Development and application of nucleic acid amplification technology (NAT) for the detection of viruses in donated blood

By

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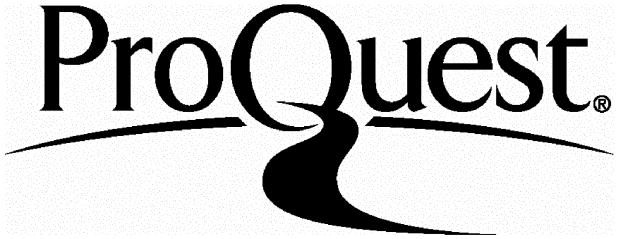

Abstract

Background and Objectives: To reduce the potential for transmission of hepatitis C virus (HCV) from an RNA positive, anti-HCV negative blood donation, the National Blood Service (NBS) decided to introduce nucleic acid amplification technology (NAT) testing of blood donations for HCV in England and Wales. The objective of this thesis was to develop an automated assay using commercial components for the detection of HCV RNA in blood donations for transfusion, and to validate sample handling and storage procedures.

Studies included in the thesis: The stability of HCV in the plasma sample was investigated to define the appropriate sample handling and storage conditions needed to preserve HCV RNA in the blood sample before analysis by NAT. HCV RNA was found to be more stable than previously thought, with HCV levels remaining stable for 120 hours in un-separated whole blood at refrigerated or room temperature. The compatibility of the Qiagen and Roche reagents were tested using manual assays and automated systems. This work has shown that it is possible to completely automate HCV NAT screening. Alternative techniques for virus detection such as TMA and HCV core antigen were also investigated and PCR was found superior to antigen and equivalent to TMA. The window period of the HCV infection was further defined in a study of window phase HCV samples from US plasma donors.

Outcome: The combination of methods developed and described in this thesis were successfully introduced into routine use within the NBS from 1999 and are now used for release of blood components with a shelf life of greater than 24 hours in England and Wales. From 1999 to the end of 2002 approximately nine million donations were tested in England and Wales of which seven were HCV infected window phase units, which would have otherwise been transfused. In addition 394 NAT positive samples were found which were also detected by anti-HCV screening tests.

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Finally I would especially like to thank my wife Cate, who not only was involved in much of the work described in this thesis but also for helpful advice and proof reading during the preparation of this manuscript.

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List of abbreviations

A	absorbance
ACD	acid citrate dextrose
AIDS	acquired immune deficiency syndrome
AL	Qiagen lysis buffer
ALT	alanine aminotransferase
AMV	avian myoblastoma virus
AST	aspartase aminotransferase
Av-HRP	avidin - horse radish peroxidase conjugate
AVL	Qiagen viral lysis buffer
AW	Qiagen wash buffer
AZT	3′-azido-3′-deoxythymidine
bDNA	branched deoxyribonucleic acid
BMV	brome mosaic virus
bp	base pair
BPL	Bio Products Laboratory
BVDV	Bovine diarrhoea virus
CBER	Center for Biologics Evaluation and Research
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CI	confidence interval
CMV	cytomegalovirus
CPDA-1	citrate phosphate dextrose adenine-one (anticoagulant)
cpm	counts per minute
CPMP	Committee for Proprietary Medicinal Products
cps	counts per second
CV	coefficient of variation
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphates
DROG	donor recipient outcome group (NBS)
DTT	dithiothreitol
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetate
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
ELONA	enzyme-linked oligonucleotide assay
EPP	exposure prone procedure
FDA	Food and Drug Administration
FFP	fresh-frozen plasma
FRET	fluorescence resonance energy transfer
g	gram
g	gravity
GVHD	graft-versus-host disease
HAART	highly active antiretroviral therapy
HAV	hepatitis A virus
HBIG	Hepatitis B immunoglobulin
HBc	hepatitis B core antigen
HBsAg	hepatitis B surface antigen

HBV	hepatitis B virus
HCV	hepatitis C virus
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HTLV-I	human T-lymphotropic virus type I
HTLV-II	human T-lymphotropic virus type II
HVR	hyper variable region
IC	Internal control
IFN	interferon
Ig	immunoglobulin
IND	investigational new drug
IRES	internal ribosome entry site
ISDR	Interferon sensitivity determining region
IU	International Unit
IVDU	Intravenous drug user
kb	kilobase
kDa	kilodalton
L	litre
LAN	local area network
LAV	lymphadenopathy associated virus
LCR	ligase chain reaction
LDL	low density lipoprotein
m	metre
M	molar
mA	milli ampere
MHC	major histocompatibility complex
min	minute
MAb	monoclonal antibody
mol	mole
MRC	medical research council
mRNA	messenger RNA
MWP	microwell plate
n	number
NA	not applicable
NAT	nucleic acid amplification technology
NANB	Non A non B hepatitis
NaOH	Sodium hydroxide
NBA	National blood authority
NBS	National blood service
NCR	noncoding region
NGI	National Genetics Institute
NHIG	normal human immunoglobulin
NIBSC	National Institute of Biological Standards and Control
NIH	National Institutes of Health
NJ	neighbour joining
NLBTC	North London blood transfusion centre
NRTI	nucleoside analogue reverse transcriptase inhibitor
NS	non structural
NT	not tested
OD	optical density

OPD	o-phenylenediamine
p	probability
PBMC(s)	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
PC	personal computer
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	Paul Ehrlich Institute
pH	negative logarithm of hydrogen ion activity
PHLS	public health laboratory service
PPT	plasma preparation tube
PTP	post transfusion purpura
QC	quality control
qPCR	quantitative polymerase chain reaction
r	correlation coefficient
RFLP	restriction fragment length polymorphism
RIA	radioimmunoassay
RIBA	recombinant immunoblot assay
RNA	ribonucleic acid
rpm	revolutions per minute
RSP	robotic sample processor
RT	reverse transcriptase
RT- PCR	reverse transcriptase- polymerase chain reaction
R*Tth* pol	recombinant *Thermus thermophilus* polymerase
SD	standard deviation
S/D	solvent/detergent
SH	serum hepatitis
SHOT	serious hazards of transfusion
SVR	sustained virological response
Taq	*Thermus aquaticus*
TRALI	transfusion-related acute lung injury
Tris	tris(hydroxyethyl) aminomethane
TMA	Transcription-mediated amplification
TMB	3,3′,5,5'-tetramethylbenzidine
TTA	tris tween azide
TTI	transfusion transmitted infection
UCL	University College London
UK	United Kingdom
UNG	uracil-N-glycosylase
US	United States
UV	ultraviolet
v-CJD	Variant Creutzfeldt-Jakob disease
VLDL	very low density lipoprotein
VZIG	Varicella zoster immunoglobulin
WHO	World Health Organization

Collaborative studies included in this thesis

While most of the work presented in this thesis is my own, some collaborative studies have been included where the work of others is presented. These studies are as follows:

Chapter 3.1 Storage of Whole Blood:
The studies described here were carried out in collaboration with NLBTC where Dr. Alan Kitchen performed blood storage and sampling.

Chapter 3.2 Storage in Minipools:
This study was performed in collaboration with BPL where Dr. Cate Sims carried out the storage and sampling.

Chapter 3.3 Freeze thaw studies:
This study was performed in collaboration with the Brentwood blood centre where Steve Tassen carried out the freeze thaw cycling and sampling.

Chapter 6: Results of NBS HCV NAT screening:
The data presented in this chapter are from the NBS NAT laboratories, however the analysis, including the writing of MS Excel macros was performed by myself. The ethanol contamination data presented in Figure 6.10 were provided by Dr. Cate Sims. The quantitative PCR, genotyping and antigen results for the window phase samples presented in Table 6.1 were performed by myself, but anti-HCV results were provided by Dr. Pat Hewitt.

List of publications arising from this thesis

Grant PR, Kitchen A, Barbara JA, Hewitt P, Sims CM, Garson JA, Tedder RS: Effects of handling and storage of blood on the stability of hepatitis C virus RNA: Implications for NAT testing in transfusion practice. Vox Sang 2000; 78:137-142

Grant PR, Sims CM, Krieg-Schneider F, Love EM, Eglin R, Tedder RS: Automated screening of blood donations for hepatitis C virus RNA using the Qiagen BioRobot 9604 and the Roche COBAS HCV Amplicor assay. Vox Sang 2002; 82:169-176

Grant PR, Sims CM, Tedder RS: Quantification of HCV RNA levels and detection of core antigen in donations before seroconversion. Transfusion 2002; 42:1032-1036

Grant PR, Busch MP: Nucleic acid amplification technology methods used in blood donor screening. Transfusion Medicine 2002; 12:229-242

Chapter 1

Introduction

1.1 Blood Transfusion

1.1.1 Development of blood transfusion, a historical perspective

The first blood transfusions in man were documented in the 17th century. In 1667 Jean-Baptiste Denis in France and Richard Lower and Edmund King in England transfused the blood from a sheep and a calf into men as a cure for madness (King and Lower, 1667). A few years later after several deaths the practice was banned throughout most of Europe. Transfusions in humans were not carried out for another 150 years.

In the early 19th century James Blundell an obstetrician at Guy's and St. Thomas's hospitals in London noted the high mortality rate from haemorrhage in women giving birth and thought about replacing the lost blood. He was also against the idea of transfusing animal blood into humans, thinking that only human blood should be given to humans. In December 1818 he carried out a blood transfusion in a patient with internal bleeding using blood collected from several assistants (Blundell, 1818). Although the patient later died, Blundell carried out transfusions in other patients of which around half survived, and his efforts led to a revival of interest in the technique.

The first major advance came at the beginning of the 20th century when an Austrian researcher, Karl Landsteiner, noticed that agglutination of the red cells would sometimes occur when mixing samples of blood in test tubes. In 1901 he published his observations of the three blood groups A, B and O (Landsteiner, 1901). The plasma from group A would cause the red cells of group B to agglutinate and vice versa, but neither would cause the cells from group O to agglutinate. A year later his colleagues discovered a fourth group that they called

AB, this reacted with plasma from both groups A and B (Von Decastello and Sturli, 1902). Landsteiner's blood grouping system was largely ignored for many years but he was later recognised for his discovery when in 1930 he was awarded the Nobel Prize for Medicine. Landsteiner later discovered the rhesus blood group (Rh) in 1940.

In the early 1900's blood transfusions were carried out directly by stitching the blood vessels between donor and recipient together, but later the technique was improved by the use of needles and rubber tubing with pumping apparatus. A major problem with this method was blood clotting, which occurred after around three to five minutes, blocking the tubing and preventing further transfusion. It was not until 1915 that the American physician Richard Lewisohn published the results of his experiments with the anticoagulant sodium citrate (Lewisohn, 1915). The anticoagulant properties of sodium citrate had been described in 1914 but at concentrations that were toxic in humans. Lewisohn had experimented with the concentration and found that a 0.2% solution of sodium citrate prevented clotting and was not toxic to the patient. However it was noted that febrile reactions occurred in around 10% of transfused patients and this was thought to be caused by the citrate anticoagulant. It was several years before the cause of these reactions was found to be bacterial endotoxins remaining after autoclaving of solutions and equipment (Seibert, 1923).

A year after Lewisohn described sodium citrate Rous and Turner noted that haemolysis of the red cells would occur after a week in the anticoagulant. However the addition of a dextrose solution to the anticoagulated blood would keep the red cells intact for up to four weeks (Rous and Turner, 1916a; Rous and Turner, 1916b).

Even with the new anticoagulant storage solutions transfusions were still carried out with the donor and the recipient present. Percy Oliver had set up a panel of donors in London in the 1920's, the donors were given a physical exam and tested for blood type and syphilis. The organisation was called the Greater London Red Cross Blood Transfusion Service. When a patient in a hospital needed a transfusion a donor was called into the hospital, bled into a bottle

containing anticoagulant and this blood was then transfused into the patient. This arrangement continued until World War II when it was realised that more blood would be needed to treat the expected number of casualties. Four blood banks were set up on the outskirts of London where blood was collected and stored in refrigerators. Later the MRC estimated that the four centres collected and distributed over 68 500 gallons of blood during the entire course of the war.

It was also during World War II that the transfusion of blood components, rather than whole blood became common. Doctors had noted during World War I that patients could die from shock due to the collapse of the circulatory system even after modest losses of blood. Experiments had been performed to try to increase the blood volume with artificial substitutes before it was shown that plasma could be used effectively to treat these patients. With America set to enter World War II it was clear that whole blood could not survive the journey across the Atlantic, but it was noted that when blood was stored for several hours it separated into three layers, the red cells at the bottom, a thin layer of white cells and platelets called the buffy coat and the top layer of plasma. The plasma could be stored for several weeks and did not require blood typing before transfusion.

1.1.2 Modern component transfusion

Today when blood donations are collected they are separated into blood components, i.e. red cells, platelets and plasma. In the UK the whole blood unit is filtered to remove the leucocytes (leucodepletion), the blood pack is then centrifuged and the components are collected into individual packs via linking tubes. The different blood components are then used to treat different conditions.

Concentrated red cells are given to patients with acute or chronic anaemia, now defined as having a haemoglobin level of less than 70g/l (Murphy *et al.*, 2001). The anaemia may be the result of massive blood loss or a chronic condition. The red cell transfusion is needed in these cases to increase the oxygen transport capacity of the blood. However, smaller blood losses (<20%) not leading to significant red cell loss but causing volume loss may be treated with volume

expanders such as saline solution to increase the volume of the blood, thereby avoiding shock.

The platelet component may be used to treat microvascular bleeding. Platelets may either be recovered from the buffy coat of whole blood donations, where the buffy coats from four units of whole blood are pooled to give one unit of platelets. Alternatively platelets may be derived from apheresis, a process where platelets or plasma are removed from the donor's blood and the red cells are returned to the donor.

The plasma component is used in patients who have suffered massive blood loss and multiple transfusions. In these cases where the entire volume of the blood has been lost and replaced the coagulation factors will be lost, leading to further bleeding. Fresh frozen plasma must be given in these cases, or cryoprecipitate, (which is rich in fibrinogen).

1.1.3 Transfusion complications

Serious hazards of transfusion (SHOT) reporting

Reporting and monitoring of the serious hazards of transfusion (SHOT) was initiated in 1996 (Williamson *et al.*, 1996). This initiative is a voluntary and confidential reporting system for transfusion related deaths and other serious complications in Britain and the Republic of Ireland. The SHOT reports are issued annually. Analysis of the data indicates that transfusion transmitted infections have diminished while the biggest cause of transfusion complication with over 50% of the reports in the first two years being attributable to the wrong blood being transfused due to errors in identification (Williamson *et al.*, 1999). Other reports were for acute transfusion reaction (15%), delayed transfusion reaction (14%), transfusion related acute lung injury (8%), post-transfusion purpura (6%), transfusion transmitted infections (3%) and graft versus host disease (2%).

Acute haemolytic transfusion reaction

This can occur with a one in three chance when ABO group incompatible red cells are transfused. The compatible blood groups are shown in Table 1.1. The recipient's anti-A or anti-B antibodies can lyse the red cells that are transfused. The lysed red cells release haemoglobin, which damages the kidneys causing acute renal failure. The cell membrane fragments activate complement causing disseminated intravascular coagulation (DIC).

Table 1.1: Blood group compatibilities

Donor \ Recipient	A	B	AB	O
A	Yes	No	Yes	No
B	No	Yes	Yes	No
AB	No	No	Yes	No
O	Yes	Yes	Yes	Yes

Blood group O is known as the universal donor because there is no anti-O, so group O cells will not be destroyed by any group. Group AB is known as the universal recipient because it contains neither anti-A nor B. The worst haemolytic reaction occurs in group O patients receiving of group A red cells.

Bacterial contamination

If the contents of the blood pack are contaminated with bacteria, a severe and often fatal bacterial septicaemia can occur. These bacterial contaminants can be introduced from the skin of the donor if it is poorly sterilised before donation. The problem is particularly found in platelets because they are stored at room temperature rather than refrigerated, and this is one of the factors that limit the shelf life of platelet packs (Engelfriet *et al.*, 2000).

Transfusion related acute lung injury (TRALI)

TRALI is a severe post transfusion reaction caused by antibodies in the donor's plasma reacting with leucocytes in the patient. This condition occurs within one to six hours of a plasma containing transfusion and is fatal in 6-10% (Engelfriet and Reesink, 2001). The symptoms include chills, fever, non-productive cough and breathlessness. In most cases of TRALI the donor will be a multiparous woman (Popovsky and Davenport, 2001).

Fluid overload

This occurs when too much fluid is transfused or the transfusion is too rapid, it can lead to pulmonary oedema and acute respiratory failure or acute left ventricular failure. Fluid overload is a particular risk with albumin solutions and in patients with chronic anaemia who may be hypervolaemic even before any transfusion.

Severe allergic reaction and anaphylaxis

These are rare but possibly life threatening conditions, caused by re-exposure to allergens in transfusions. If the patient has a severe IgA deficiency, anaphylaxis can occur when they are exposed to IgA in a transfusion.

Delayed haemolytic reaction

This can occur when a patient develops antibodies to blood groups from past transfusions or pregnancy, a re-transfusion can raise the levels of these antibodies causing delayed haemolysis. This can particularly occur with the Kidd (Jk) system. Other red cell antibodies such as Rhesus, S, Kell, Duffy and Kidd antigen systems can cause a slow haemolysis in the liver and spleen.

Transfusion-associated graft-versus-host disease (TA-GvHD)

This is caused by transfused T-lymphocytes, which recognise the host as foreign. It can occur when the patient is immuno-compromised but has also occurred in immuno-competent patients receiving donations from relatives. It is almost always fatal and is not prevented by leucodepletion, however it can be prevented by gamma-irradiation of the donation (Williamson, 1998).

Post transfusion purpura (PTP)

This is caused by platelet specific alloantibodies, and is potentially fatal. It is more usually found in female patients who develop a low platelet count and bleeding about 5-9 days after a red cell or platelet transfusion (Mueller-Eckhardt, 1986).

Transfusion transmitted infections

The three major transfusion transmitted infections; hepatitis C virus (HCV), hepatitis B virus (HBV) and human immunodeficiency virus (HIV) are discussed in section 1.2. *Treponema pallidum* is also screened for by serological testing in all blood donations. Human T-lymphotropic virus (HTLV) type I and II screening by serology has recently been introduced (July 2002). Cytomegalovirus (CMV) screening is carried out on donations in special circumstances such as donations for premature infants and CMV seronegative bone marrow allograft recipients in which primary CMV infection can be fatal. With leucodepletion the risk of CMV and HTLV infection is reduced, as these viruses are mostly cell associated.

Bacterial contamination of blood can cause serious or fatal reactions as discussed above, cryophilic strains such as *Pseudomonas fluorescens* or *Yersinia enterocolitica* can proliferate in red cell packs in refrigerated storage (McDonald *et al.*, 1996). Platelet donations are stored at 20-22°C and therefore open to

contamination with Staphylococci present on donor skin, so they cannot be stored for more than five days.

A total of 38 transfusion-transmitted infections were reported to SHOT between 1995 and 2001 (Figure 1.1), of these 22 (58%) were bacterial, six of which were fatal. The only other fatal infection was from malaria. Interestingly only two cases of post transfusion HCV were reported during this period, and yet it is HCV that has been the infection targeted for NAT screening.

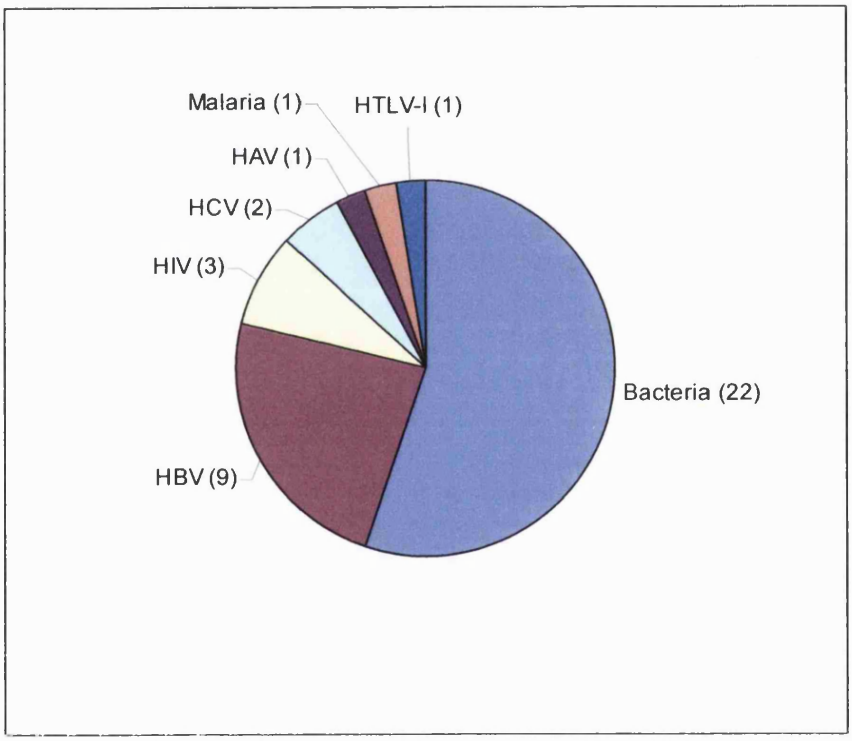

Figure 1.1: Reported transfusion transmitted infections in the UK 1995 to 2001.

