.398	0.408
20	717	C100, C33, C22	Pos.	NS4, NS3, Core	2.032	0.822
21	718	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	1.687	0.613
22	719	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	1.670	1.683
23	722	C22	Neg/Ind.	Core only	1.403	0.169
24	725	C100, C33, C22	Pos.	NS4, NS3, Core	0.204	0.081
25	727	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	2.144	1.125
26	728	C33, C22	Pos.	NS3, Core	0.666	0.417
27	729	C100, C33, C22	Pos.	NS4, NS3, Core	2.408	0.900
28	731	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	0.764	0.092
29	736	C33, C22	Pos.	NS3, Core	1.313	0.956
30	738	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	1.921	0.373
31	739	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	2.480	0.430
32	741	C33, C22	Pos.	NS3, Core	2.193	0.225
33	742	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	Over	0.121
34	743	C33, C22	Pos.	NS3, Core	1.556	0.214
35	746	C33, C22	Pos.	NS3, Core	1.856	0.815
36	748	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	1.845	0.286
37	750	C33, C22	Pos.	NS3, Core	1.678	0.171

No.	*IEI No.*	*RIBA Band*	*Int.*	*Viral Region(s)*	*New reading*	*Full pool*
38	753	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	2.040	0.405
39	754	5-1-1, C100, C33, C22	Pos.	NS5, NS4, NS3, Core	1.839	0.211
40	762	C22	Neg/Ind.	Core only	0.586	0.121
41	767	C22	Neg/Ind.	Core only	0.450	0.112
42	779	C100, C33, C22	Pos.	NS4, NS3, Core	1.119	0.670
43	859	C100, C33, C22	Pos.	NS4, NS3, Core	1.383	0.316
44	865	C33, C22	Pos.	NS3, Core	1.552	1.468
45	894	C22	Neg/Ind.	Core only	0.592	0.122
46	903	C22	Neg/Ind.	Core only	0.287	0.123
47	904	C22	Neg/Ind.	Core only	0.107	0.065
48	913	C22	Neg/Ind.	Core only	0.483	0.076
49	935	C22	Neg/Ind.	Core only	0.142	1.103
50	937	C22	Neg/Ind.	Core only	0.535	0.078
51	943	C100, C33	Pos.	NS4, NS3	2.364	0.214
52	944	C22	Neg/Ind.	Core only	0.114	0.087
53	945	C33, C22	Pos.	NS3, Core	0.942	0.303
54	946	C100, C33, C22	Pos.	NS4, NS3, Core	1.649	0.845
55	947	C22	Neg/Ind.	Core only	0.133	0.882
56	950	C33, C22	Pos.	NS3, Core	1.875	0.894
57	951	C33, C22	Pos.	NS3, Core	1.632	0.348
58	953	C22	Neg/Ind.	Core only	0.477	0.102
59	958	C33, C22	Pos.	NS3, Core	0.198	0.082
60	959	C22	Neg/Ind.	Core only	0.189	0.060
61	961	C33, C22	Pos.	NS3, Core	1.934	1.589
62	963	C33, C22, 5-1-1	Pos.	NS3, Core, NS5	2.144	0.748

No.	IEI No.	RIBA Band	Int.	Viral Region(s)	New reading	Full pool
63	981	C33, C22	Pos.	NS3, Core	0.201	0.190
64	985	C22	Neg/Ind.	Core only	0.237	0.095
65	991	C22	Neg/Ind.	Core only	0.296	0.133
66	992	C22	Neg/Ind.	Core only	0.154	0.083
67	993	C22	Neg/Ind.	Core only	0.503	0.164
68	995	C33, C22	Pos.	NS3, Core	1.673	0.916
69	996	C100, C33, C22	Pos.	NS4, NS3, Core	0.902	0.112
70	997	C22	Neg/Ind.	Core only	0.519	0.158
71	998	C22	Neg/Ind.	Core only	0.385	0.197
72	999	C22	Neg/Ind.	Core only	0.135	0.091
73	1013	C22	Neg/Ind.	Core only	0.739	0.114
74	1025	C22	Neg/Ind.	Core only	0.307	0.134
75	1039	C22	Neg/Ind.	Core only	1.110	0.194
76	1053	C100, C33, C22, 5-1-1	Pos.	NS4, NS3, Core, NS5	1.989	0.619
77	1055	C22	Neg/Ind.	Core only	0.179	0.090
78	1059	C22	Neg/Ind.	Core only	0.415	0.129
79	1070	C33	Neg/Ind.	NS3 only	0.576	0.085
80	1072	C22	Neg/Ind.	Core only	0.827	0.282
81	1076	C22	Neg/Ind.	Core only	0.192	0.074
82	1077	C33	Neg/Ind.	NS3 only	0.761	0.074
83	1081	C22	Neg/Ind.	Core only	1.412	0.220
84	1086	C22	Neg/Ind.	Core only	0.371	0.116
85	1110	C22	Neg/Ind.	Core only	0.495	0.287
86	1112	C22	Neg/Ind.	Core only	0.236	0.113
87	1115	C33, C22	Pos.	NS3, Core	0.463	0.108
88	1265	C33	Neg/Ind.	NS3 only	0.571	0.263