Data collected from 1995 to 2001 and published in SHOT annual report for 2000 – 2001 (Asher *et al.*, 2002). Number of cases shown in brackets. There may have been under-reporting of cases before SHOT reporting began in October 1996

1.1.4 Plasma fractionation

During World War II with plasma being shipped across the Atlantic from the USA to the allied front in Europe, an American protein chemist Edwin Cohn began work on purifying the proteins in plasma. He knew of the value of plasma transfusion as an anti-shock treatment for soldiers wounded in battle, but that it could easily become contaminated with bacteria. He also knew that the plasma was made up of several proteins and thought that if these could be purified they might also confer the anti-shock properties of whole plasma.

Cohn used ethanol to precipitate the plasma proteins and by adding the ethanol in several stages, each time adjusting the temperature, salt content and pH, different protein fractions could be extracted from the plasma. This became known as the Cohn method of plasma fractionation (Cohn *et al.*, 1946), (Figure 1.2).

During his studies Cohn determined that the first fraction (I) consisted mainly of fibrinogen but also some factor VIII. Fractions II and III contained immunoglobulins, and fraction IV contained cholesterol and immune agents. It was the final fraction (V) that was of most interest as it contained albumin. This protein could be further purified and when injected into shock victims had the capacity to draw fluid into the blood vessels from the surrounding tissues, thereby increasing the volume of the blood. The benefits of the albumin solution produced at Cohn's Harvard laboratory were first shown in treatment of casualties after the attack on Pearl Harbour in December 1941. After this the production of albumin on an industrial scale was initiated at several pharmaceutical companies.

The Cohn method of fractionation is still the basis of the processes in use today for the production of albumin, clotting factors and immunoglobulins from plasma.

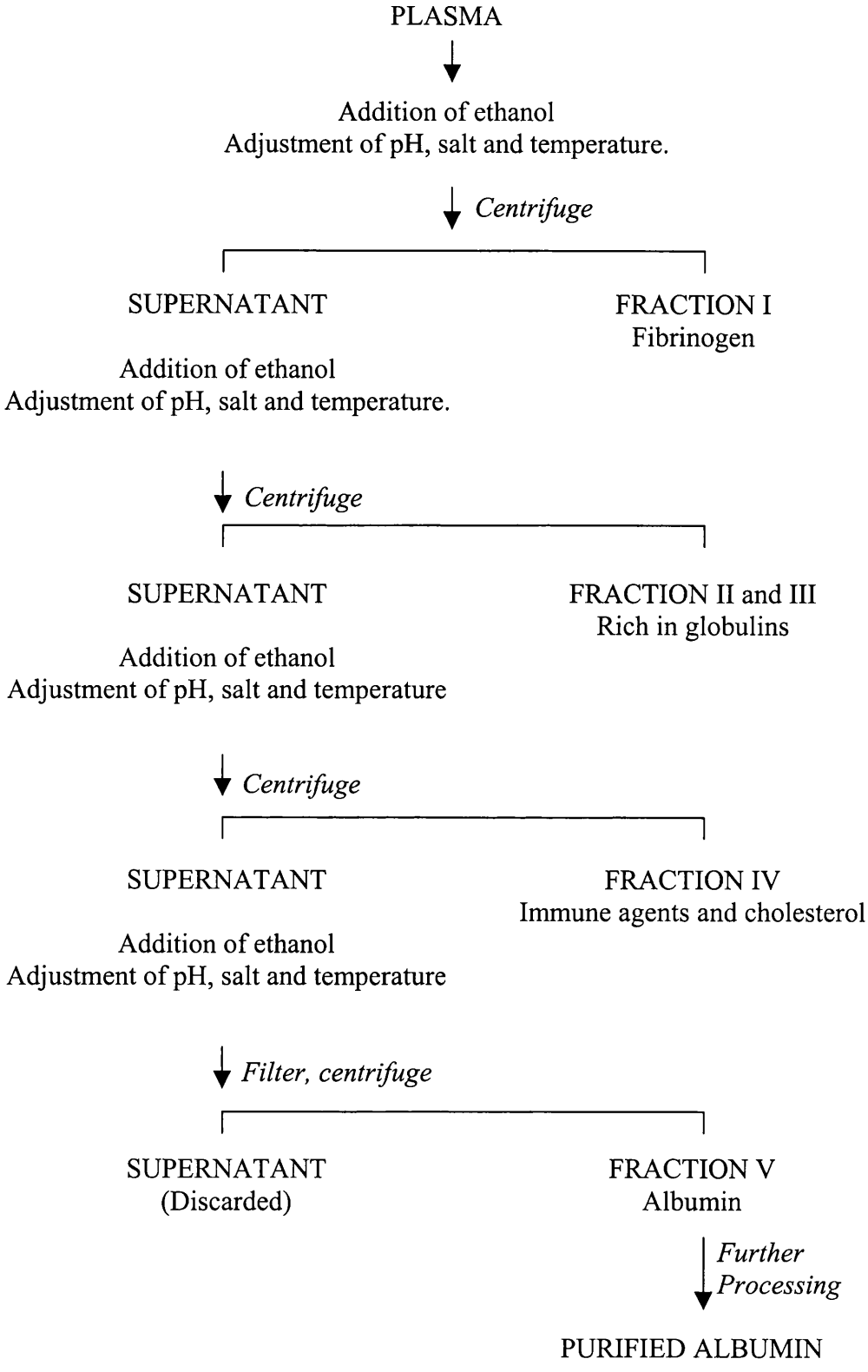

Figure 1.2: Plasma fractionation - Cohn method

(Diagrammatic representation)

Apart from the production of albumin, modern plasma fractionation is probably best known for the production of anti-haemophilic factors such as factor VIII which is deficient in haemophilia A, and factor IX which is deficient in haemophilia B. There are also a number of other clotting factors that can be used to treat other disorders such as von Willebrand's disease.

The other plasma products are immunoglobulins, which may be used intravenously in the treatment of immunological disorders such as immune cytopaenias and primary hypogammaglobulinaemia. Normal human immunoglobulin (NHIG) and specific immunoglobulin are also used intramuscularly in the prevention of infection by passive immunisation in situations when a vaccine cannot be used or in combination with a vaccine.

NHIG may be used to give short-term protection against hepatitis A virus (HAV) in travellers and contacts of HAV cases, protection against measles in immuno-compromised patients coming into contact with measles and in pregnant women who come into contact with rubella.

Specific immunoglobulin products made from selected donors have a higher level of antibodies against certain infectious organisms. Tetanus immunoglobulin is used with active immunisation in injuries with a high risk of tetanus. Hepatitis B immunoglobulin (HBIG) is used with HBV vaccine in accidental inoculation of HBV containing blood and other exposures such as of babies newborn to high risk mothers. Varicella Zoster Immunoglobulin (VZIG) is used in immuno-compromised neonates and in non-immune pregnant women who come into contact with cases of chickenpox.

1.2 Transfusion transmitted viral infections

Many infections can be blood borne but will not usually be transmitted by transfusions because they cause obvious illness which precludes donation or because the viraemia is only transient. Infections that cause most transfusion transmitted infections (TTI's) are generally asymptomatic or subclinical with long lasting viraemia and which are stable in stored blood and prevalent in the population such as HBV, HCV and HIV.

The three main viruses associated with transfusion transmission are described in the following sections. Although these are not the only viruses that are transmitted by transfusion they are considered the most clinically important.

1.2.1 History of transfusion transmitted viral infections

The infectious nature of hepatitis has long been known, with infectious hepatitis being described by Hippocrates. Other outbreaks of hepatitis have been described with terms such as 'epidemic catarrhal jaundice'. It was thought that this disease was transmitted by contaminated food or water, airborne or spread by biting insects.

Hepatitis associated with blood has been described more recently; in 1883 an outbreak of hepatitis among shipyard workers in Bremen was described that was caused by inoculation with smallpox vaccine containing glycerated human lymph (Lurman, 1885). More cases of this disease occurred in the early 20th century when non-sterilised needles were used for repeated injections in large numbers of patients such as salvarsan therapy for syphilis.

The link between hepatitis and the transfusion of blood or blood products was further established in the early 1940s (Beeson, 1943; Morgan and Williamson, 1943). This was after cases of hepatitis in those receiving measles convalescent serum (Findlay and MacCallum, 1937) or mumps serum (Beeson, 1943). The

largest outbreaks occurred in World War II troops who had received yellow fever vaccine containing human serum (Findlay *et al.*, 1944). These cases became known as 'homologous serum jaundice' (Ministry of Health, 1943) or 'serum hepatitis' and it was recognised that this was a different type of hepatitis than the 'infectious hepatitis' that was food borne or caused by close contact with jaundiced patients. The terms hepatitis A and hepatitis B were introduced in 1947 to distinguish between the short incubation period infectious hepatitis (A) and the longer incubation period serum hepatitis (B); (MacCallum, 1947). In the following years it had become suspected that post transfusion hepatitis had a viral cause.

The distinction between the two types of hepatitis was defined in a study of the children at the Willowbrook State School (Krugman *et al.*, 1967; Krugman and Giles, 1970). They isolated two strains, which they called MS-1 and MS-2. These were isolated from the same patient with MS-1 being taken during an attack of infectious type hepatitis and MS-2 being taken during a second episode of hepatitis occurring six months later and resembling type B hepatitis. The first isolate MS-1 caused hepatitis with a short incubation period averaging 32 days after oral or parenteral exposure. The second isolate MS-2 caused hepatitis with a long incubation period of approximately 65 days after a parenteral exposure and 98 days after an oral exposure.

The agent responsible for serum or type B hepatitis began to be elucidated with the discovery of an antigen 'in foci of hepatic parenchymal cells' by Prince and colleagues (Prince *et al.*, 1964). Further evidence was found by Blumberg and colleagues when they discovered an antigen in the blood of an Australian Aborigine that precipitated with antibodies in the serum from a haemophiliac, they named this Australia antigen (Blumberg *et al.*, 1965). In the original paper Blumberg postulated that Australia antigen was linked to leukaemia, but subsequently showed a link with hepatitis (Blumberg *et al.*, 1967).

Using immunodiffusion, Prince showed that the serum hepatitis (SH) antigen was present in the incubation period of cases of post transfusion serum hepatitis, but not in patients with infectious or type A hepatitis (Prince, 1968a). Prince also

showed that antibody to SH antigen could be found in multiply transfused patients. Prince later proved that the SH antigen and the Australia antigen were similar or identical (Prince, 1968b). The Australia or SH antigen is now known to be the surface protein of HBV and has been re-named hepatitis B surface antigen (HBsAg).

The viral source of the Australia antigen, hepatitis B virus was identified by Dane and colleagues in 1970 (Dane *et al.*, 1970). The 42 nm viral particle was distinguished from the 22 nm spherical or filamentous forms of HBsAg using immune electron microscopy on sera from patients with the Australia antigen, and became known as the Dane particle.

The viral cause of type A hepatitis was discovered in 1973, by immune electron microscopy, in the stools of volunteers who had been inoculated with the MS-1 strain of HAV (Feinstone *et al.*, 1973). The 27 nm particles were associated with an antigen allowing the development of a screening test. Look back studies showed that HAV was not strongly associated with transfusion transmission (Feinstone *et al.*, 1975; Dienstag *et al.*, 1977; Stevens *et al.*, 1978).

Although the introduction of HBsAg testing dramatically reduced the level of post transfusion hepatitis (Alter *et al.*, 1972), there were still cases that were not caused by either HAV or HBV. Studies carried out on cases of post transfusion hepatitis with blood screened for HBsAg showed that up to 90% of these cases were not due to HAV or HBV, but were due to other infectious agents as yet undiscovered (Prince *et al.*, 1974; Feinstone *et al.*, 1975; Alter *et al.*, 1975; Dienstag *et al.*, 1977).

Although the term type C hepatitis was suggested (Prince *et al.*, 1974) it was thought that there may be more than one causative agent and therefore these became known as non-A non-B (NANB) hepatitis.

The long sought after cause of most non-A non-B post transfusion hepatitis - HCV was finally identified in 1989 (Choo *et al.*, 1989; Kuo *et al.*, 1989; Choo *et*

al., 1990), and in September 1991 screening for anti-HCV was introduced in the NBS (MacLennan *et al.*, 1994).

A major transfusion transmitted infection crisis had hit the blood services of the world in the 1980's. Acquired immunodeficiency syndrome (AIDS) was first recognised in 1981 as a cluster of cases of *Pneumocystis carinii* and Kaposi's sarcoma in young homosexual men (Gottlieb *et al.*, 1981; Masur *et al.*, 1981; Siegal *et al.*, 1981; CDC, 1981). These diseases were not usually associated with young and otherwise healthy people, and at first the new condition was associated with the lifestyle of these homosexual men. When AIDS cases began to be found among injecting drug users (Gold *et al.*, 1982), haemophiliacs (Davis *et al.*, 1983) and transfusion recipients (Jett *et al.*, 1983; Curran *et al.*, 1984; Jaffe *et al.*, 1984) an infectious agent began to be suspected (Francis *et al.*, 1983).

In 1983 a French group isolated a virus related to human T-cell lymphotropic virus (HTLV) from a patient with AIDS, they named this virus lymphadenopathy associated virus (LAV); (Barré-Sinoussi *et al.*, 1983). Further evidence came a year later when Gallo and colleagues, who had first described HTLV, also isolated a virus from AIDS patients that they called HTLV-III (Popovic *et al.*, 1984; Gallo *et al.*, 1984; Schupbach *et al.*, 1984; Sarngadharan *et al.*, 1984). These later turned out to be the same virus, which is now known as HIV-1.

1.2.2 Hepatitis C Virus

Hepatitis C virus was first described in 1989 by Choo and colleagues at Chiron Corporation (Choo *et al.*, 1989; Kuo *et al.*, 1989). Due to difficulties isolating the virus by more conventional methods Choo and colleagues decided to try to isolate the virus from the acute phase serum of a chimpanzee that had been infected with NANB. The nucleic acid was isolated from the serum, reverse transcribed and the cDNA library was cloned into a bacteriophage expression vector. The cDNA library was then screened with a series of NANB sera and negative controls, and a clone designated 5-1-1 was isolated and shown to hybridise with a single strand positive sense RNA of around 10 000 nucleotides in length (Choo *et al.*, 1989). Using this 5-1-1 clone in a serological assay it was shown that the vast majority of NANB post transfusion hepatitis was caused by this virus which they designated hepatitis C virus (Kuo *et al.*, 1989; Choo *et al.*, 1990).

Genomic Structure

Choo and colleagues went on to sequence the entire genome of HCV (Choo *et al.*, 1991). These studies have shown that HCV is a positive sense RNA virus of approximately 9.6 kb in length with an open reading frame coding for a polyprotein of around 3000 amino acids. It has similarities to the viruses in both the genus *Pestivirus* and the genus *Flavivirus* and was therefore grouped as a third genus - *Hepacivirus* within the virus family *Flaviviridae.*

The structure of the HCV genome is shown in Figure 1.3.

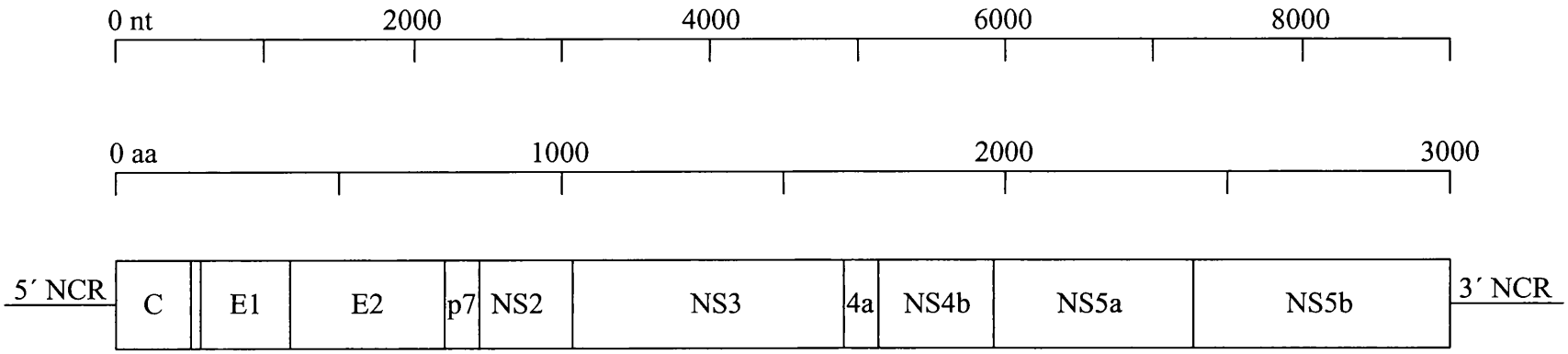

Figure 1.3: Genomic structure of HCV

The single polyprotein is flanked by two non coding regions (NCR). The polyprotein is cleaved into the nucleocapsid core protein (C), the envelope glycoproteins (E1 and E2), a protein of unknown function (p7) and the non-structural proteins NS2 to NS5.

The genome contains a 5′ non coding region (5′ NCR) which has a strong sequence similarity with the pestiviruses with a 48% similarity to hog cholera virus (now known as classical swine fever virus) and a 45% similarity to bovine viral diarrhoea virus (BVDV); (Han *et al.*, 1991). The 5′ NCR also shows conservation between different isolates of HCV from around the world (Takeuchi *et al.*, 1990). The 5′ NCR contains an internal ribosome entry site (IRES) which requires specific conformation of the secondary structure, this requirement tends to conserve the sequence in this region (Tsukiyama-Kohara *et al.*, 1992; Wang *et al.*, 1993).

The IRES initiates translation of the single open reading frame into a polyprotein of 3011 amino acids (Choo *et al.*, 1991). The polyprotein is then cleaved into ten distinct products, starting at the amino terminus with the core nucleocapsid protein C and the two envelope glycoproteins E1 and E2. The three structural proteins are cleaved from the polyprotein by host cell pepsidases. The envelope protein E2 contains two hyper-variable regions (HVR 1 and 2); (Weiner *et al.*, 1991). These areas show a very high mutation rate thought to be caused by selective pressure from the host immune system. E2 also contains the binding site for CD81 which has been suggested as a cellular receptor for the virus (Pileri *et al.*, 1998). Another candidate receptor for HCV, the low or very low density lipoprotein (LDL / VLDL) has also been associated with the envelope protein (Monazahian *et al.*, 1999; Agnello *et al.*, 1999).

The function of the next protein p7 (Lin *et al.*, 1994) is still unclear. The rest of the proteins are non structural and are cleaved from the polyprotein by HCV encoded enzymes. NS2 and the amino terminus of NS3 form a protease that catalyses cleavage at the NS2/NS3 junction (Grakoui *et al.*, 1993). The amino terminus of NS3 contains a serine protease that cleaves the rest of the non-structural proteins (Bartenschlager *et al.*, 1993). The remaining carboxy terminus of NS3 acts as a helicase (Kim *et al.*, 1995). NS4a acts as co-factor to the NS3 protease (Failla *et al.*, 1994). The functions of NS4b and NS5a are still unclear, although NS5a contains the interferon sensitivity determining region (ISDR) which has been associated with resistance to interferon (Enomoto *et al.*, 1995;

Gale *et al.*, 1997). Lastly, NS5b is an RNA dependant RNA polymerase enzyme (Behrens *et al.*, 1996).

There is another non coding region of at the 3′ end the genome which consists of a variable sequence of around 40 bases then a poly-U region of variable length followed by a highly conserved region of 98 bases (Tanaka *et al.*, 1995).

Sequence variation

The average mutation rate for HCV is 10^{-3} to 10^{-4} base substitutions per genome per year (Ogata *et al.*, 1991) due to errors in the incorporation of nucleotides by the viral RNA polymerase which has no proof reading function. This high mutation rate is one of the mechanisms by which the virus evades the host immune system (Kato *et al.*, 1993). However the rate of change is not constant throughout the genome; the 5′ and 3′NCRs are relatively conserved (Takeuchi *et al.*, 1990; Tanaka *et al.*, 1995), whereas there are hyper variable regions of sequence in the envelope gene E2 (Weiner *et al.*, 1991).

The high mutation rate has resulted in sequence diversity between different strains of HCV with variants showing only around 70% sequence homology. Phylogenetic analysis has shown that there are six distinct groups or genotypes of HCV (Simmonds *et al.*, 1993). Viruses within each genotype can be grouped into subtypes. A common nomenclature of the HCV genotypes has been proposed by Simmonds and colleagues (Simmonds *et al.*, 1994). The different genotypes of HCV are related to the geographical location of the isolate (McOmish *et al.*, 1994). Types 1 to 3 are found most commonly in the UK, with type 4 associated with the Egypt and the Middle East, type 5 associated with South Africa and type 6 associated with Hong Kong (Davidson *et al.*, 1995). The HCV genotype is also related to the response rate to antiviral therapy, with type 1 showing significantly less response to interferon alpha treatment than types 2 and 3 (Garson *et al.*, 1995).

Disease progression

Acute infection with hepatitis C virus is asymptomatic in around 80% of cases, the remaining 20% are icteric with symptoms including malaise and nausea. In very rare cases fulminant hepatitic failure can occur (Farci *et al.*, 1996), although this may be more common in cases where there is a super-infection with hepatitis A virus (Vento *et al.*, 1998). Approximately 80% of acute HCV infections result in chronic HCV, which can last up to 30 years before the onset of symptoms. Chronic HCV infection can result in varying degrees of liver disease during a lifetime but in around 30% of cases severe progressive liver disease occurs which can lead to liver cirrhosis and hepatocellular carcinoma (HCC) which is ultimately fatal (Di Bisceglie *et al.*, 1991; Seeff *et al.*, 1992). There are several factors that have been associated with an increased risk of development of liver cirrhosis in chronic HCV, these include being male, being over 50 years old when infected, alcohol consumption and co-infection with HIV-1 or HBV (Poynard *et al.*, 1997; Soto *et al.*, 1997; Zarski *et al.*, 1998). The liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST) are released into the peripheral blood when the liver is damaged and can therefore be used as markers of liver disease.

Treatment

The aim of therapy is a sustained virological response (SVR) usually defined as viral RNA being undetectable 24 weeks after the end of the treatment. This is because the viral RNA may often become undetectable during treatment only to return after the treatment period has ended, this is known as relapse. Those patients who attain a SVR have remained HCV uninfected when followed up long term (Lau *et al.*, 1998). The current standard antiviral treatment for chronic HCV is interferon alpha-2b in combination with a synthetic guanosine nucleoside analogue ribavirin (Brillanti *et al.*, 1994). This combination when given for 48 weeks has resulted in SVR rates of 38 to 47% (Poynard *et al.*, 1998; McHutchison *et al.*, 1998; Manns *et al.*, 2001). These percentage response rates represent an average, but if the patients are sorted into different genotype groups

it has been noted that those with HCV genotype 2 or 3 have a significantly better SVR rate than those with genotype 1, in a recent trial this was 79% for type 2 and 3 versus 33% for type 1 (Manns *et al.*, 2001). This is an improvement over interferon monotherapy which only resulted in an average of 17% SVR rate (Thevenot *et al.*, 2001).

Recent trials have shown that pegylation of the interferon alpha-2b can double the SVR in comparison to the standard non-pegylated interferon (Zeuzem *et al.*, 2000; Heathcote *et al.*, 2000; Lindsay *et al.*, 2001). The addition of the poly ethylene glycol (PEG) molecule to the drug decreases its half life in the body allowing it to remain active for longer so that it can be given once a week instead of the more usual three times a week. When pegylated interferon alpha-2b is used in combination with ribavirin SVR rates of 54% and 56% have been shown after 48 weeks (Manns *et al.*, 2001; Fried *et al.*, 2001). In these recent studies it appears to be the patients with genotype 1 virus that have shown the greatest increase in SVR from around 30% with standard interferon to around 40% with pegylated interferon.

Treatment is usually only given to patients with chronic infection however a recent study showed the benefit of treatment in the acute phase of infection (Jaeckel *et al.*, 2001). In this study 44 patients with acute HCV were treated with interferon alpha-2b for 24 weeks, a further 24 weeks after the end of treatment 43 (98%) had cleared HCV RNA.

1.2.3 Hepatitis B virus (HBV)

The hepatitis B virus was first identified in 1970 by immune electron microscopy (Dane *et al.*, 1970). Although its surface antigen HBsAg had been discovered some years earlier (Blumberg *et al.*, 1965) and linked with type B hepatitis (Blumberg *et al.*, 1967; Prince, 1968a) as described in section 1.2.1.

HBV is a member of the family *Hepadnaviridae*; this family includes mammalian viruses in the genus *Orthohepadnavirus* such as HBV and the woodchuck hepatitis virus and avian viruses in the genus *Avihepadnavirus* such as duck hepatitis virus.

Viral characteristics and genome structure

The 42nm Dane particle is the infectious virus, which contains a nucleocapsid core (HBcAg) enclosing the nucleic acid. The envelope material or surface antigen is present in vast excess of the virus in the form of 22nm spheres and filaments and may be detected in the plasma by serological assays. The genome is circular partially double stranded DNA of around 3.2kb in length (Figure 1.4).

The genome has four overlapping open reading frames S, C, P and X genes, the S and C genes have flanking regions termed *preS1*, *preS2* and *preC*. The surface or envelope protein can be expressed in three forms, termed the large, middle and major forms. The type of surface protein expressed depends on whether transcription starts at *preS1*, *preS2* or just the S gene alone. The nucleocapsid core protein (HBcAg) is expressed from the C gene, an alternative protein HBeAg can be expressed from the preC/C gene. The large P gene encodes the viral polymerase enzyme and the X gene encodes two proteins which are transcriptional transactivators.

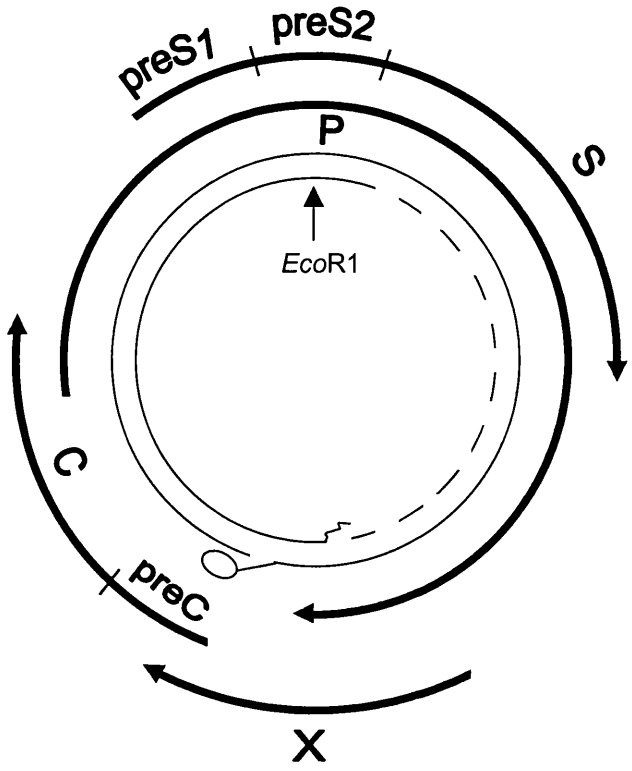

Figure 1.4: The hepatitis B genome.

HBV has a 3.2kb, partially double stranded circular DNA genome. The relative positions of the four viral genes (preS1/preS2/S, preC/C, P and X) are shown. A protein is linked to the 5′ end of the full length DNA strand, and an oligonucleotide is linked to the 5′ end of the short strand.

Epidemiology

It is estimated that there are over 350 million carriers of HBV worldwide. In some areas of the world such as Southeast Asia, Africa and China, the virus is endemic and up to 50% of the population in these areas may be infected with the virus at some point in their lives (Kane, 1995). In those areas where the virus is highly endemic over 8% of the population are carriers and transmission generally occurs at childbirth (vertical transmission) or in infancy as a result of horizontal transmission from child to child. In areas of the world where the virus is less prevalent such as Western Europe, North America and Australia, transmission is

horizontal between young adults. Infections can result from intravenous drug use, sexual activity, blood transfusion and haemodialysis. The virus may also be transmitted by health care workers undertaking exposure prone procedures (EPP), (Sundkvist *et al.*, 1998). However, measures are now underway to ensure that carriers of the virus are do not carry out EPP's if they have a viral load greater than 1000 copies/ml (Gilbert *et al.*, 2002).

Disease Progression

Most HBV infections in adults result in an acute infection with only 5% developing chronic infection, the infection results in icteric disease in around a third of those infected. The situation is reversed in neonates and infants in whom the majority of infections result in chronic disease (Peters *et al.*, 1991). The infection starts with an incubation phase of around 45 to 120 days, during this phase the virus replicates prolifically with viral titres reaching 10^{10} copies/ml in the plasma. Viral DNA, HBsAg and HBeAg can be detected during this period and later anti-HBc develops.

In the second stage of the infection the host immune response to the virus reduces the viral levels as well as causing inflammation of the liver. There is a short prodromal phase of around a week characterized by mild fever, fatigue, malaise and nausea, after which the icteric disease begins in the patient, (co-incident with anti-HBc) lasting for four to six weeks. As a result of the hepatic injury the aminotransferase levels become elevated. Fulminant hepatic failure can also occur although it is rare (Berk and Popper, 1978; Lee, 1993).

In the acute infection the host immune system begins to clear infected hepatocytes effectively stopping active replication. During this convalescent phase HBeAg disappears and anti-HBe becomes detectable. The level of viral DNA drops but may still remain detectable by NAT methods, aminotransferase levels drop back to normal. Eventually HBsAg disappears and anti-HBs becomes detectable marking the final immune phase of the disease. At this stage the

infection has been completely cleared and HBV DNA is no longer detectable, the anti-HBs response is also protective, preventing re-infection with the virus.

In chronic HBV infection, the HBsAg and HBeAg are not cleared quickly but remain for many years indicating continued viral replication, which can lead to liver cirrhosis and hepatocellular carcinoma. The chronic carrier state is usually defined as HBsAg remaining detectable for more than six months. The carrier state can spontaneously resolve with the disappearance of HBeAg and the detection of anti-HBe. This signals a decline in HBsAg which eventually becomes undetectable. Anti-HBs may or may not appear after loss of HBsAg in chronic carriers resolving infection.

In both acute and chronic cases anti-HBc becomes detectable a few weeks after the appearance of HBsAg and remains detectable throughout the course of infection and for many years afterwards. At the time of writing anti-HBc detection in donated blood is not performed in the NBS, however this is currently under review and may be implemented in pooled donations in the future. Anti-HBc is important in transfusion screening because it remains detectable at the end of the infection in the period between loss of HBsAg and development of anti-HBs during which time the blood may still be infectious (Allain *et al.*, 1999).

Prevention and therapy

The first vaccines for HBV were HBsAg preparations purified from human serum (Maupas *et al.*, 1978). However concerns over safety of these vaccines particularly after the discovery of HIV-1 in the early 1980's led to the development of recombinant HBsAg expressed from yeast cells (McAleer *et al.*, 1984).

For those with chronic HBV infection treatment with drugs is aimed at shifting the course of infection from the active or stage two disease to the convalescent or

stage three disease, i.e. HBeAg to anti-HBe sero-conversion, a small percentage may even clear HBsAg. Standard treatment has been a four month course of interferon alpha 2b (Hoofnagle and Di Bisceglie, 1997) which can result in a sustained response in approximately a third of patients (Wong *et al.*, 1993). Long term follow up of the responders to interferon treatment show improved clinical outcome (Niederau *et al.*, 1996).

Recent studies have shown that the nucleoside analogue Lamivudine given in combination with interferon alpha may give greater response rates (Schalm *et al.*, 2000; Barbaro *et al.*, 2001).

1.2.4 Human immunodeficiency virus (HIV)

Human immunodeficiency virus, the causative agent of acquired immunodeficiency syndrome (AIDS) was discovered in 1983 (Barré-Sinoussi *et al.*, 1983) and was later shown to be transmitted by blood transfusion (Jett *et al.*, 1983).

Genome structure and sequence variation

HIV-1 is a member of the genus *Lentivirus* within the family *Retroviridae*, it is an enveloped virus of 120nm in diameter with a diploid RNA genome of 9.2 kb. The genome shares the attributes of the other retroviruses with the main genes being *gag*, *pol* and *env* (Figure 1.5). The *gag* gene codes for several structural proteins including those used in the nucleocapsid. The second gene *pol* codes for a protein which is cleaved into the integrase (IN), reverse transcriptase (RT) and protease (PR) proteins. The *env* gene codes for the gp120 and gp41 envelope glycoproteins. The genes *tat* and *rev* code for proteins with regulatory functions, and the genes *nef*, *vif*, *vpu* and *vpr* also code for proteins that may have accessory functions some of which are still unclear.

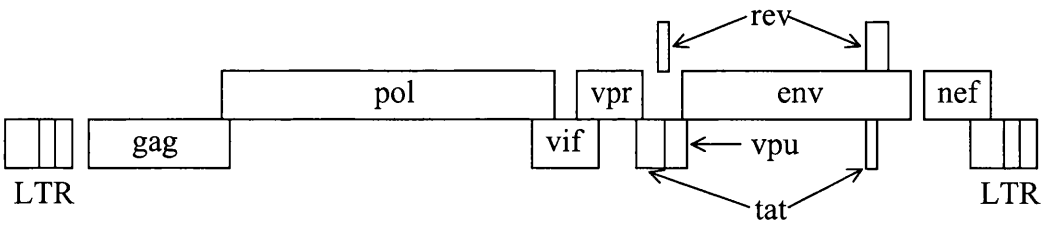

Figure 1.5: HIV-1 genome

The HIV-1 genome shows the same layout of the structural genes *gag*, *pol* and *env* as other retroviruses. In addition there are the regulatory genes *tat* and *rev*, and the accessory genes *nef*, *vif*, *vpu* and *vpr*.

A second virus known as HIV-2 was isolated from patients in West Africa in 1986 (Clavel *et al.*, 1986). The two viruses are related but have a sequence similarity of only around 50%. HIV-2 has mostly remained confined to West Africa and is generally thought to be less transmissible and pathogenic than HIV-1 (Pepin *et al.*, 1991).

HIV-1 sequence diversity in isolates from different areas of the world have led to its classification into groups and further classification into subtypes or clades. There are three groups of HIV-1 based on sequence diversity, these are; group M (main), group O (outlier), and group N (new). The main group of HIV-1, group M is classified into subtypes A to K which can vary by 20-25% in their envelope sequences. The more distantly related strains of the outlier group O were first isolated from a patient in the Cameroon, with a sequence similarity to HIV-1 group M of only 65% (Gurtler *et al.*, 1994). Another group of viruses also isolated in the Cameroon have been described which do not cluster with either HIV-1 group M or O, these have been designated group-N (Simon *et al.*, 1998).

Epidemiology

At the end of 2001 more than 40 million people were living with HIV, and over 60 million people have been infected with the virus since the epidemic began (UNAIDS/WHO, 2001). The vast majority of those affected are in Sub-Saharan Africa, with South and South-East Asia the second highest affected. HIV-1 group M viruses are responsible for the majority of the epidemic, with HIV-1 groups N and O and HIV-2 largely confined to West Africa. HIV-1 subtype C viruses represent the majority worldwide, being prevalent in Africa and India. However subtype B is the most common HIV-1 subtype in western countries. Subtype E is present in a rapidly expanding epidemic in South East Asia. In the UK the predominant subtype is B, however the other subtypes are also present (Clewley *et al.*, 1996).

The most common route of transmission worldwide is heterosexual intercourse, however the early epidemic in Western countries was due mainly to homosexual

intercourse. Recently however, heterosexual transmission has been found to have overtaken homosexual as the main route of spread in the UK (Kent, 2001). The virus is also spread by parenteral transmission particularly intravenous drug use, but also by needle stick injuries in healthcare workers, transfusion of blood or blood products and organ transplantation. Another route of infection is perinatal from an infected mother to her baby either through the placenta, during birth or through breast feeding.

Disease progression

The acute primary infection of HIV-1 may be asymptomatic in around half of the cases, but where there are symptoms they include a mild flu or mononucleosis like illness with fever, rash, pharyngitis and lymphadenopathy lasting for about three weeks (Kinloch-de Loes *et al.*, 1993). The symptoms usually coincide with sero-conversion two weeks to three months after exposure. There then follows a variable asymptomatic period of eight to eleven years (Collaborative group on AIDS Incubation and HIV Survival including the CASCADE EU Concerted Action, 2000). During this period there may still be symptoms of generalised lymphadenopathy and fatigue.

As the disease progresses, the CD4+ cell count slowly declines with further symptoms of reduced immunity occurring when the CD4+ cell count falls below 500 cells per μl. When the CD4+ cell count falls below 200 cells per μl a variety of opportunistic infections and other conditions associated with a depressed cell mediated immunity characterise the symptomatic stage defined as full blown AIDS (CDC, 1993). This final symptomatic stage often presents with *Pneumocystis carinii* pneumonia or Kaposi's sarcoma and ultimately leads to death (Adler, 2001).

Treatment

The first anti retroviral drug to be used for HIV therapy in 1985 was the nucleoside analogue reverse transcriptase inhibitor (NRTI) 3′-azido-3′-deoxythymidine (AZT); (Fischl *et al.*, 1987). However, the use of this drug for prolonged periods selected viral mutants with reduced drug susceptibility. When further NRTI's were developed the use of these drugs in combination gave superior results to monotherapy. In the mid 1990's the first protease inhibitors were developed and when used in combination with the NRTI's gave a further improvement. The highly active antiretroviral therapy (HAART) triple combination therapies in use today are able to reduce viral loads to undetectable levels and significantly reduce the rate of progression to AIDS (Palella *et al.*, 1998).

1.3 Screening for viral infections transmitted by transfusion

One way in which transfusion transmitted infections (TTI's) can be reduced is by careful donor selection. It has been noted that the risk of post transfusion hepatitis is greater in blood from paid donors than voluntary donors (Muller-Breitkreutz, 2000). In the UK, blood donors are voluntary, non-remunerated and have to provide lifestyle details before donating blood. Another measure to reduce the incidence of TTI's is the screening of all donations for the presence of infectious agents. The first screening of blood donations for an infectious agent by the NBS was introduced by 1950 for antibodies to *Treponema pallidum* (syphilis). The identification and development of screening methods for viral TTI's are described below.

1.3.1 Introduction of serological screening assays for viral TTI's

In 1965 Blumberg and colleagues at the US National Institutes of Health (NIH) discovered an antigen in the serum of an Australian aborigine that precipitated with serum from a haemophiliac (Blumberg *et al.*, 1965). Further studies linked this Australia antigen, now known as the hepatitis B surface antigen (HBsAg), with post transfusion hepatitis (Blumberg *et al.*, 1967; Prince, 1968a).

Retrospective studies using an HBsAg screening test showed that the use of this test could cut the rate of post transfusion hepatitis by up to 25% (Gocke *et al.*, 1970). HBsAg screening of blood donations for HBV was introduced in the UK in 1971 and over the next few years the sensitivity of the tests was increased from the first generation assays using countermigration immunoelectrophoresis (CIEP) to the third generation radioimmuno assays (RIA), (Barbara *et al.*, 1977).

HIV-1 the causative agent of acquired immunodeficiency syndrome (AIDS) was discovered in 1983 (Barré-Sinoussi *et al.*, 1983) and was later shown to be transmitted by blood transfusion (Jett *et al.*, 1983). This led to the introduction in the autumn of 1985 of donor selection in the NBS with all male blood donors

required to read a leaflet asking them not to donate if they had ever had sex with another man. Further high risk groups were defined later and the leaflet expanded to all donors. Donors at the North London Blood Transfusion Centre (NLBTC) were also given the option to donate blood but confidentially indicate that it should not be used for transfusion, so that any relatives or work colleagues would not become suspicious (Barbara *et al.*, 1986). Serological tests for anti-HIV-1 soon became available and screening of all blood donations in the UK for anti-HIV-1 was introduced in October 1985 (Barbara *et al.*, 1986). In June 1990 screening for both anti-HIV-1 and anti-HIV-2 was introduced (Flanagan *et al.*, 1995).