Appendix 3.

Absorbance values of a number of IEI positive and Cork negative samples on ELISA solid phase with directly coated combinations 1 to 6. Represented in Figure 3.5.2a.

Sample	*Region*	*1*	*2*	*3*	*4*	*5*	*6*
678	NS3, Core	2.216	1.554	2.122	2.002	2.221	1.961
682	NS3, Core	1.709	1.945	Over	2.387	Over	1.629
687	NS3, Core	1.745	1.423	2.461	2.211	2.076	1.804
694	NS3, Core	2.115	1.945	Over	2.234	2.338	1.881
696	NS3, Core	2.181	2.053	2.442	2.311	2.295	2.221
699	NS3, Core	2.323	2.061	Over	2.354	2.421	2.281
700	NS3, Core, NS4	2.347	2.003	2.406	2.189	2.338	2.198
701	NS3, Core, NS4	1.251	1.041	1.591	1.574	1.842	1.096
703	NS3, Core, NS4	2.046	2.447	2.442	2.459	Over	Over
706	NS3, Core	2.163	1.971	Over	2.311	2.385	2.255
708	NS3, Core	1.944	1.911	Over	2.201	2.421	2.438
709	NS3, Core, NS4	2.463	Over	Over	Over	Over	Over
710	NS3, Core	1.574	2.029	1.574	2.281	1.029	2.353
711	NS3, Core, NS4	1.937	2.094	Over	Over	1.744	2.131
712	NS3, Core, NS4	2.016	1.838	Over	2.254	2.323	2.172
713	NS3, Core, NS4	1.981	1.993	2.341	2.173	2.338	2.182
714	NS3, Core, NS4	1.573	2.331	2.341	2.308	2.267	2.322
717	NS3, Core, NS4	2.145	2.149	2.407	2.323	2.405	2.462
718	NS3, Core, NS4	2.481	1.767	2.032	2.087	2.371	2.069
719	NS3, Core, NS4	2.145	1.682	2.063	2.105	2.113	1.591
729	NS3, Core, NS4	2.113	2.248	2.296	2.388	2.424	2.442
731	NS3, Core, NS4	2.098	1.973	Over	Over	Over	2.386
727	NS3, Core, NS4	2.055	2.085	Over	1.922	2.086	2.052
736	NS3, Core	1.164	0.364	1.322	1.053	1.149	1.017
738	NS3, Core, NS4	2.287	2.173	2.455	Over	1.608	1.722
739	NS3, Core, NS4	2.002	2.342	2.317	Over	2.481	2.452
741	NS3, Core	1.655	2.159	Over	2.454	2.481	2.452
742	NS3, Core, NS4	1.365	2.031	2.262	2.167	2.378	2.334
743	NS3, Core	1.097	0.951	2.115	1.651	1.976	1.226
746	NS3, Core	2.035	1.795	2.484	2.482	Over	2.378