The long sought after cause of most non-A, non-B post transfusion hepatitis, (HCV) was finally identified in 1989 (Choo *et al.*, 1989; Kuo *et al.*, 1989; Choo *et al.*, 1990), and in September 1991 screening for anti-HCV was introduced in the NBS (MacLennan *et al.*, 1994).

The first generation of anti-HCV screening enzyme immuno assays (EIA) was based on the c100-3 antigen from the NS4 region of the genome which included the initial clone 5-1-1 (Kuo *et al.*, 1989). In look back studies of blood donations, which had been shown to cause HCV infection in the recipients, this test had the ability to detect around 80% of the infected donations. However, look back studies and comparison with confirmation tests identified a very high false positive rate of 50-60% in the first generation assays (van der Poel *et al.*, 1991; Tobler *et al.*, 2000).

There was a marked improvement in serological testing for HCV with the introduction of multi-antigen second generation EIA's (Chien *et al.*, 1992). In these new tests two epitopes were added, c22-3 from the core protein and c33c from NS3, increasing the detection rate to around 90% (Bresters *et al.*, 1992). Antibodies to these epitopes appear much earlier in the infection.

The third generation EIA's in use today have added NS5 and reconfigured the NS3 and NS4 epitopes into the recombinant protein c200, allowing HCV to be identified even earlier in the infection (Couroucé *et al.*, 1994; Barrera *et al.*,

1995). A recent study has shown that 71% of the window phase HCV samples that were RNA positive by minipool NAT and anti-HCV negative by a second generation EIA were anti-HCV positive when re-tested with a third generation EIA (Galel *et al.*, 2002).

1.3.2 Window phase and residual risk of TTI

The introduction of screening the blood supply for markers of viral infection dramatically reduced the incidence of TTI'sover the last three decades. Screening assays have further reduced TTI's due to the population of repeat donors being identified and excluded from further donation if found positive, and the tests themselves becoming more sensitive.

However, there remains a period between the donor being infected and the development of detectable serological markers, this is known as the 'window phase' of infection. The window phase for HCV has been estimated to average 82 days with 2nd generation ELISA's (Schreiber *et al.*, 1996) and can be reduced to 66 days with 3rd generation ELISA's (Barrera *et al.*, 1995). However, rare cases have been described which are HCV RNA positive but have not sero-converted in excess of one year (Soldan and Barbara, 1999; Peoples *et al.*, 2000). HBV and HIV have window phases of 59 and 22 days respectively (Schreiber *et al.*, 1996). The serological window phase for HCV has now been greatly reduced with the advent of an HCV core antigen assay (discussed in Chapter 5). However at the time the NBS NAT project started this assay was not available.

Several cases of viral infection in patients who received blood or blood products from donors in the window phase of the infection have been documented (Williamson *et al.*, 1999; Ling *et al.*, 2000). Thus, even with proficient antibody serological screening there is a residual infectious risk (Busch *et al.*, 1992; Wolcott, 1992; Busch *et al.*, 1997b). This risk can be estimated by compilation of the window phase estimates with viral marker incidence rates in donors (Kleinman and Busch, 2000).

The residual risk of HCV positive donations being transfused with anti-HCV screening was estimated to be 1 in 103 000 donations in the US (Schreiber *et al.*, 1996), 1 in 200 000 donations in Germany (Koerner *et al.*, 1998) and between 1 in 225 000 (Soldan and Barbara, 1999) and 1 in 250 000 donations in the UK (Snape and Flanagan, 1998). The difference in the estimated risk between Europe and the US was probably due to the more sensitive third generation serological tests used in Europe, which were not licensed in the US at the time. The risk of HIV transmission by sero-negative blood transfusions in the US was estimated to be 1 in 493 000, compared with 1 in 63 000 for HBV (Schreiber *et al.*, 1996; Kleinman and Busch, 2000).

Early studies using reverse-transcription polymerase chain reaction (RT-PCR) assays on serial samples from post-transfusion hepatitis cases showed that HCV RNA could be detected in plasma an average of 10-12 days after infection (Busch *et al.*, 1997a). Studies of samples from infected patients in high risk cohorts identified HIV RNA by about 11 days after infection, whereas HBV DNA could not be detected until 20-30 days after infection (Schreiber *et al.*, 1996). The large (approximately 50 day) reduction in the HCV window phase by PCR over serological testing indicated that NAT screening of donors would give the greatest benefit for this virus, a prediction born out by subsequent experience with prospective NAT screening.

It is important to note that for each virus there is time between the exposure leading to infection and the presence of detectable virus in the peripheral blood even using highly sensitive NAT assays. This period is known as the 'eclipse phase', and is due to the early replication of the virus in target organs such as lymph nodes and liver, prior to systemic dissemination of the virus in the blood. Recent evidence from HIV-1 infection studies in an animal model has indicated that blood from a donor in the pre-NAT eclipse phase of infection may not be infectious (Murthy *et al.*, 1999). Consequently, although NAT testing may not be able to close the diagnostic window (which begins at the time of exposure) completely, this technology may be able to close the infectious window period that is critical to blood safety. However, this goal will require very high

sensitivity NAT systems, and probably application to individual samples rather than pooled donations (Schuttler *et al.*, 2000).

1.3.3 Introduction of NAT testing of donated blood

The use of NAT for blood screening was first formally considered after there was a report to the US Food and Drug Administration (FDA) of several cases of HCV infection resulting from the use of an intravenous immunoglobulin preparation called 'Gammagard' which was manufactured by Baxter Healthcare Corporation (CDC, 1994). Baxter withdrew Gammagard from the market on the 23rd of February 1994. The preparation was later found to contain HCV RNA but was HCV antibody negative i.e. this was the result of a window phase donation (Gomperts, 1996). Most modern fractionated products have various viral inactivation steps in their manufacture, such as solvent detergent, which are capable of destroying enveloped viruses like HCV, HIV-1 and HIV-2, however the early intravenous immunoglobulin products such as Gammagard did not employ these technologies.

This episode led to the FDA's Center for Biologics Evaluation and Research (CBER) in the USA to establish a requirement that all finished products manufactured without a virus inactivation procedure should be tested for the presence of HCV RNA before release. The European regulatory authority, the European Committee for Proprietary Medicinal Products (CPMP) followed this in 1995 with a guideline for HCV RNA testing of the start pool before fractionation, rather than finished products, for release of immunoglobulin products manufactured without the use of a virus inactivation procedure (CPMP, 1995). Latterly the CPMP extended this guideline to the release of all plasma derived medicinal products, effective throughout the European Union from July 1st 1999 (CPMP, 1998).

The CPMP guideline requires the use of a run control of 100 WHO International Units per millilitre (IU/ml), and for an assay to be considered valid this control must test positive. This sensitivity requirement relates to the assay used to test the fractionation start pool, which may contain thousands of plasma donations.

However, the CPMP strongly recommended testing smaller pools "mini-pools" before the larger start pool is made, to avoid large volumes of plasma being destroyed if found to be contaminated.

Importantly, the development of sensitive NAT systems has required parallel development of appropriate standards. The World Health Organisation (WHO) International Unit (IU) for HCV RNA was established in 1997 after a collaborative study to assess the suitability of three candidate materials was coordinated by the National Institute for Biological Standards and Control (NIBSC, UK); (Saldanha *et al.*, 1999). The WHO HCV RNA International Standard (code 96/790) contains a lyophilised preparation of HCV genotype 1 at 100 000 IU/ml. An International Unit has been shown to be equivalent to approximately 2 to 6 genome equivalents depending on the assay used (Saldanha *et al.*, 2000). WHO international standards have now also been established for HIV-1 RNA (Holmes *et al.*, 2001), for HBV DNA (Saldanha *et al.*, 2001) and for B19 DNA (Saldanha *et al.*, 2002).

The requirement issued by the CPMP in 1998 did not govern the transfusion of cellular components. However, because the plasma for fractionation was recovered from the same donations from which cellular components would be transfused, ethical concerns over releasing plasma products but not cellular components on the basis of the NAT test led to many countries, including the UK to begin the development of NAT screening programs for the release of cellular components for transfusion (Engelfriet and Reesink, 2002).

In England a two-phase approach to HCV NAT testing was taken: Phase 1 was designed to enable NAT testing of UK plasma for fractionation to start as quickly as possible to meet the CPMP guidelines. It involved all donations from the England being NAT tested at the Bio Products Laboratory (BPL), the UK plasma fractionator in Elstree, Hertfordshire, and also allowed the NAT release of long shelf life frozen components for transfusion. During the development of the screening program BPL stopped fractionating English plasma as a response to the theoretical risk of transmission of variant Creutzfeld-Jakob disease (v-CJD); (Department of health, 1998; Hunter *et al.*, 2002). All BPL blood products are

now manufactured from plasma sourced in the United States, and the FDA require that this is NAT tested in the US by the National Genetics Institute (NGI).

Therefore BPL only needed to NAT test the plasma start pools, and the screening of the UK blood donations was no longer required to comply with the CPMP. However, it was widely believed at the time that there would be a requirement for component release using a NAT result in the near future and the facility to NAT test all donations in England and Wales, around 2.5 million per year, was already established at BPL before the shift to fractionating US plasma, therefore the NAT screening program continued to develop within the NBS in England.

Phase 2 (now complete) was designed to allow the release of all cellular components with a shelf life of greater than 24 hours on the basis of a negative NAT test. This has involved moving the NAT testing from BPL to three NBS laboratories around the country. This was done to cut down the transportation time, allowing rapid testing of labile components, such as platelets which have a shelf life of only five days. Three NAT testing laboratories also allow redundancy so that if one laboratory is shut down due to an incident such as a fire, then the other two can take on the extra work.

There is currently only one mandate within the European Union for NAT testing of blood components prior to release, which is from the Paul Ehrlich Institute of Germany (PEI, 1998). This requires platelet and red cell components to have a valid NAT test for HCV before release in Germany. It also states that the sensitivity of the test used must be equivalent to 5000 IU/ml per donation. This means that if the plasma samples are pooled before testing, the dilution of any positive donation must be taken into account, i.e. to test a pool of 100 plasma samples the assay sensitivity must be at least 50 IU/ml.

The NBS decided that the use of commercial reagents was preferable to an 'in house' assay, to avoid excessive quality control (QC) testing of the assay reagents. At the time the most sensitive commercially available assay for the detection of HCV RNA in plasma was the Roche HCV Amplicor. There was an

automated amplification and detection available for this assay, the COBAS Amplicor (DiDomenico *et al.*, 1996), but no automated sample extraction. The only high throughput automated sample extraction available was the BioRobot 9604 from Qiagen. It was decided to investigate the compatibility of HCV RNA extracted from plasma using the BioRobot with amplification and detection using the Roche COBAS. Although the initial investigations took place using the Qiagen manual spin column method and the Roche manual micro well plate (MWP) method, to determine if the technologies were compatible. It was felt necessary to combine Qiagen and Roche methods to achieve a completely automated assay for several reasons: namely, with the large number of donations requiring testing, the BioRobot had the ability to process samples faster than the manual Roche method. The robotic methods also had electronic sample tracking available throughout and therefore the possibility for human errors was greatly reduced. However, the disadvantage in substituting the Roche extraction with the Qiagen extraction is that it contravenes the manufacturer's instructions and nullifies their liability for the assay; in this case the user must take on the manufacturer's role and become responsible for the assay as a whole.

It was decided to test plasma pools containing 96 donations each, which were generated with a Tecan Genesis robotic sample processor (RSP). The RSP was also used to add an aliquot of each plasma sample to an 8 by 12 format deep well plate, which was then kept as an archive. The 96 donation minipools were tested by NAT and if any positive mini-pools were detected, a cross pooling system was used to identify the individual positive donation within the pool (Mortimer, 1997). The positive pool was referred back to the pooling laboratory where cross-pools were made from the archive plate. The rows and columns of the 96 well plate were pooled giving 12 cross-pools of 8 donations each, and 8 cross-pools of 12 donations each. These 20 cross-pools were then NAT tested, and the positive row and positive column pools were used to locate the position in the archive plate of the positive sample, which was then checked by NAT individually. Using this system the positive sample in a minipool of 96 could be identified using 20 tests, rather than testing all 96 samples. The details of this testing system are discussed further in Chapter 6.

1.4 NAT Methods

The various NAT methods have three steps in common: 1) sample preparation, including viral concentration and nucleic acid extraction; 2) amplification of the target DNA or RNA; and 3) detection of the amplified product. The various extraction methods generally have a viral lysis step in which the protein coat of the virus is disrupted and the nucleic acid is released. The viral nucleic acid is then purified by biochemical methods, captured by either sequence specific probe capture methods or non-specific binding of nucleic acids to silica particles. Once the nucleic acid is captured in this way the lysis buffer and other impurities are washed away and the nucleic acid is re-suspended. Amplification is then achieved by one of the several methods that will be discussed later. Typically these will generate up to a billion-fold amplification of the target nucleic acid sequence, enabling detection of ten or fewer copies of viral nucleic acid in the starting sample. The amplified sequences can then detected by a variety of methods including colourimetric, chemiluminescent or fluorescent technologies.

1.4.1 Polymerase Chain Reaction (PCR)

PCR was the first DNA amplification method devised and is still the most versatile and widely used NAT technique. It was devised by Kary Mullis of Cetus Corporation (Emeryville, CA USA); (Mullis *et al.*, 1986; Mullis and Faloona, 1987) who received the 1993 Nobel Prize in chemistry in recognition. PCR was first described for the amplification of the beta globin gene in the diagnosis of sickle cell anaemia (Saiki *et al.*, 1985). The polymerase enzyme was destroyed by the temperature required for denaturation and therefore had to be replaced after each PCR cycle, however, the discovery of a thermostable polymerase enzyme from a bacteria that lives in hot pools, *Thermophilus aquaticus* (*Taq*) allowed the PCR process to be repeated for many cycles without the need to replace the enzyme (Saiki *et al.*, 1988).

The main steps of PCR are shown in Figure 1.6. Target DNA or cDNA molecules are denatured into single strands by heating the reaction mixture to around 94°C, the temperature is then reduced to allow synthetic oligonucliotide primers complimentary to the area of interest to bind to each of the single-stranded DNA molecules. The temperature is then raised to the optimum for strand extension by *Taq* DNA polymerase; (Mullis and Faloona, 1987). Two such primers, binding to the sense and antisense strands are used to copy the complimentary sequence of the target DNA. By cycling the temperature between denaturation, primer annealing and extension steps for 30 or more cycles, a greater than 10^9 fold amplification of the target sequence can be achieved. The amplified sequences (amplicons) can be detected by a number of methods such as visualisation with ethidium bromide on an agarose gel or by using sequence specific probes labelled with radioactive or chemiluminescent tags with enzymes which can catalyse colour change or light production reactions.

PCR may be used to detect the DNA sequences of viruses directly (Ou *et al.*, 1988; Larzul *et al.*, 1988; Kaneko *et al.*, 1989), however for detection of RNA viruses such as HCV a reverse transcription step must be used (Murakawa *et al.*, 1988; Garson *et al.*, 1990). Reverse transcription of an RNA target is carried out to generate complimentary DNA (cDNA) which can then be used as a template for PCR (Kawasaki *et al.*, 1988).

Roche Molecular Systems Inc. (Pleasanton, CA, USA) purchased the patent rights to PCR from Cetus in 1991 and has developed commercial assays for the detection or quantification of several viruses in peripheral blood mononuclear cells, plasma or other fluids (Young *et al.*, 1993; Vrielink *et al.*, 1997; Long *et al.*, 1998; Sun *et al.*, 1998; Gerken *et al.*, 1998). Each assay is capable of detecting as few as one to 100 molecules of target DNA or RNA in the processed sample volume, and each uses primers and probes recognising the conserved sequences of one or more viral genes to ensure the detection of most known variants.

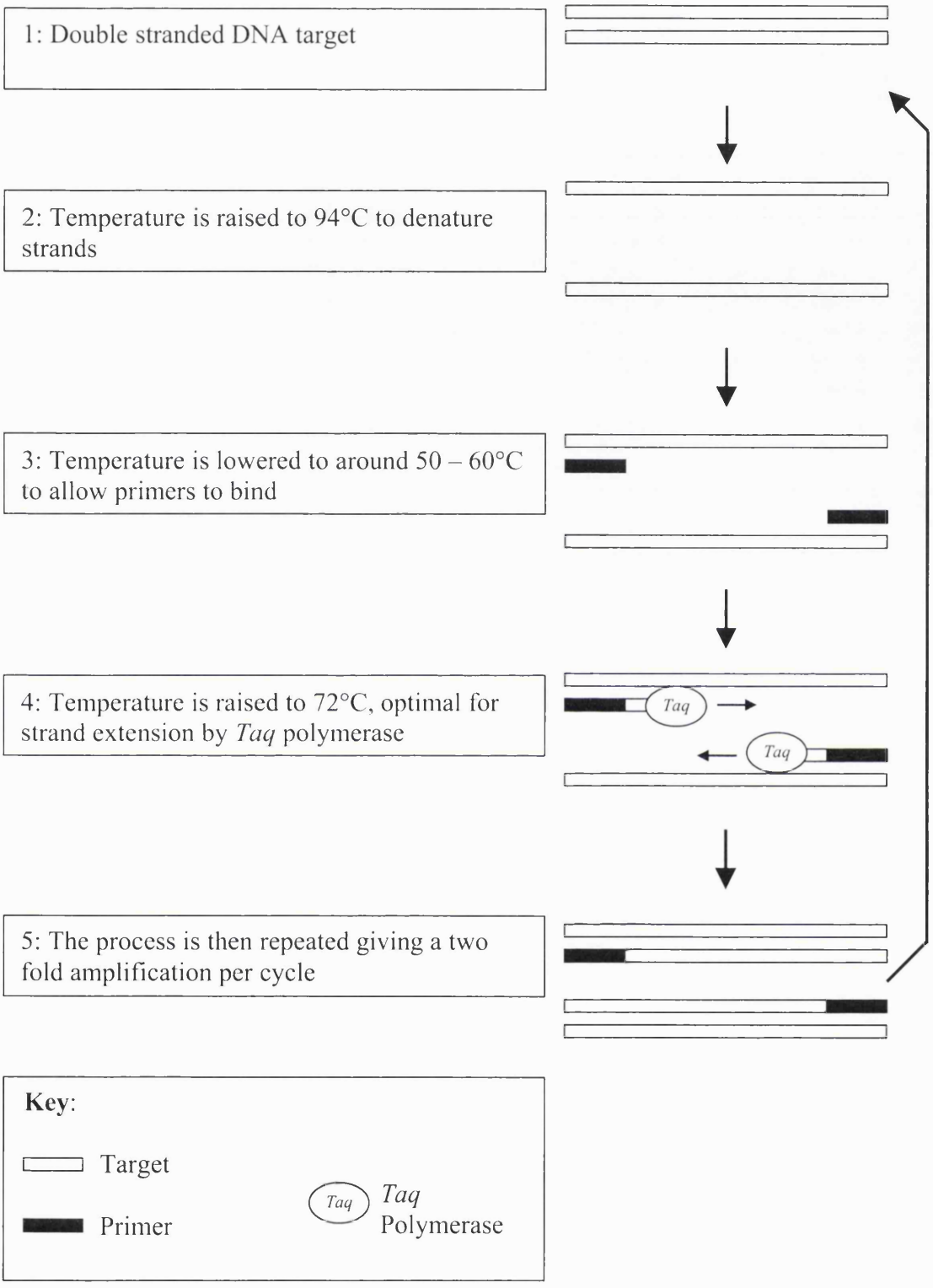

Figure 1.6: Polymerase Chain Reaction (PCR)

Real-Time PCR

Real time PCR is based on the conventional PCR reaction for the amplification of target molecules; the difference is that the amount of PCR amplicon is measured during the reaction i.e. in real time, rather than at the end of the reaction. There are several methods by which this can be achieved but the most well known is TaqMan PCR (Lee *et al.*, 1993).

In a TaqMan PCR the amount of amplicon is measured using a dual fluorescent-labelled oligonucleotide probe. The probe has a fluorescent dye with a different excitation and emission wavelength attached to each end, these dyes are designated reporter and quencher. The excitation wavelength of the quencher is similar to the emission wavelength of the reporter and so when the reporter and quencher are held in close proximity the energy emitted by the reporter is absorbed by the quencher in a process known as fluorescence resonance energy transfer (FRET); (Förster, 1948).

The dual labelled probe is designed so that its melting temperature is higher than that of the two primers. This ensures that when the temperature is reduced between the denaturation and annealing steps of the PCR the probe will bind to the target sequence before the primers. During primer extension the bound dual labelled probe is hydrolysed by the 5′ exonuclease activity of the *Taq* polymerase (Holland *et al.*, 1991). As the dual labelled probe is hydrolysed the reporter and quencher dyes are released and so FRET no longer occurs and the light is emitted by the reporter fluorophore. The dual labelled probe is hydrolysed during each round of PCR and so the fluorescence emitted by the un-quenched reporter increases. The increase in fluorescence at the emission wavelength of the reporter dye is measured after each cycle of PCR and is proportional to the amount of amplicon generated in the reaction.

A variation called kinetic PCR (kPCR) has also been developed which is based on the intercalation of ethidium bromide or SYBR Green dyes (included in each PCR reaction tube) into the double-stranded DNA that accumulates during each

PCR cycle (Higuchi *et al.*, 1993). This results in exponentially increasing fluorescence in the tube that parallels PCR product formation and can be monitored in real time.

The advantage of these systems is that amplification and detection of nucleic acid is fully automated, occurring in a closed tube. There is no need for post PCR amplicon manipulation which reduces the time taken and minimises the possibility of contamination of other samples by amplicons.

1.4.2 Transcription mediated amplification (TMA)

TMA is an isothermal amplification method that is patented by Gen Probe Inc (San Diego, CA, USA). It uses two enzymes, reverse transcriptase and RNA polymerase to achieve greater than one billion fold amplification without thermal cycling (McDonough *et al.*, 1998). A primer with an RNA polymerase promoter sequence attached is used to anneal to the RNA template and prime cDNA synthesis with reverse transcriptase, the RNA is then degraded with RNAse H activity to leave a single strand of DNA which includes the promoter sequence. Another primer is then used to generate the complementary strand. The RNA polymerase enzyme generates 100 to 1000 RNA copies from the DNA template which go through the process again (Figure 1.7).

As both enzymes require the same temperature to function the reaction occurs auto-catalytically under isothermal conditions, with the majority of the amplicon being RNA transcripts.

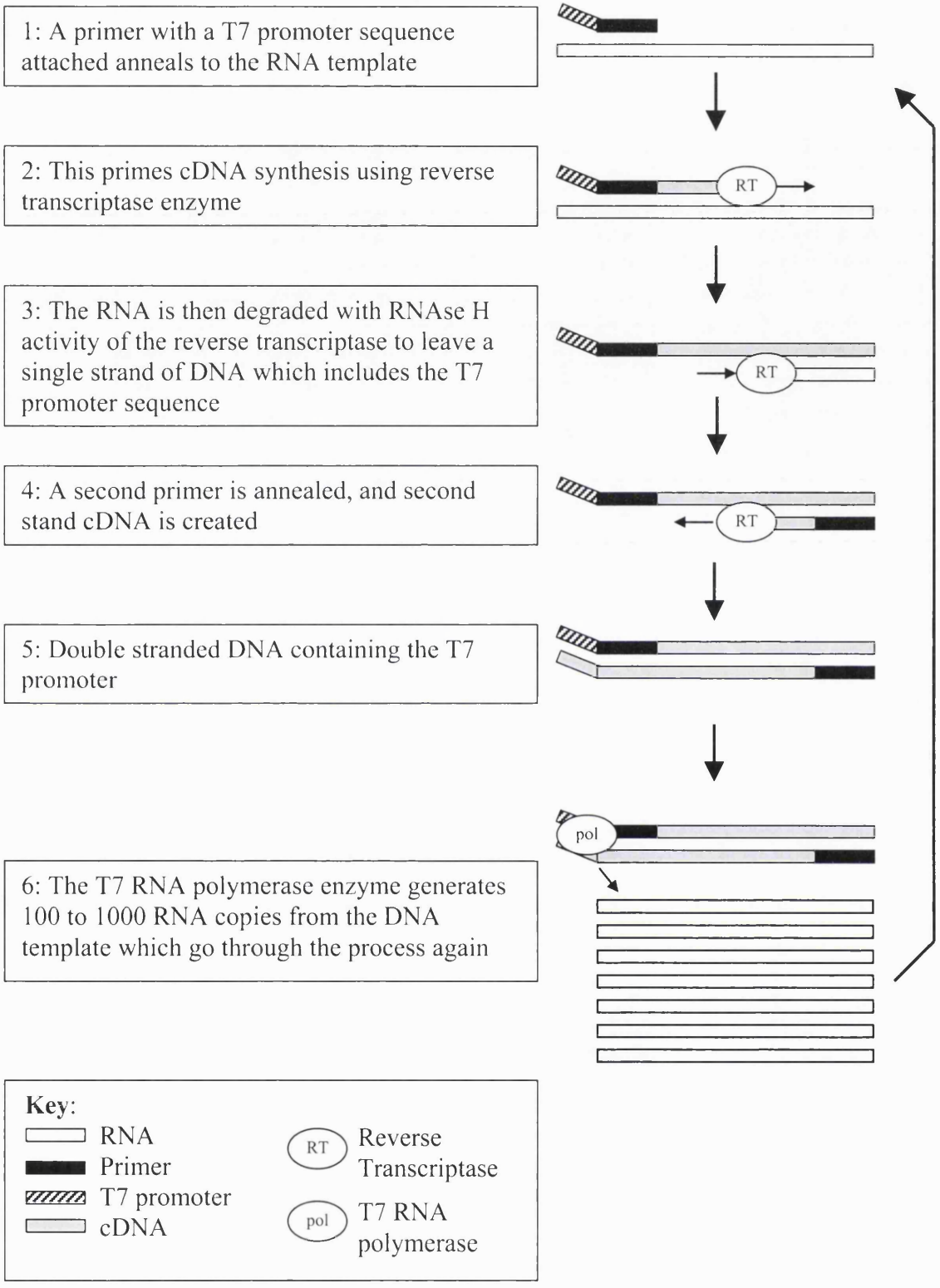

Figure 1.7: Transcription Mediated Amplification (TMA)

1.4.3 Nucleic acid sequence based amplification (NASBA)

Another isothermal amplification system, nucleic acid sequence-based amplification (NASBA) is patented by Organon Teknika (Boxtel, The Netherlands); (Kievits *et al.*, 1991; Compton, 1991). NASBA kits are available for HIV-1 and CMV as well as a basic kit that can be adapted to other viruses by the user (Deiman *et al.*, 2002). NASBA is not used for blood screening, but the extraction technology which uses the chaotropic salt guanidinium isothiocyanate and silica particles (Boom *et al.*, 1990), may be automated with the NucliSens Extractor (van Buul *et al.*, 1998). This extraction method has been used by the blood services of several countries for in house developed NAT methods as the NucliSens Extractor will extract any DNA or RNA from up to 2 ml sample volume, which can then be used in a variety of amplification and detection methods.

1.4.4 Ligase Chain Reaction (LCR)

Ligase chain reaction uses two pairs of probes complementary to each other that hybridise adjacent to each other on each strand of the target nucleic acid sequence, each pair is then joined together by the nick repair mechanism of DNA ligase (Wu and Wallace, 1989). After denaturation both the single stranded target sequence and the ligated probes can act as a template for annealing of further probe sets. Using a thermostable DNA ligase, the reaction can be cycled as in PCR to denature the strands and re-anneal the probes (Figure 1.8), achieving exponential amplification (Barany, 1991).

Abbott Laboratories (Abbott Park, IL) currently markets LCR assays for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using an automated instrument the LCx analyser, but at present LCR is not used for screening for blood borne pathogens.

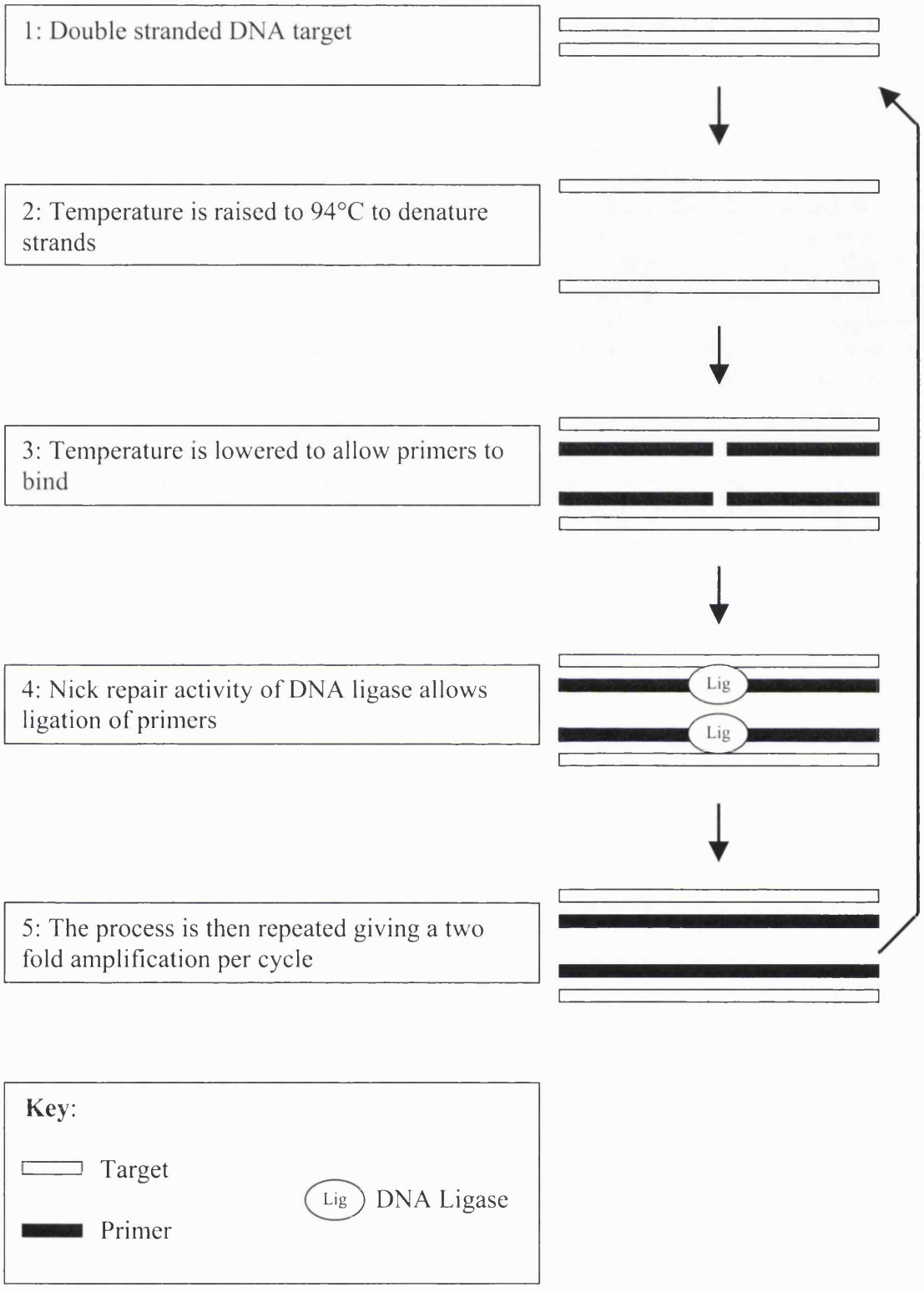

Figure 1.8: Ligase Chain Reaction (LCR)

1.4.5 Branched DNA Signal Amplification Assay (bDNA)

The branched DNA (bDNA) technique was developed by Chiron Corporation (Emeryville, CA), but this technology is now owned and marketed by Bayer Corporation (Emeryville, CA, US). The bDNA assays are based on the principle of amplifying the signal from annealed DNA probes through the formation of controlled network of synthetic oligonucleotide probes, rather than amplification of the target nucleic acid (Urdea, 1997). The method involves a series of hybridisations of target probes specific to the sequence of interest (e.g., the HIV assay uses 39 binding sites to *gag* or *pol* sequences); (Figure 1.9). A subset of the probes mediate capture of the target molecules to a capture oligo modified microplate, while the second set bind to target as well as to a bDNA amplifier molecule. The bDNA amplifier is a chemically synthesized structure containing 45 copies of a sequence that can bind a labelled probe, but just one copy of a sequence that binds to target probes. Multiple copies of bDNA amplifiers are bound to the probe-target complex on the microplate to incorporate up to 1,800 labelled (alkaline phosphatase) probes on each target sequence. A chemiluminescent substrate, dioxetane, is used to generate a visible light signal that is quantified with the use of a luminometer.

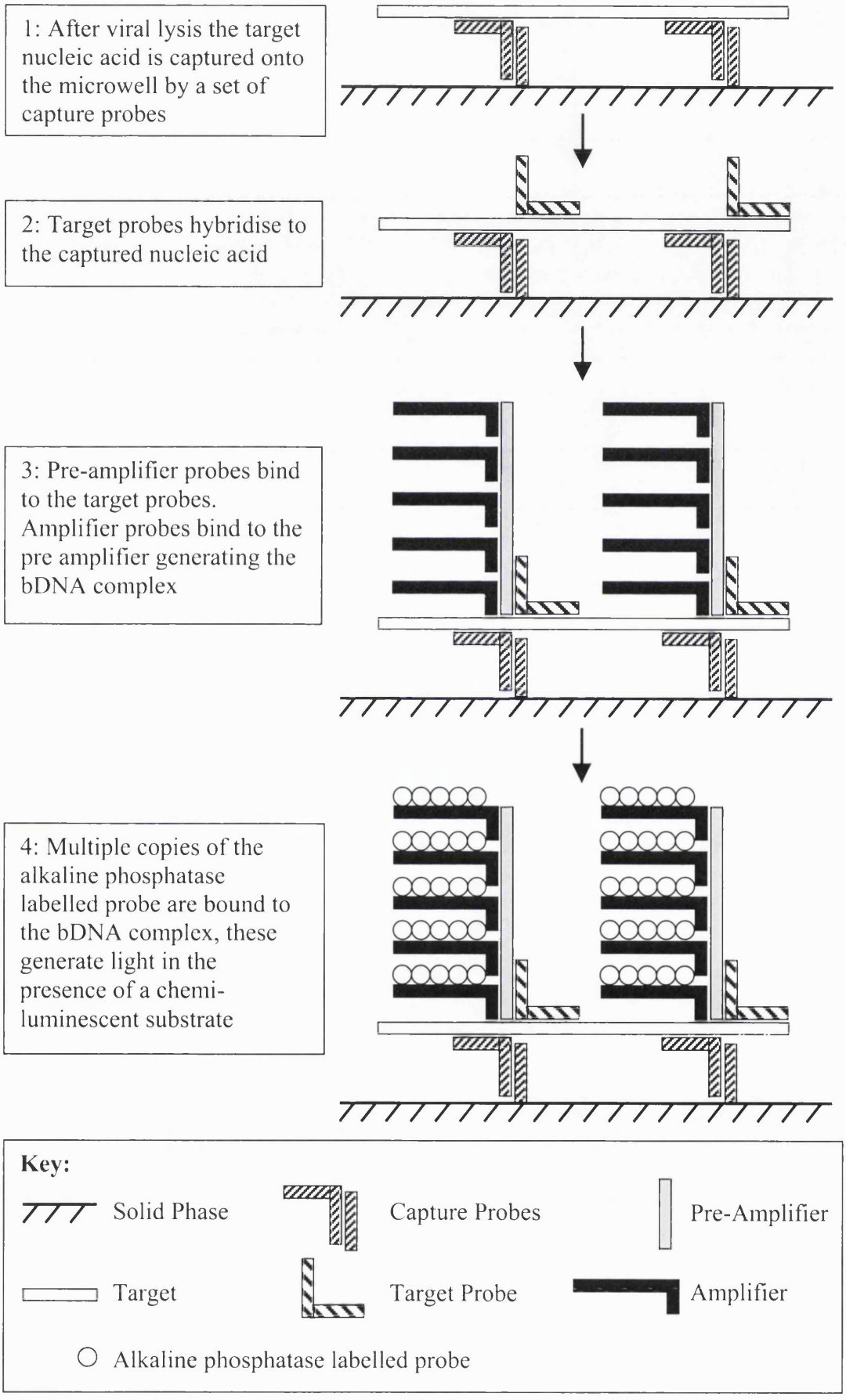

Figure 1.9: Branched DNA signal amplification.

1.5 Aims of the Thesis

This thesis will describe the research that was performed to allow NAT testing to be put into practice within the NBS, and the development of the assay after testing began. The main objective of this thesis was to develop an automated assay using commercial components for the detection of HCV RNA in blood donations for transfusion, and to validate sample handling and storage procedures. This work was undertaken in collaboration with the NBS to develop a system for the national screening blood donations for HCV by NAT.

Studies in the thesis include investigations into:

- The stability of HCV in the plasma sample to define the appropriate sample handling and storage conditions needed to preserve the virus in the blood sample before analysis by NAT.
- The compatibility of the Qiagen and Roche reagents starting with manual assays and then expanding to include fully automated systems.
- The sensitivity, specificity and robustness were defined for the final assay combinations.
- Alternative techniques for virus detection were investigated such as TMA and HCV core antigen.
- Further definition of the window period of the HCV infection in a study of window phase HCV samples from US plasma donors.
- Monitoring of the performance of the assay systems in routine use within the NBS laboratories including development of Microsoft Excel spreadsheets with macros to automate the analysis of raw data.
- Development of in house assays such as quantitative HCV for use in the studies described above, as well as for use in a reference role.

Chapter 2

General Methods and Development of Research Techniques

This chapter describes the general methods used in this thesis and also the development of research assays used in the studies described hereafter. However the main aim of this thesis, the development of an automated HCV NAT assay is described fully in Chapter 4.

2.1 General Methods

2.1.1 Extraction of viral RNA from plasma using spin columns – Qiagen QIAamp Viral RNA purification protocol

The QIAamp Viral RNA spin column purification kit was obtained from Qiagen Ltd, Crawley, West Sussex, UK. This extraction method is based on the silica and guanidinium thiocyanate method of Boom (Boom *et al.*, 1990). In the QIAamp method the silica is bound into a membrane within the spin column so that the method is more straightforward to perform. The principles behind the QIAamp method are further explained in section 4.1.1.

The manufacturer's original instructions (April 1997) were as follows: 140 µl of plasma were added to 560 µl of viral lysis buffer (AVL), containing 26.6 µg/ml carrier RNA. This mixture was incubated at room temperature (15 to 25°C) for 10 minutes after which 560 µl of 99.7-100% AnalaR ethanol (BDH, Dorset, UK) were added. Half (630 µl) of this mixture was spun through the column into a collection tube by centrifugation at 6000 x *g* for 1 min, this eluate was discarded and the step repeated with the remaining 630 µl.

The spin column was then washed with 500 µl of wash buffer (AW) centrifuged through the column into a collection tube at 6000 x *g* for 1 minute. The wash buffer was discarded and the step repeated with 500 µl AW at 20 000 x *g* for 3 minutes to ensure that all the wash buffer had cleared through the column. The RNA was eluted from the column in 50 µl of RNAse free water (preheated to 80°C) by centrifugation at 6000 x *g* for 1 minute. The manufacturer claimed that a 5-10% increase in RNA yield may be achieved by performing a second elution step using the eluate from the first step and therefore this extra step was routinely performed.

The manufacturer changed the above protocol in January 1999 as follows; the amount of carrier RNA in buffer AVL was reduced to 10 µg/ml. The two wash steps with buffer AW were replaced by a wash step with buffer AW1 and a wash step with buffer AW2. A spin with a dry collection tube after the second wash was added as an optional step to ensure that no wash buffer was left on the outside of the column. The elution was changed to 60 µl buffer AVE at room temperature.

The composition of the buffers used in the above method are proprietary, and therefore cannot be given in this thesis, however information available in the material safety data sheets (MSDS) indicates the following: Lysis buffer AVL contains the chaotropic salt guanidinium thiocyanate. The first wash buffer AW1 contains the chaotropic salt guanidinium chloride. The original wash buffer AW and the second wash buffer AW2 do not contain chaotrope.