Sample	Region	1	2	3	4	5	6
748	NS3, Core, NS4	1.771	0.847	1.655	1.268	1.331	1.217
750	NS3, Core	1.928	1.409	1.979	2.035	2.402	2.044
753	NS3, Core, NS4	1.333	1.282	2.197	2.025	2.077	1.722
754	NS3, Core, NS4	2.035	1.948	2.484	2.316	2.427	2.355
859	NS3, Core, NS4	1.213	0.537	1.682	1.081	1.537	0.988
865	NS3, Core	2.053	1.551	2.359	2.127	2.295	1.764
935	Core only	0.225	0.196	0.191	0.209	0.248	0.141
937	Core only	0.191	0.185	0.724	0.296	0.355	0.242
943	NS4, Core	1.743	2.171	2.476	2.389	2.267	Over
944	Core only	0.122	0.139	0.503	0.157	0.239	0.137
945	NS3, Core	1.533	2.153	2.194	2.474	Over	2.491
950	NS3, Core	2.214	1.904	Over	2.234	2.331	2.135
951	NS3, Core	0.702	0.532	1.463	1.125	1.338	1.061
958	NS3, Core	1.841	1.981	Over	2.494	Over	Over
959	Core only	0.237	0.184	0.199	0.264	0.261	0.196
961	NS3, Core	0.246	0.251	0.495	0.404	0.298	0.241
981	NS3, Core	1.968	1.523	1.946	2.088	2.267	2.014
993	Core only	0.446	0.231	1.331	0.311	0.691	0.471
995	NS3, Core	0.831	2.091	1.520	1.597	1.982	2.443
996	NS3, Core, NS4	0.411	0.426	0.782	0.578	0.673	0.357
997	Core only	0.256	0.381	0.385	0.279	0.503	0.473
998	Core only	0.191	0.215	0.547	0.279	0.437	0.208
999	Core only	0.135	0.149	0.108	0.199	0.192	0.168
1112	Core only	1.608	0.562	1.521	0.997	1.461	0.373
C1	Negative	0.215	0.236	0.204	0.197	0.186	0.198
C2	Negative	0.222	0.185	0.288	0.262	0.202	0.251
C3	Negative	0.264	0.222	0.221	0.253	0.212	0.257
C4	Negative	0.262	0.251	0.282	0.298	0.243	0.276
C5	Negative	0.216	0.123	0.211	0.209	0.206	0.222
C6	Negative	0.199	0.142	0.279	0.144	0.129	0.163
C7	Negative	0.206	0.237	0.238	0.204	0.195	0.227
C8	Negative	0.188	0.075	0.188	0.176	0.168	0.169
C9	Negative	0.178	0.186	0.162	0.181	0.151	0.164
C10	Negative	0.194	0.229	0.166	0.158	0.158	0.185

Sample	Region	1	2	3	4	5	6
C11	Negative	0.257	0.169	0.175	0.163	0.161	0.177
C12	Negative	0.209	0.136	0.159	0.185	0.156	0.168
C13	Negative	0.183	0.137	0.198	0.175	0.172	0.186
C14	Negative	0.273	0.251	0.281	0.205	0.201	0.279
C15	Negative	0.161	0.128	0.178	0.171	0.157	0.175
C16	Negative	0.251	0.174	0.251	0.262	0.245	0.255
C17	Negative	0.187	0.096	0.186	0.204	0.172	0.189
C18	Negative	0.239	0.107	0.121	0.176	0.168	0.173
C19	Negative	0.184	0.115	0.166	0.186	0.145	0.146
C20	Negative	0.179	0.141	0.158	0.163	0.164	0.157

Appendix 4.

Absorbance values of IEI samples on conjugated and directly coated peptide for final evaluation of HCV ELISA. Represented in Figure 3.6.1a.

No.	IEI Lot No.	Int.	Viral Region(s)	Conjugated Peptide	Directly Coated
1	473	Pos.	NS4, NS3	0.472	0.598
2	678	Pos.	NS3, Core	1.696	1.669
3	682	Pos.	NS3, Core	2.006	2.332
4	687	Pos.	NS3, Core	2.109	2.057
5	694	Pos.	NS3, Core	2.055	2.282
6	696	Pos.	NS3, Core	2.025	2.459
7	699	Pos.	NS3, Core	1.890	2.499
8	700	Pos.	NS5, NS4, NS3, Core	2.141	2.387
9	701	Pos.	NS5, NS4, NS3, Core	1.605	1.257
10	702	Neg./Ind.	Core only	0.612	0.432
11	703	Pos.	NS5, NS4, NS3, Core	2.143	2.387
12	706	Pos.	NS3, Core	2.013	2.331
13	708	Pos.	NS3, Core	1.733	2.150