2.1.2 Automated extraction of viral RNA from plasma using the BioRobot 9604 - Qiagen QIAamp96 Viral RNA purification protocol

BioRobot reagents for these studies were kindly provided by Qiagen GmbH, Hilden, Germany. The protocol supplied with the BioRobot (the QIAamp 96 viral RNA protocol) for HCV RNA extraction from plasma was performed as follows; 200 µl of plasma were added to 800 µl viral lysis buffer AVL,

containing carrier RNA at a concentration of 7.1 μg/ml, and incubated in a plastic square well block at room temperature for 10 minutes.

After the incubation 800 μl of 100% ethanol were added. Half of this mixture was then transferred to a 96 well QIAamp plate on a vacuum manifold and a vacuum was applied to the lysate. This step was repeated with the other half of the lysate mixture. The plate was washed with 900 μl wash buffer AW1 and then with 900 μl AW2. The QIAamp plate was then transferred to a centrifuge by the user and spun at 6000 rpm for 10 minutes to dry the plate. The QIAamp plate was placed back on the BioRobot fitted onto a collection microtube rack and 60 μl RNAase free water were added, this was spun through the membrane by centrifugation at 6000 rpm for 3 minutes.

2.1.3 Automated extraction of viral nucleic acid from plasma using the BioRobot 9604 – Qiagen QIAamp96 Virus purification protocol

BioRobot reagents for these studies were kindly provided by Qiagen GmbH, Hilden, Germany. The manufacturer's protocol (06/2000) was performed as follows: 40 μl of protease were added to a square well block, 200 μl plasma sample were then added followed by 200 μl lysis buffer AL containing 28 μg/ml carrier RNA. The lysis mixture was incubated on a heating block for 10 minutes at 56°C and then transferred to 275 μl ethanol in another square well block at room temperature.

The mixture was transferred to the QIAamp plate on a vacuum manifold, and 250 μl of wash buffer AW1 were added on top to reduce foaming as the mixture was passed through the membrane by vacuum. The wells of the QIAamp plate were washed with 360 μl of wash buffer AW1 by vacuum, followed by two washes with wash buffer AW2, the first with 1000 μl and the second with 1100 μl. The QIAamp plate was then covered with porous tape and transferred to a centrifuge by the user and spun at 6000 rpm for 10 minutes to dry. The QIAamp plate was placed back on the BioRobot, fitted onto a collection microtube rack and 87 μl

elution buffer AVE were added. After sealing the plate with plastic tape to avoid aerosol contamination, the eluate was collected from the membrane by centrifugation at 6000 rpm for 3 minutes.

2.1.4 Roche Amplicor HCV test (versions 1.0 and 2.0)

The Roche Amplicor HCV test was obtained from Roche Diagnostics Ltd. Lewis, East Sussex, UK or kindly provided by Roche Molecular Systems Inc. Pleasanton CA, USA. The manufacturer's instructions (01/1997) for the Amplicor v1.0 test were performed as follows: 100 µl of plasma sample were added to 400 µl HCV lysis buffer in labelled tubes. For the controls 50 µl HCV (+) or HCV (-) control were added to 100 µl NHP in 400 µl HCV lysis buffer.

All tubes were mixed by vortexing and incubated for 10 minutes at 60°C. Propanol (500 µl BDH-100% pure) was then added to each tube, mixed by vortexing and incubated at room temperature for 2 minutes. Samples were centrifuged at between 13 000 and 16 000 x *g* for 15 minutes at room temperature with an orientation mark on the tube facing outwards to show the position of the pellet.

The supernatant was discarded using a fine tip plastic transfer pipette with care being taken to avoid the pellet. The pellets were washed with 1 ml of 70% ethanol (freshly prepared daily from 99.7-100% AnalaR ethanol and sterile water) by vortexing briefly. The tubes were centrifuged at between 13 000 and 16 000 x *g* for 5 minutes at room temperature with the orientation mark facing outwards. The supernatant was removed as before and the pellet was re-suspended in 1 ml of specimen diluent containing internal control (IC) by scraping the pellet from the side of the tube with a pipette tip.

At this point the RNA was either stored frozen at -80°C for up to a month or amplified and detected in the following way: 50 µl of the processed specimen or control were added to 50 µl PCR master mix in 0.2 ml thin walled PCR tubes

(ABgene, Epsom, Surrey, UK). The PCR was performed in a GeneAmp PCR system 9600 (Applied Biosystems, Warrington, Cheshire. UK.) in a post-PCR containment laboratory.

Initially the tubes were held at three temperatures as follows: 50°C for 2 minutes, 60°C for 30 minutes, 95°C for 1 minute. This was followed by 2 cycles of 95°C for 15 seconds and 60°C for 20 seconds, and then by 38 cycles of 90°C for 15 seconds and 60°C for 20 seconds. Finally the reactions were held at 72°C for no more than 15 minutes, before being removed from the machine. All reactions were immediately denatured with the addition of 100 µl of NaOH (denaturation solution) followed by an incubation for 10 minutes at room temperature.

At this point the reactions were held for up to a week at 4°C or continued into the detection reaction as follows: 100 µl hybridisation buffer were added to the appropriate number of wells in the microwell plate (MWP). MWPs coated with HCV or IC capture probes were used for the detection of HCV and IC respectively. The denatured PCR amplicons (25 µl) were added to the HCV and IC MWPs and mixed by gently tapping the plate until the colour changed to a pale yellow. The MWP was covered and incubated at 37°C for 1 hour to allow the amplicons to bind to the MWP.

After this incubation the MWP was washed in an automated washer with wash buffer diluted to working strength in deionised water. The wells were filled with 350 µl wash buffer, allowed to soak for 30 seconds and then aspirated dry. The wash was repeated five times in total. After tapping dry on a paper towel 100 µl of avidin-horse radish peroxidase (AV-HRP) conjugate were added to the MWP, which was then incubated at 37°C for 15 minutes. The plate was washed as before and 100 µl of working TMB substrate were added. This was prepared by adding 0.25 ml of TMB concentrate to 1.0 ml of substrate diluent per 8 well MWP strip. The colour was allowed to develop for 10 minutes in the dark at room temperature, after which the reaction was stopped with the addition of 100 µl sulphuric acid (stop reagent).

The optical density was read at 450nm in a spectrophotometer and the results interpreted as in Table 2.1 and Table 2.2. However in the case of the very low level HCV standards analysed for determination of endpoint sensitivity HCV, A_{450} values ≥0.25 with a valid IC result were considered HCV positive.

The protocol for version 2.0 of the test (03/1998) was changed from the above in the following ways: the internal control was added to the lysis reagent instead of the sample diluent with the addition of 100 µl of internal control to a bottle of lysis reagent (enough for 12 specimens).

The amount of sample added to the working lysis reagent was increased to 200 µl. Positive and negative controls used 200 µl NHP with 20 µl of the HCV (+) or HCV (-) reagent. The amount of 100 % propanol added after the 10 minute incubation was increased to 600 µl. The centrifugation and wash steps were carried out as before, but the amount of sample diluent was reduced to 200 µl.

For the amplification step the master mix required activation with the addition of 100 µl manganese solution to one tube of master mix. The 100 µl reactions were made up by adding 50 µl of the processed specimens or controls to 50 µl of the activated PCR master mix in 0.2 ml thin walled PCR tubes (ABgene). These were taken into the post-PCR containment laboratory and cycled in a GeneAmp PCR system 9600. Initially the tubes were held at two temperatures as follows: 50°C for 5 minutes, 62°C for 30 minutes. This was then followed by 37 cycles of 90°C for 10 seconds and 58°C for 25 seconds. Finally the reactions were held at 91°C until they were removed from the machine (after no more than 3 hours) and denatured as before.

The rest of the detection reaction was performed as before with the interpretation of the results as in Table 2.1 and Table 2.2. However as before, in the case of the very low level HCV standards analysed for determination of endpoint sensitivity HCV, A_{450} values ≥0.25 with a valid IC result were considered HCV positive.

Table 2.1: Interpretation of Amplicor MWP results

Amplicor MWP v1.0		Amplicor MWP v2.0		Interpretation
HCV OD_{450}	IC OD_{450}	HCV OD_{450}	IC OD_{450}	
<0.25	≥0.6	<0.3	≥0.3	HCV negative
<0.25	<0.6	<0.3	<0.3	Invalid / inhibitory
≥0.6	Any	≥1.0	Any	HCV positive
≥0.25, <0.6	Any	≥0.3, <1.0	Any	Equivocal result [a]

[a]Equivocal results were retested in duplicate and the results were interpreted as in Table 2.2

Table 2.2: Interpretation of equivocal Amplicor MWP results

Amplicor MWP v1.0		Amplicor MWP v2.0		Interpretation
HCV OD_{450}	IC OD_{450}	HCV OD_{450}	IC OD_{450}	
2/3 results ≥0.4	Any	3/3 results ≥0.3	Any	HCV positive
2/3 results <0.4	2/3 results ≥0.6	1/3 or 2/3 results <0.3	2/3 results ≥0.3	HCV Negative
2/3 results <0.4	2/3 results <0.6	1/3 or 2/3 results <0.3	2/3 results <0.3	Indeterminate

The COBAS version of the Amplicor HCV v2.0 test was the same as above except the amplification and detection stages were performed in the COBAS instrument. The preparation of the master mix was the same as above but the PCR was run in 12 tube A-rings, two of which can be amplified in one COBAS at one time. The denaturation reagent, HCV and IC magnetic microparticle mixtures, conjugate, substrate reagents were located in reagent cassettes loaded onto the instrument.

The HCV and IC magnetic microparticle mixtures and the substrate reagent were prepared before use with the addition of 2.5 ml HCV and IC concentrate or 5 ml of the substrate concentrate, to the respective diluent buffer in the cassettes. Once the HCV and IC reagents were made up they were used within one month, the working substrate reagent was used within 16 hours. The other reagents were ready to use as supplied. The results were interpreted by the COBAS and printed.

2.1.5 Roche COBAS Amplicor HCV Monitor test version 2.0

The procedure for the COBAS Amplicor HCV Monitor test is similar to the Amplicor HCV test, but the Monitor test is a quantitative assay. The protocol (05/2000 rev 3.1) was performed as follows: 100 µl of each test sample or control were added to 400 µl of working lysis reagent (made by adding 100 µl quantitation standard (QS) to one bottle of lysis reagent) in a 1.5 ml tube. The high positive H(+), low positive L(+) and negative (-) controls were used as follows: 100 µl negative human plasma were added to 400 µl working lysis reagent, and 100 µl of the H(+), L(+) or (-) control were added. The rest of the extraction procedure was the same as that described for the Amplicor HCV v1.0 test (Section 2.1.4) with the exception of the re-suspension of the pellet in 1 ml of specimen diluent, which did not contain IC as in the v1.0 Amplicor HCV test.

For the amplification step the master mix required activation with the addition of 100 µl manganese solution to one tube of master mix. The 100 µl reactions were made up by adding 50 µl of the processed specimens or controls to 50 µl of the

activated PCR master mix in a COBAS A-ring, and placed in the COBAS instrument for amplification and detection. The COBAS reagents were made up as described for the HCV COBAS Amplicor test, although the Monitor test required different COBAS cycling and detection parameters supplied by Roche as bar codes. The results were calculated by the COBAS using the concentration of the QS and the acceptable values for the high and low positives that were entered by the user.

2.1.6 Roche COBAS AmpliScreen HCV test version 2.0

The COBAS AmpliScreen HCV test version 2.0 protocol (dated 04/2000) is intended for blood screening purposes and as such contains a validated pooling protocol based on pools of 24 samples. Two extraction procedures are given in the kit insert, the multiprep procedure is for extraction of nucleic acid from pooled samples and the standard procedure is intended for single samples and is used in the resolution of positive pools. As this assay was only assessed for its sensitivity rather than being used to detect HCV in blood donations the pooling protocol was not used, but both the multiprep and standard extraction procedures were investigated.

The multiprep extraction was performed as follows: 1000 µl of sample, or NHP for the controls, were centrifuged at 23 600 x *g* for an hour at 4°C in a pre-cooled centrifuge. Working lysis reagent was made by adding 100 µl of IC to one bottle of lysis reagent and mixing. After the centrifugation of the samples, 900 µl of supernatant were discarded and 600 µl working lysis reagent were added to the 100 µl specimen remaining and mixed by vortexing. Controls were made by adding 20 µl of positive or negative control reagent to the appropriate tube containing NHP and working lysis reagent and mixed by vortexing. After an incubation for 10 minutes at room temperature, 700 µl of 100% propanol were added to each tube and the contents mixed by vortexing. Samples were then centrifuged at between 14 250 ± 1750 x *g* for 15 minutes at room temperature with an orientation mark on the tube facing outwards to show the position of the

pellet. The supernatant was discarded using a fine tip transfer pastette being careful to avoid the pellet. The pellets were washed with 1 ml of freshly prepared 70% ethanol by vortexing briefly. The tubes were centrifuged for 5 minutes at room temperature at 14 250 ± 1750 x *g* with the orientation mark facing outwards. The supernatant was removed as before and the pellet was re-suspended in 200 µl of specimen diluent, by scraping the pellet from the side of the tube with a pipette tip and vortexing.

The standard extraction procedure was the same as the multiprep procedure with the following exceptions: There was no centrifugation of the samples before processing. The extraction was performed with 200 µl of sample or control. The volume of propanol added was increased to 800 µl. The rest of the procedure was the same as the multiprep method.

The amplification and detection steps were performed as described for the COBAS version of the Amplicor HCV v2.0 test in Section 2.1.4, although the AmpliScreen test required different COBAS cycling and detection parameters as supplied by Roche.

2.1.7 The Ortho HCV core antigen ELISA assay.

The prototype HCV core antigen enzyme linked immunosorbent assay (ELISA) assay (Ortho Clinical Diagnostics, Raritan, NJ, USA) detects free HCV core antigen and therefore may only be used for the detection of HCV during the window phase of infection. The protocol was performed as follows: 100 µl sample or control were added to 100 µl of specimen diluent in a microtitre plate leaving well A1 as a blank. Three negative and two positive controls (as supplied) were run on each assay. The plate was incubated for 90 minutes at 37°C in a shaking incubator (supplied by Ortho) with the shaking set at 700 +/- 25 rpm. The plate was then washed by an automated plate washer using Ortho 1x wash buffer (made up from 20x stock in deionised water) with six cycles of 300 µl/well with a 20 second soak time between washes. After tapping the plate dry,

200 µl conjugate were added to all wells except A1, the plate was incubated in the Ortho incubator at 37°C for 30 minutes, and then washed as before.

Working substrate solution was prepared during the 30 minute incubation by adding four tablets of o-phenylenediamine (OPD) to 24 ml Ortho substrate buffer and allowing to dissolve for five minutes. After the incubation and wash steps 200 µl substrate were added to all wells, including A1 and the colour was allowed to develop at room temperature for 30 minutes in the dark. The reactions were stopped with 50 µl Ortho stop solution (2M sulphuric acid) and the plate was read at 492 nm using a 620 nm reference filter.

The results were interpreted as follows: The run was considered valid if the reagent blank absorbance value was between ≥0.000 and ≤0.050, at least 2/3 negative calibrator absorbance values were between ≥-0.010 and ≤0.012, and both of the positive control absorbance values were between ≥0.083 and ≤2.500. The cut off for the assay was then calculated as the mean of the negative calibrators + 0.040.

2.1.8 The Ortho Trak-C quantitative core antigen assay.

This assay has an additional dissociation step and therefore measures total antigen rather than free antigen i.e. it is able to detect HCV core antigen in the presence of anti-HCV antibodies and therefore may be used for testing post sero-conversion samples. An HCV antigen standard was also supplied to allow quantification.

The manufacturer's protocol (07/02/02 v3.0) was performed as follows: 100 µl sample or control (high positive, low positive and negative), but not the standard curve dilutions, were added to an uncoated microtitre plate and 50 µl pre-treatment buffer were added to each. The standard curve was made up of dilutions of a purified antigen standard and therefore did not require dissociation. The plate was covered with an adhesive plate sealer and mixed in an Ortho

shaking incubator for 1 minute (with the shaking set at 900 rpm). The plate was incubated at 56°C for 30 minutes during which the antibody-antigen complexes were dissociated. After incubation the plate was removed, allowed to cool to room temperature for 10 minutes and then mixed for 1 minute in the Ortho incubator.

The capture antibody coated assay plate was removed from its pouch and 100 µl reaction buffer were added to the required wells except well A1 which was left as a blank. The diluted standard curve, and the pre-treated samples and controls (100 µl of each) were added to the assay plate, which was then sealed and incubated at 25°C for 60 minutes in the Ortho shaking incubator. After the incubation the assay plate was washed by an automated plate washer using Ortho 1x wash buffer and urea (made up from 20x wash buffer stock in deionised water and adding 81g urea [supplied] to 3 litres) with 6 cycles of 300 µl/well with a 20 second soak time between washes.

After tapping the plate dry, 200 µl working conjugate (10 µl conjugate concentrate to 1 ml conjugate diluent) were added to all wells except A1, the plate sealed and incubated at 25°C for 30 minutes without shaking. The plate was washed as before and 200 µl working OPD solution (1 OPD tablet to 6 ml substrate buffer) added to all wells of the plate including A1. The colour was allowed to develop for 30 minutes at room temperature in the dark, after which the reaction was stopped with the addition of 50 µl 2M sulphuric acid.

The plate was read at 492 nm with a 620 nm reference filter. The run was considered valid if the reagent blank absorbance value was <0.030, and with the blank subtracted from all other values: the 0 pg/ml standard ≤ 0.050, the 100 pg/ml standard ≥ 0.900 and ≤ 2.200, and the difference between the 1.5 pg/ml standard and the 0 pg/ml standard was ≥ 0.010. The high and low positives were also required to be within set ranges specified for each reagent lot. To calculate the quantitative antigen values for the samples, linear regression was performed on concentration versus the OD of the standard curve using Microsoft Excel

2000. The ODs of the unknown samples were then calculated using the Trend worksheet function of Microsoft Excel 2000.

2.1.9 The Bayer TMA HCV assay

The TMA assay (section 1.4.2) was performed according to the manufacturer's protocol as follows: 500 µl of plasma sample or calibrator was added to 400 µl of target capture reagent (containing internal control) in a ten tube unit (TTU). The TTU was sealed, vortexed for 20 seconds and incubated for 20 minutes at 60°C in a waterbath. After the incubation the rack was allowed to cool to room temperature for 15 minutes before being placed onto the magnetic target capture system. The magnetic beads were allowed to separate in the magnetic field for 10 minutes, after which the supernatant was aspirated and 1ml of wash solution was added. The magnetic beads were re-suspended in the wash solution by vortexing for 20 seconds and the rack was placed back on to the magnetic target capture system. After five minutes the supernatant was aspirated and the wash step was repeated as before.

After the aspiration of the second wash the rack was removed from the target capture system and 75 µl of amplification reagent was added and covered with 200 µl of mineral oil. The tubes were sealed and vortexed for 20 seconds after which they were incubated at 60°C for 10 minutes in a waterbath. After this incubation the rack was transferred to a 45°C waterbath 25 µl enzyme was added the tubes were re-sealed and incubated for 60 minutes to allow the TMA reaction to occur.

After the amplification was complete the rack was moved to another part of the laboratory for the detection reaction, where 100µl of probe was added. The tubes were sealed, vortexed for 20 seconds and incubated at 60°C for 15 minutes in a waterbath. After the incubation 250 µl of selection reagent was added and the tubes were sealed, vortexed for 20 seconds and incubated at 60°C for 10 minutes in a waterbath. The tubes were then transferred to a room temperature (19°C to

27°C) waterbath and allowed to cool for at least 10 minutes. The tubes were then transferred to the Leader HC luminometer (Gen Probe inc.) and the light output was measured after the automatic injection of auto detect reagents. The results were interpreted by software on a PC connected to the luminometer.

2.1.10 Detection of PCR products by Enzyme Linked OligoNucleotide Assay (ELONA)

The principle of the Enzyme Linked OligoNucleotide Assay (ELONA) assay is as follows: biotin labelled PCR products are added to streptavidin coated microtitre plates and incubated to allow amplicon capture onto the solid phase. The bound products are then denatured with 0.15M NaOH leaving the biotin labelled strands bound to the plate and allowing the unbound strands to be washed away. Alkaline phosphatase labelled oligonucleotides with complimentary internal sequences are then hybridised to the captured products. Any un-hybridised probe is washed away and the substrate (Lumiphos 530; Lumigen Inc. Southfield, MI. USA) is added. The alkaline phosphatase catalyses a reaction that generates light, which can be measured in a luminometer.