No.	IEI Lot No.	Int.	Viral Region(s)	Conjugated Peptide	Directly Coated
14	709	Pos.	NS4, NS3, Core	2.001	Over
15	710	Pos.	NS3, Core	1.983	2.046
16	711	Pos.	NS5, NS4, NS3, Core	2.244	2.338
17	712	Pos.	NS5, NS4, NS3, Core	1.612	2.145
18	713	Pos.	NS5, NS4, NS3, Core	2.372	2.168
19	714	Pos.	NS5, NS4, NS3, Core	2.398	2.274
20	717	Pos.	NS4, NS3, Core	2.032	2.168
21	718	Pos.	NS5, NS4, NS3, Core	1.687	2.055
22	719	Pos.	NS5, NS4, NS3, Core	1.670	1.917
23	722	Neg./Ind.	Core only	1.403	0.465
24	725	Pos.	NS4, NS3, Core	0.204	1.234
25	727	Pos.	NS5, NS4, NS3, Core	2.144	2.168
26	728	Pos.	NS3, Core	0.666	1.320
27	729	Pos.	NS4, NS3, Core	2.408	2.435
28	731	Pos.	NS5, NS4, NS3, Core	0.764	2.435
29	736	Pos.	NS3, Core	1.313	1.810
30	738	Pos.	NS5, NS4, NS3, Core	1.921	2.170
31	739	Pos.	NS5, NS4, NS3, Core	2.480	2.428
32	741	Pos.	NS3, Core	2.193	2.155
33	742	Pos.	NS5, NS4, NS3, Core	Over	2.155
34	743	Pos.	NS3, Core	1.556	1.942
35	746	Pos.	NS3, Core	1.856	2.114
36	748	Pos.	NS5, NS4, NS3, Core	1.845	1.129
37	750	Pos.	NS3, Core	1.678	2.101
38	753	Pos.	NS5, NS4, NS3, Core	2.040	1.579
39	754	Pos.	NS5, NS4, NS3, Core	1.839	2.155
40	762	Neg./Ind.	Core only	0.586	0.489
41	767	Neg./Ind.	Core only	0.450	0.313
42	779	Pos.	NS4, NS3, Core	1.119	2.196
43	859	Pos.	NS4, NS3, Core	1.383	1.162

No.	IEI Lot No.	Int.	Viral Region(s)	Conjugated Peptide	Directly Coated
44	865	Pos.	NS3, Core	1.552	2.016
45	894	Neg./Ind.	Core only	0.592	0.459
46	903	Neg./Ind.	Core only	0.287	0.362
47	904	Neg./Ind.	Core only	0.107	0.458
48	913	Neg./Ind.	Core only	0.483	0.426
49	935	Neg./Ind.	Core only	0.142	0.341
50	937	Neg./Ind.	Core only	0.535	0.231
51	943	Pos.	NS4, NS3	2.364	2.317
52	944	Neg./Ind.	Core only	0.114	0.114
53	945	Pos.	NS3, Core	0.942	2.220
54	946	Pos.	NS4, NS3, Core	1.649	2.141
55	947	Neg./Ind.	Core only	0.133	0.231
56	950	Pos.	NS3, Core	1.875	0.661
57	951	Pos.	NS3, Core	1.632	1.134
58	953	Neg./Ind.	Core only	0.477	0.323
59	958	Pos.	NS3, Core	0.198	2.462
60	959	Neg./Ind.	Core only	0.189	0.147
61	961	Pos.	NS3, Core	1.934	1.814
62	963	Pos.	NS3, Core, NS5	2.144	2.145
63	981	Pos.	NS3, Core	0.201	2.056
64	985	Neg./Ind.	Core only	0.237	1.340
65	991	Neg./Ind.	Core only	0.296	0.354
66	992	Neg./Ind.	Core only	0.154	0.189
67	993	Neg./Ind.	Core only	0.503	0.620
68	995	Pos.	NS3, Core	1.673	2.100
69	996	Pos.	NS4, NS3, Core	0.902	0.494
70	997	Neg./Ind.	Core only	0.519	0.522
71	998	Neg./Ind.	Core only	0.385	0.440
72	999	Neg./Ind.	Core only	0.135	0.256
73	1013	Neg./Ind.	Core only	0.739	0.597

No.	IEI Lot No.	Int.	Viral Region(s)	Conjugated Peptide	Directly Coated
74	1025	Neg./Ind.	Core only	0.307	0.256
75	1039	Neg./Ind.	Core only	1.110	0.911
76	1053	Pos.	NS4, NS3, Core, NS5	1.989	1.519
77	1055	Neg./Ind.	Core only	0.179	0.398
78	1059	Neg./Ind.	Core only	0.415	0.589
79	1070	Neg./Ind.	NS3 only	0.576	0.356
80	1072	Neg./Ind.	Core only	0.827	0.697
81	1076	Neg./Ind.	Core only	0.192	0.418
82	1077	Neg./Ind.	NS3 only	0.761	0.269
83	1081	Neg./Ind.	Core only	1.412	0.894
84	1086	Neg./Ind.	Core only	0.371	0.258
85	1110	Neg./Ind.	Core only	0.495	0.564
86	1112	Neg./Ind.	Core only	0.236	1.203
87	1115	Pos.	NS3, Core	0.463	0.958
88	1265	Neg./Ind.	NS3 only	0.571	0.212

Appendix 5.

Absorbance values of negative samples assayed on directly coated and conjugated peptide for final evaluation of HCV ELISA. Represented in Figure 3.6.1b.

Sample	Conjugated Peptide	Directly Coated	Sample	Conjugated Peptide	Directly Coated
C1	0.151	0.106	*C9*	0.133	0.095
C2	0.174	0.076	*C10*	0.127	0.070
C3	0.153	0.106	*C11*	0.099	0.061
C4	0.256	0.122	*C12*	0.139	0.088
C5	0.190	0.111	*C13*	0.160	0.118
C6	0.103	0.062	*C14*	0.182	0.090
C7	0.147	0.083	*C15*	0.150	0.074
C8	0.148	0.087	*C16*	0.232	0.147

Sample	Conjugated Peptide	Directly Coated	Sample	Conjugated Peptide	Directly Coated
C17	0.167	0.072	*C50*	0.175	0.069
C18	0.136	0.090	*C51*	0.166	0.078
C19	0.149	0.082	*C52*	0.241	0.132
C20	0.127	0.068	*C53*	0.152	0.084
C21	0.113	0.057	*C54*	0.147	0.093
C22	0.122	0.062	*C55*	0.198	0.104
C23	0.143	0.101	*C56*	0.213	0.109
C24	0.153	0.073	*C57*	0.154	0.051
C25	0.184	0.124	*C58*	0.189	0.120
C26	0.143	0.111	*C59*	0.222	0.099
C27	0.165	0.108	*C60*	0.135	0.048
C28	0.163	0.095	*C61*	0.164	0.101
C29	0.116	0.057	*C62*	0.192	0.122
C30	0.172	0.087	*C63*	0.178	0.083
C31	0.187	0.101	*C64*	0.155	0.085
C32	0.132	0.065	*C65*	0.163	0.076
C33	0.198	0.102	*C66*	0.178	0.056
C34	0.212	0.132	*C67*	0.215	0.118
C35	0.102	0.059	*C68*	0.159	0.087
C36	0.155	0.078	*C69*	0.137	0.059
C37	0.111	0.046	*C70*	0.164	0.097
C38	0.141	0.068	*C71*	0.196	0.088
C39	0.165	0.116	C72	0.144	0.081
C40	0.125	0.063	C73	0.172	0.069
C41	0.194	0.089	C74	0.219	0.108
C42	0.191	0.072	C75	0.233	0.117
C43	0.185	0.069	C76	0.121	0.058
C44	0.197	0.113	C77	0.148	0.047
C45	0.181	0.088	C78	0.193	0.071
C46	0.201	0.123	C79	0.127	0.065
C47	0.165	0.084	C80	0.099	0.049
C48	0.132	0.057	C81	0.158	0.096
C49	0.206	0.114	C82	0.166	0.059

Sample	*Conjugated Peptide*	*Directly Coated*	*Sample*	*Conjugated Peptide*	*Directly Coated*
C83	0.209	0.131	**C89**	0.184	0.081
C84	0.189	0.092	**C90**	0.216	0.094
C85	0.158	0.107	**C91**	0.167	0.057
C86	0.167	0.059	**C92**	0.129	0.068
C87	0.210	0.140	**C93**	0.236	0.119
C88	0.181	0.115			

Appendix 6.