The ELONA was adapted from the method of Whitby and Garson (Whitby and Garson, 1995) and was performed as follows: 100 µl ELONA diluent (Appendix A) were added to the PCR products using a multichannel pipette. ELONA diluent (80 µl) was also added to each well of a black streptavidin coated plate (Thermo Labsystems, Ashford, Middlesex, UK). After mixing by aspirating contents up and down three times, 20 µl of the diluted PCR product were added to each well of the streptavidin coated plate in duplicate across the plate using fresh tips for each transfer. The plate was incubated in a plastic box containing damp tissue paper at 45°C for one hour.

After incubation, the product and diluent were aspirated from the wells using a plate washer and 100 µl of 0.15M NaOH were added to each well using a multichannel pipette. The plate was incubated at room temperature for 2 to 5

minutes and then washed in an automated plate washer set to wash each well with 350 µl Tris/Tween/Azide (TTA; Appendix A) 10 times. After the plate was tapped dry on tissue paper, 100 µl of the appropriate alkaline phosphatase labelled oligonucleotide probe diluted in hybridisation buffer (Appendix A) were added. The plate was incubated for 30 minutes in a damp box at 45°C.

After the incubation the plate was washed 20 times as before with TTA in an automated plate washer. The plate was again tapped dry on tissue paper and 100 µl of Lumiphos 530 were added to each well, the plate was then covered with a clear plastic adhesive cover (Anachem Ltd, Luton, Bedfordshire, UK) and incubated in the dark for one hour.

The light output was measured using an automated microtitre plate luminometer (TopCount, Canberra Packard Inc.) in single photon counting mode at 20°C for 6 seconds per well. The data output from the TopCount luminometer was automatically transferred to a PC connected to the departmental local area network (LAN), this data was then transferred to a PC outside the post PCR containment laboratory.

2.1.11 Detection of PCR products by agarose gel electrophoresis:

PCR amplicons were detected by horizontal agarose gel electrophoresis with ethidium bromide staining. A 100 ml 2.5% agarose gel (Appendix A) was prepared and allowed to cool to around 50°C before adding 5 µl 0.5 mg/ml ethidium bromide solution (Sigma-Aldrich Company Ltd. Dorset, UK.). This was then mixed carefully by gentle swirling to avoid bubbles, and poured into a gel tray with tape on the open ends. Gel combs were inserted and the gel was allowed to set on a level surface.

After the gel was set the combs and tape were removed, 10-15 µl of PCR product were added to each well. The DNA size marker ΦX174 digested with *Hae*III or *Hinf* I in blue orange dye; (Promega UK, Southampton, UK) was run on each

row at a concentration of 1 μg. The gel was placed in a horizontal gel running tank and 1X TAE running buffer (Appendix A) was carefully added until just covering the gel. This was added slowly to avoid washing the PCR products out of the wells. The gel was run at 100V with 60 to 70 mA current for 1 hour. After running the gel was removed and visualised on a UV transilluminator. Electronic images of the gel were captured to a PC and exported from the post-PCR containment laboratory via a local area network (LAN).

2.2 Development of HCV quantitative PCR with internal control

2.2.1 Introduction

This method is based on HCV RNA extraction using Qiagen QIAamp technology with either spin columns or the BioRobot 9604. The amplification utilises the Titan one tube RT-PCR system (Roche) which contains a mixture of enzymes for the RT and PCR reactions that are active with a single buffer system so that the entire process can be performed in a single step (Mallet *et al.*, 1995).

The enzymes used are avian myoblastoma virus (AMV) reverse transcriptase for cDNA synthesis from the viral RNA and a mixture of *Taq* and *Pwo* DNA polymerases for the PCR reaction.

Amplicons were detected using the ELONA method (Whitby and Garson, 1995) described in section 2.1.10.

Brome Mosaic Virus (BMV) RNA (Ahlquist *et al.*, 1984) was used as an internal control introduced at the extraction stage in the lysis buffer. BMV is a single stranded positive sense RNA plant virus for which the purified RNA is commercially available (Promega).

Quantification was achieved by comparison of the HCV values or HCV/BMV ratios of the test samples with those of an external standard curve using a Microsoft Excel 2000 spreadsheet with macros for automation of results analysis.

2.2.2 Development of HCV Quantitative PCR

HCV RNA was extracted using the Qiagen QIAamp spin column method (Section 2.1.1). The RNA was reverse transcribed and amplified using the Titan RT-PCR system (Roche) containing primers to the 5′ non-coding region of HCV. The 50 µl reactions were made by adding 10 µl of the Qiagen eluate to 40 µl of Titan RT-PCR mix. The amounts of the components for RT-PCR in the final reaction mix were as follows: 10 µl 5x RT-PCR buffer (as supplied) giving 1.5 mM $MgCl_2$, 0.8 mM of each deoxynucleotide triphosphate (Amersham Pharmacia Biotech UK Ltd. Little Chalfont, Buckinghamshire, UK.), 0.4 µM of the 5′ non-coding region primers PT3BIO and PT4 (Table 2.3), (Garson *et al.*, 1991), 5 mM dithiothreitol (DTT; supplied), 10 units recombinant RNAsin (Promega), and 1 µl Titan enzyme mix containing AMV reverse transcriptase with *Taq* and *Pwo* polymerases.

Table 2.3: Primer and probe sequences.

Name	Sequence	Label	Positions[a]
PT3BIO	5′ AGTGTCGTGCAGCCTCCAGG	5′ Biotin	-242 to -224
PT4	5′ CGGTTCCGCAGACCACTATG	None	-184 to -203
INTAP Probe	5′ ATGGCTCTCCCGGGAGGG	Alkaline Phosphatase	-201 to -218
BMV1.1BIO	5′ TCGAGCACGAAAGAGCCCGG	5′ Biotin	364 to 381
BMV2.1	5′ GCGCCCTTGGAGATAGGAGG	None	415 to 434
BMVAP Probe	5′ GGTTCAACAGTTCACCGATAG	Alkaline Phosphatase	386 to 409

[a]Primer positions are shown for HCV primers (PT3BIO, PT4 and INTAP) with the numbering system of (Choo *et al.*, 1991); Genbank accession No. M62321. Primer positions for the BMV sequences use the numbering from (Ahlquist *et al.*, 1984); Genbank accession No. X01678. Primers and probes were obtained from Oswel, Southampton UK.

The reactions were cycled in a GeneAmp PCR System 9600 thermal cycler in a post PCR laboratory using the following parameters: 50°C for 30 minutes and 94°C for 2 minutes followed by 40 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 68°C for 2 seconds. After a final extension at 68°C for 7 min the reactions were held at 4°C.

PCR products were quantified by a chemiluminescent enzyme linked oligo-nucleotide assay (ELONA), (Whitby and Garson, 1995) described in section 2.1.10 using the INTAP probe (Table 2.3) with an external calibration curve based on an 'in-house' standard. The 'in-house' standard was calibrated in copies per ml against an HCV RNA reference standard (96/586) provided by the National Institute for Biological Standards and Control (NIBSC), (Saldanha and Minor, 1996).

Plotting photon output in counts per second (cps) against HCV level gave a standard curve ranging from 40 copies/ml to 4 000 000 copies/ml (Figure 2.1). The HCV level in test samples was quantified by comparison to the standards using curve fitting software (Genesis, Life Sciences (UK) Ltd).

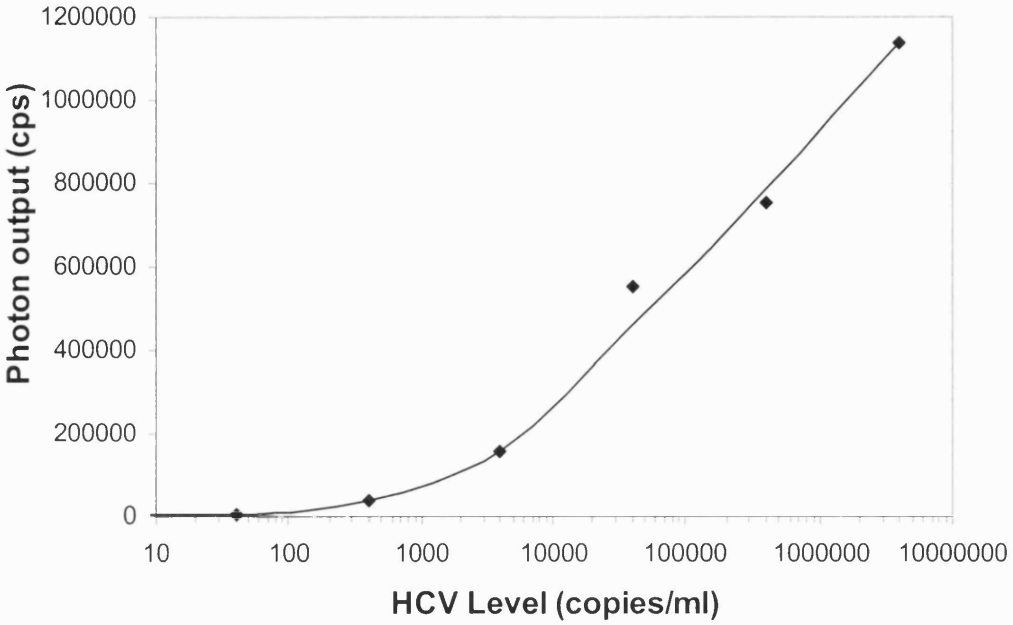

Figure 2.1: HCV Standard Curve with spin column extraction

Using the automated BioRobot QIAamp 96 viral RNA extraction method described in section 2.1.2 in place of the manual QIAamp spin column extraction resulted in similar performance (Figure 2.2).

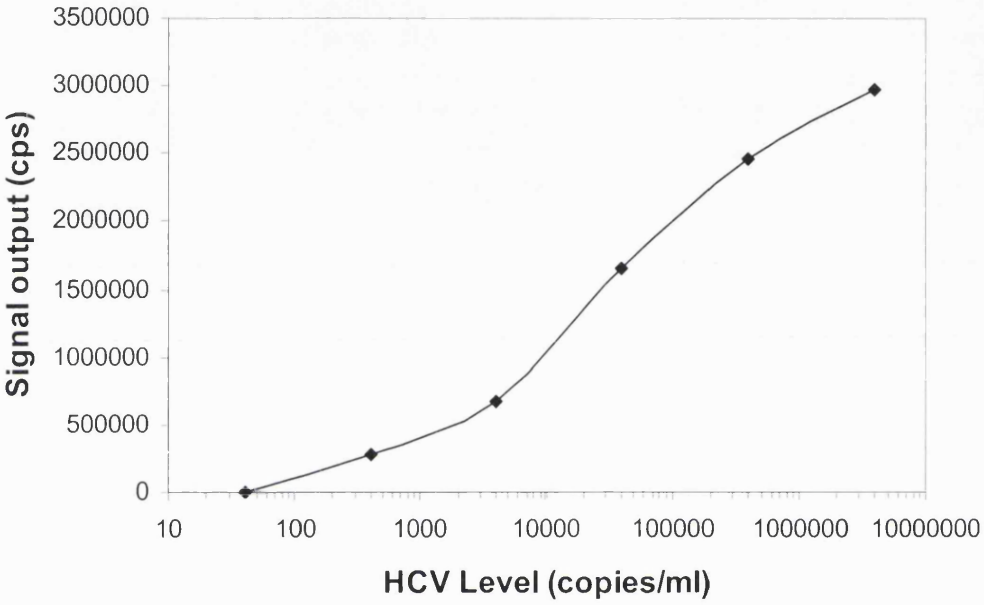

Figure 2.2: HCV standard curve with BioRobot extraction

To determine the variability of quantification using this method 24 replicates of an HCV positive sample were tested and the values compared. The values for the 24 replicates ranged from 51 900 copies/ml to 115 000 copies/ml with a median of 75 900 copies/ml. This represents a difference of 2.2 fold with a coefficient of variation of 22.9%.

2.2.3 Introduction of BMV internal control

BMV RNA was added to the lysis buffer of the QIAamp extraction to act as an internal control. The primers BMV1.1BIO and BMV2.1 (Table 2.3) were added to the reaction mix at a concentration of 0.4 μM to allow co-amplification of both the target HCV and the BMV internal control.

Initially on the addition of the BMV primers to the RT-PCR mix, the BMV failed to amplify. A titration of the magnesium concentration was then performed to optimise the co-amplification of BMV with HCV (Figure 2.3).

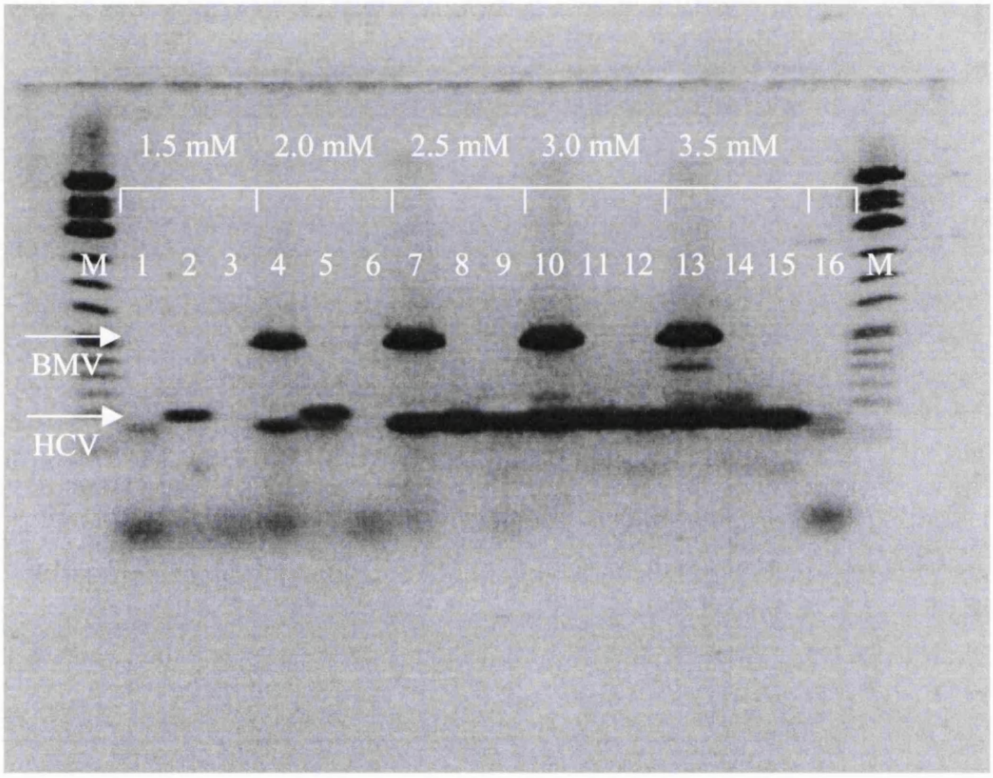

Figure 2.3: Gel photograph showing effect of magnesium concentration.

The BMV product is missing at 1.5 mM $MgCl_2$ (lane 1) but is present at higher $MgCl_2$ concentrations (lanes 4, 7, 10 and 13). The HCV product is present at 1.5 mM $MgCl_2$ (lane 2) but gets more intense at higher $MgCl_2$ concentrations (lanes 5, 8, 11 & 14). The HCV product from the alternative primers was missing at lower $MgCl_2$ concentrations (lanes 3 and 6) therefore this primer pair was not used.

Key: M = marker (ΦX174 DNA/*Hin*f I). Lanes 1, 4, 7, 10 & 13 contain BMV primers 1.1 and 2.1. Lanes 2, 5, 8, 11 & 14 contain HCV primers PT3 & PT4. Lanes 3, 6, 9, 12 & 15 contain alternative HCV primers PT2 & PT4. Lane 16 is a negative control.

Lanes 1, 2 & 3 had 1.5 mM $MgCl_2$. Lanes 4, 5 & 6 had 2.0 mM $MgCl_2$. Lanes 7, 8 & 9 had 2.5 mM $MgCl_2$. Lanes 10, 11 & 12 had 3.0 mM $MgCl_2$. Lanes 13, 14 & 15 had 3.5 mM $MgCl_2$.

The BMV was only visible on the gel at a magnesium concentration of 2.0 mM and above. Using 2.0 mM $MgCl_2$ a range of BMV dilutions were amplified to find the optimal level (Figure 2.4). Bands could be seen on the gel down to a 10^6 dilution i.e. 5 pg/ml BMV. The ELONA was able to detect BMV at 0.5 pg/ml i.e. 10 fold more dilute than gel electrophoresis. The lower bands in Figure 2.3 and Figure 2.4 are primer dimers probably caused by the excess primer seen as a lower smeared band, the concentration of the BMV primer in the PCR reaction was therefore reduced from 0.4 μM to 0.2 μM.

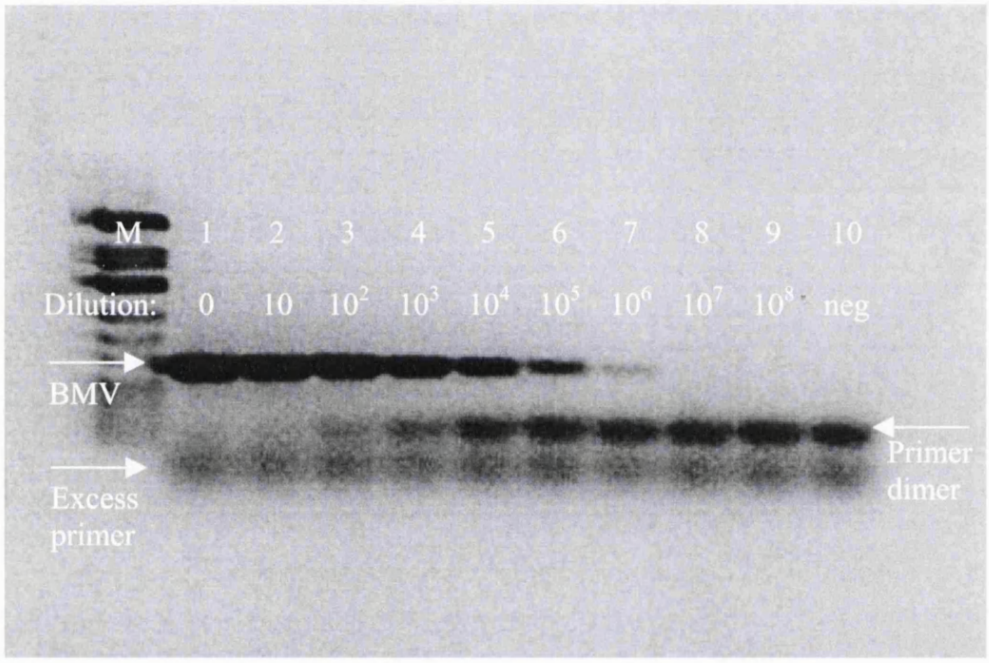

Figure 2.4: Gel photograph showing titration of BMV.

A ten fold dilution series was run from BMV RNA stock (5μg/ml). BMV was detected down to a concentration of 5 pg/ml, representing a million fold dilution of the stock. Excess primers can be seen as a lower band causing primer dimer formation; therefore the primer concentration was reduced from 0.4 μM to 0.2 μM.

Key: M = marker (ΦX174 DNA/*Hin*f I). Lane 1 = stock BMV 5 μg/ml, lane 2 = 0.5 μg/ml, lane 3 = 50 ng/ml, lane 4 = 5 ng/ml, lane 5 = 0.5 ng/ml, lane 6 = 50 pg/ml, lane 7 = 5 pg/ml, lane 8 = 0.5 pg/ml, lane 9 = 50 fg/ml, lane 10 = water control.

The effect of the increased magnesium level on the HCV standard curve was investigated and better HCV sensitivity was found with 2.0 mM magnesium compared with the standard 1.5 mM (Figure 2.5).