Details of the procedural protocols of the HCV ELISA and rapid competitors, showing assay incubation times, temperatures and reagent preparation necessary. Discussed in section 5.4.

HCV ELISA competitors

Test	*Number of incubations*	*Reagent preparation required*	*Assay time*	*Sample medium*	*Sample volume*	*Performance data/comments*
① Abbott Diagnostics HCV EIA 3.0	3	Conjugate and substrate	2 hours	Serum or plasma	10µls	Incubation temperature at 40°C. Bead assay. No performance data.
②General Biologicals Corp. NANBASE C-96 anti-HCV EIA	3	Conjugate, substrate and wash	1 ½ hours	Serum or plasma	10µls	Pre-dilution of sample necessary. Humidified incubations.
③ General Biologicals Corp. NANBDINE^{125} C anti-HCV RIA	3	Conjugate and wash	2 hours	Serum or plasma	10µls	Radioactive label has safety implications.
④INNOGENETICS INNOTEST HCV Ab III	3	Conjugate and wash	2 ½ hours	Serum or plasma	10µls	Performance data given. 3rd generation assay.
⑤Murex anti-HCV (Version III)	3	Conjugate, substrate and wash	2 hours	Serum or plasma	20µls	Good performance data available. Colour on sample addition.
⑥Ortho Chiron HCV 3.0 ELISA	3	Wash and substrate	2 ½ hours	Serum or plasma	10µls	Heat treated samples cannot be used. No performance data given.
⑦Sanofi Diagnostics MONOLISA® anti-HCV EIA	3	Wash and substrate	2 hours	Serum	20µls	No performance data given.
⑧Syntron Bioresearch Inc., Microwell HCV EIA	2	Wash	1 ¼ hours	Serum or plasma	10µls	No performance data given. 2nd generation test not as sensitive.
⑨United Biomedical HCV EIA	3	Conjugate, substrate and wash	1 hour	Serum or plasma	15µls	Heat treated samples cannot be used.

HCV Rapid competitors

Test	*Number of incubations*	*Reagent preparation required*	*Assay time*	*Sample medium*	*Sample volume*	*Performance data/comments*
① Bionike Laboratories	1	None	10 min.	Serum or plasma	1 drop	Product not yet launched.
② Genelabs Diagnostics	6	None	5 min.	Serum or plasma	1 drop	Multiple steps. Performance data given
③ SERO-Med® Rapid Test	4	None	5 min.	Serum or whole blood	1 drop	Fresh samples necessary.

Appendix 7.

Comparison of both assays, developed in this research, with Murex anti-HCV Version III EIA and General Biologicals Corp. NANBASE C-96 EIA. A total of 75 samples of unknown status were assayed. Discussed in section 5.4.

Sample Number	Murex	General Biologicals	ELISA	SeroCard	Test correlated	Sample Number	Murex	General Biologicals	ELISA	SeroCard	Test correlated
1	Pos.	Pos.	Pos.	Ind.	ELISA	**15**	Pos.	Pos.	Pos.	Pos.	Both
2	Pos.	Neg.	Pos.	Neg.	Ind.	**16**	Pos.	Pos.	Pos.	Pos.	Both
3	Neg.	Neg.	Pos.	Neg.	SeroCard	**17**	Pos.	Pos.	Pos.	Pos.	Both
4	Pos.	Pos.	Pos.	Pos.	Both	**18**	Pos.	Pos.	Pos.	Pos.	Both
5	Neg.	Neg.	Pos.	Ind.	None	**19**	Pos.	Pos.	Pos.	Pos.	Both
6	Pos.	Pos.	Pos.	Pos.	Both	**20**	Pos.	Pos.	Pos.	Pos.	Both
7	Pos.	Pos.	Pos.	Pos.	Both	**21**	Pos.	Pos.	Pos.	Pos.	Both
8	Pos.	Pos.	Pos.	Pos.	Both	**22**	Pos.	Pos.	Pos.	Pos.	Both
9	Pos.	Pos.	Pos.	Pos.	Both	**23**	Pos.	Pos.	Pos.	Neg.	ELISA
10	Pos.	Pos.	Pos.	Pos.	Both	**24**	Pos.	Pos.	Pos.	Pos.	Both
11	Pos.	Pos.	Pos.	Pos.	Both	**25**	Neg.	Neg.	Neg.	Neg.	Both
12	Pos.	Pos.	Pos.	Pos.	Both	**26**	Pos.	Pos.	Pos.	Pos.	Both
13	Pos.	Pos.	Pos.	Pos.	Both	**27**	Pos.	Pos.	Pos.	Pos.	Both
14	Pos.	Pos.	Pos.	Pos.	Both	**28**	Pos.	Pos.	Pos.	Pos.	Both

Sample Number	Murex	General Biologicals	ELISA	SeroCard	Test correlated
29	Pos.	Pos.	Pos.	Pos.	Both
30	Pos.	Pos.	Pos.	Pos.	Both
31	Pos.	Pos.	Pos.	Pos.	Both
32	Neg.	Neg.	Neg.	Neg.	Both
33	Pos.	Pos.	Pos.	Pos.	Both
34	Pos.	Pos.	Pos.	Pos.	Both
35	Pos.	Pos.	Pos.	Pos.	Both
36	Pos.	Pos.	Pos.	Pos.	Both
37	Pos.	Pos.	Pos.	Pos.	Both
38	Pos.	Pos.	Pos.	Pos.	Both
39	Neg.	Neg.	Neg.	Neg.	Both
40	Pos.	Pos.	Pos.	Pos.	Both
41	Pos.	Neg.	Neg.	Neg.	Both
42	Neg.	Neg.	Neg.	Neg.	Both
43	Neg.	Neg.	Neg.	Neg.	Both
44	Pos.	Pos.	Pos.	Pos.	Both
45	Neg.	Neg.	Neg.	Neg.	Both
46	Pos.	Pos.	Pos.	Pos.	Both

Sample Number	Murex	General Biologicals	ELISA	SeroCard	Test correlated
47	Pos.	Pos.	Pos.	Pos.	Both
48	Neg.	Neg.	Neg.	Neg.	Both
49	Neg.	Neg.	Neg.	Neg.	Both
50	Neg.	Neg.	Pos.	Ind.	None
51	Pos.	Neg.	Neg.	Neg.	Both
52	Pos.	Pos.	Pos.	Pos.	Both
53	Neg.	Neg.	Neg.	Neg.	Both
54	Pos.	Pos.	Pos.	Pos.	Both
55	Pos.	Pos.	Pos.	Pos.	Both
56	Pos.	Pos.	Pos.	Pos.	Both
57	Pos.	Pos.	Pos.	Pos.	Both
58	Pos.	Pos.	Pos.	Pos.	Both
59	Neg.	Neg.	Neg.	Neg.	Both
60	Neg.	Pos.	Neg.	Neg.	Both
61	Neg.	Neg.	Neg.	Neg.	Both
62	Neg.	Pos.	Neg.	Neg.	Both
63	Neg.	Neg.	Neg.	Neg.	Both
64	Neg.	Neg.	Neg.	Neg.	Both

Sample Number	Murex	General Biologicals	ELISA	SeroCard	Test correlated	Sample Number
65	Neg.	Neg.	Neg.	Neg.	Both	**71**
66	Neg.	Neg.	Neg.	Neg.	Both	**72**
67	Neg.	Pos.	Neg.	Neg.	Both	**73**
68	Neg.	Neg.	Neg.	Neg.	Both	**74**
69	Neg.	Neg.	Neg.	Neg.	Both	**75**
70	Neg.	Neg.	Neg.	Neg.	Both	

Murex	General Biologicals	ELISA	SeroCard	Test correlated
Neg.	Neg.	Neg.	Neg.	Both
Neg.	Neg.	Neg.	Neg.	Both
Neg.	Neg.	Neg.	Neg.	Both
Neg.	Neg.	Neg.	Neg.	Both
Neg.	Neg.	Neg.	Neg.	Both