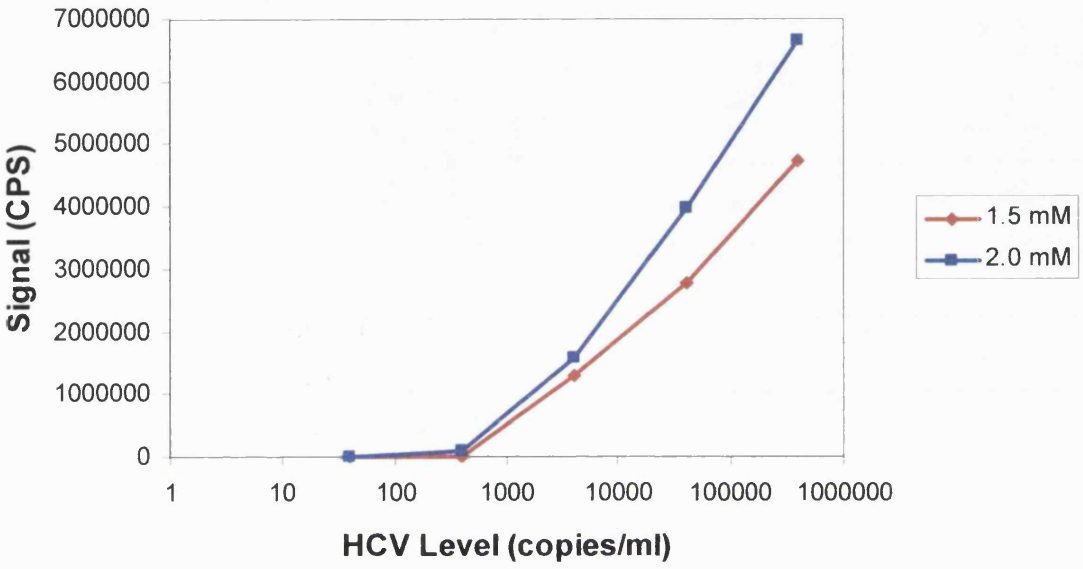

Figure 2.5: Effect of magnesium concentration on HCV standard curve

A higher HCV signal was obtained at 2.0 mM $MgCl_2$ compared to 1.5 mM therefore 2.5 mM $MgCl_2$ was assessed (Figure 2.6).

Further optimisation of the $MgCl_2$ level was performed initially with a comparison of 2.0 and 2.5 mM $MgCl_2$ on the HCV standard curve with and without BMV (Figure 2.6). A reduced HCV signal was found at all dilutions with 2.5 mM $MgCl_2$ compared to 2.0 mM although the addition of the BMV did not reduce performance any further. When 2.0 and 2.25 mM $MgCl_2$ were compared, better performance was found at 2.25 mM for both HCV (Figure 2.7) and BMV (Figure 2.8), and therefore 2.25 mM was chosen as the optimal $MgCl_2$ level.

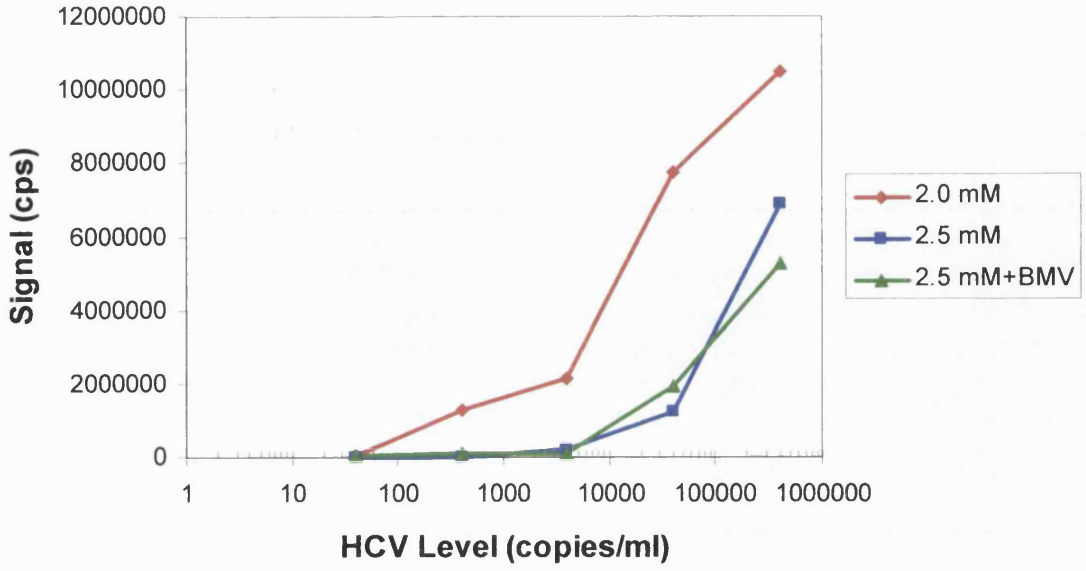

Figure 2.6: Effect of $MgCl_2$ concentration.

2.5 mM $MgCl_2$ resulted in a decreased HCV signal compared to 2.0 mM.

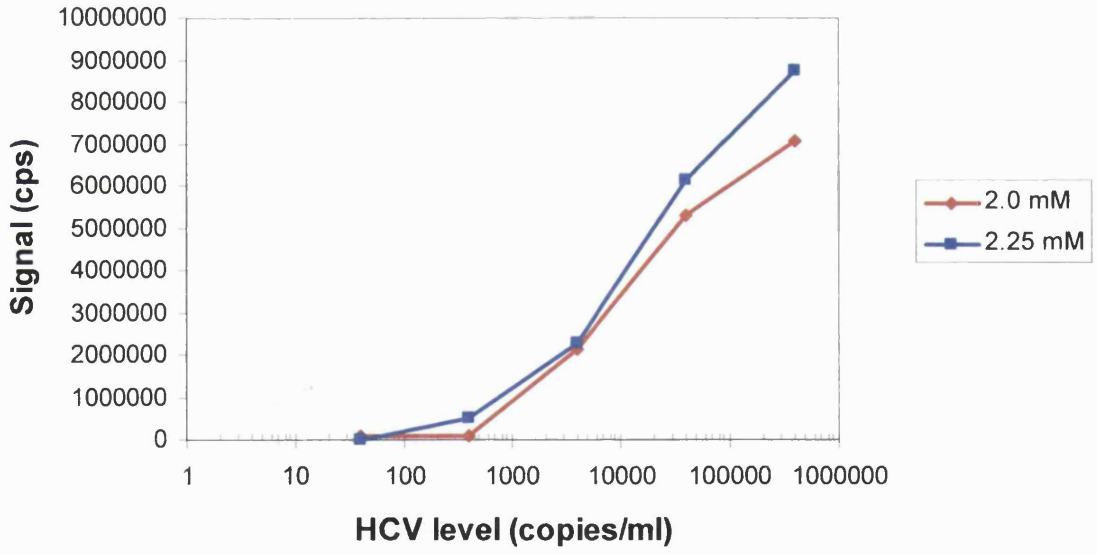

Figure 2.7: Effect of $MgCl_2$ concentration on HCV qPCR.

2.25 mM $MgCl_2$ resulted in an increased HCV signal compared to 2.0 mM and therefore 2.25 mM was assessed with BMV (Figure 2.8).

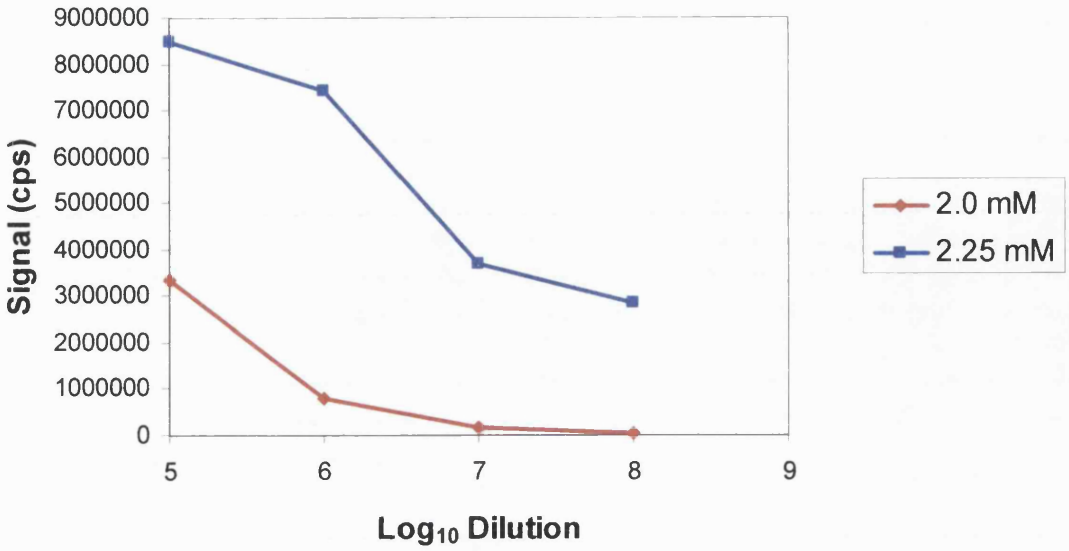

Figure 2.8: Effect of $MgCl_2$ concentration on BMV.

2.25 mM $MgCl_2$ also resulted in an increased BMV signal compared to 2.0 mM and therefore 2.25 mM was chosen as the optimum $MgCl_2$ level.

The effect of reducing the dNTP concentration from 800 μM to a more standard 200 μM resulted in reduced signal (Figure 2.9). Therefore 800 μM was maintained.

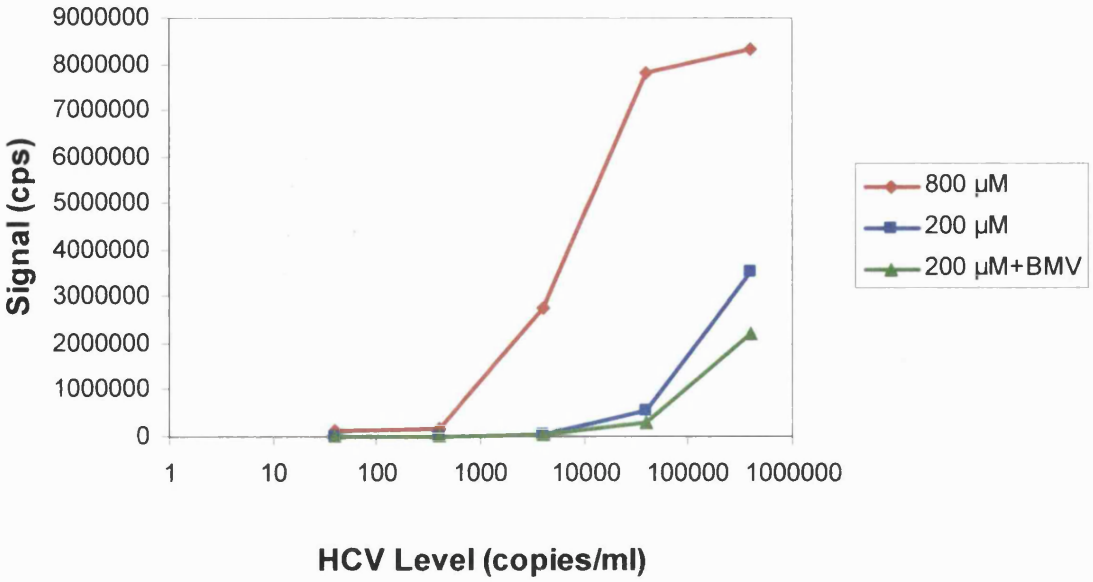

Figure 2.9: Effect of dNTP concentration.

The reduced dNTP concentration resulted in decreased HCV signal therefore 800 μM was maintained.

Stock BMV RNA was diluted in RNAase free water and extracted using the QIAamp spin column method. The spin column RNA extracts were amplified with the equivalent levels of un-extracted BMV RNA i.e. the dilutions of stock in water not extracted by spin column. The signals from the dilutions of extracted and non-extracted RNA were very similar (Figure 2.10) showing that the extraction method was efficient.

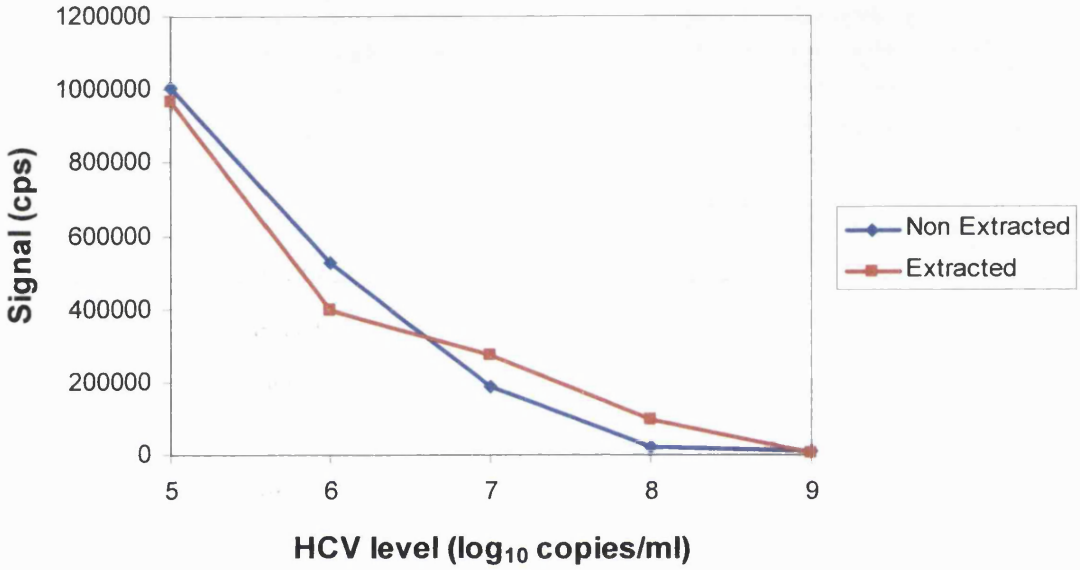

Figure 2.10: Titration of extracted & non extracted BMV RNA.

A 10^7 dilution of the 5 µg/ml stock BMV RNA in the Qiagen lysis buffer AVL was found to give the most appropriate levels in the ELONA at approximately 200 000 cps. However, in order to use the BMV: HCV signal ratio for quantification the BMV had to give a positive signal throughout the whole range of HCV levels used in the standard curve. To determine what effect a high level of HCV would have on the BMV signal, various dilutions of BMV were co-extracted with 1 million copies/ml of HCV per ml (Figure 2.11).

The 10^7 BMV dilution still gave sufficient signal at this high level of HCV and was therefore chosen as the BMV level to be routinely used.

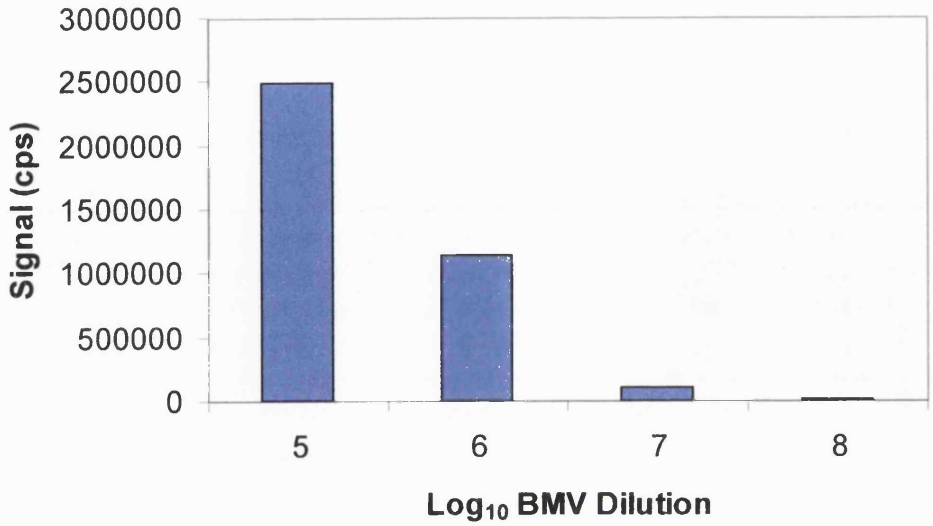

Figure 2.11: BMV signal with 1 000 000 copies/ml HCV.

The effect of co-extraction and amplification of BMV with HCV on the sensitivity of HCV detection was then investigated by extraction of five replicates of a 1 in 10 dilution of the NIBSC standard (i.e. 400 copies or 71 IU per ml) with and without BMV at a dilution of 10^7 in the AVL. The average light output from the HCV ELONA was 42 000 cps with BMV and 41 000 cps without BMV. The BMV output at a low HCV level (NIBSC 1:10 dilution) averaged 228 000 cps and at a high HCV level (4×10^6 HCV copies/ml) it was reduced to 26 887 cps. The BMV level at this high HCV load was still acceptable with a signal to noise ratio of 25:1.

To test the system an HCV standard curve was extracted with and without the BMV at a dilution of 10^7. The HCV standard curve with the BMV had slightly reduced signals (Figure 2.12) but was still positive at all the dilutions expected.

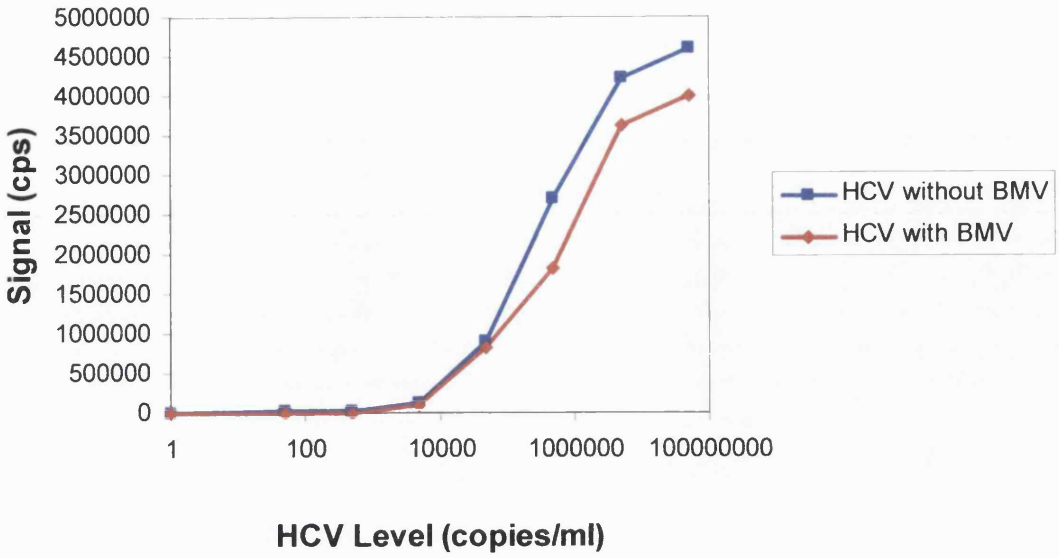

Figure 2.12: HCV standard curve with and without BMV.

2.2.4 Calibration of an in house HCV standard against the WHO International HCV Standard

An HCV genotype 1a donation in the window phase of infection was chosen as an in house standard, this had the advantage of containing a very high level of HCV. In order to assign this standard an HCV level in International Units it was calibrated against the WHO International HCV Standard 96/790 (Saldanha *et al.*, 1999) supplied by the National Institute of Biological Standards and Control (NIBSC).

The in house standard "X" was tested in parallel with the WHO standard at several dilutions (Table 2.4) using the BioRobot QIAamp 96 Virus protocol with the Roche COBAS HCV AmpliScreen assay described in section 4.7.

Table 2.4: Calibration of the in house standard 'X'.

WHO International HCV RNA Standard			In House Standard X		
HCV Level [a] IU/ml	No. Positive / No. Tested	Percentage	Dilution	No. Positive / No. Tested	Percentage
25	24 / 24	100%	1: 1 million	24 / 24	100%
10	23 / 24	95.8%	1: 2 million	23 / 24	95.8%
5	17 / 24	70.8%	1: 5 million	16 / 24	66.7%
2	4 / 16	25.0%	1: 10 million	12 / 24	50.0%
0	0 / 16	0.0%	diluent	0 / 16	0.0%

[a] Values are based on the dilution of the WHO 96/790 international HCV standard diluted in NHP.

As the 50% end point occurred at a 1:10 000 000 dilution of the in house standard an arbitrary value of 10 000 000 IU/ml was set. Probit analysis was used to find the 50%, 75% and 95% end points for both standards. The arbitrary value for the in house standard was then adjusted so that the end points matched those of the WHO standard. The arbitrary value of 10 000 000 set for the in house standard gave end points approximately 2.5 fold less than the WHO standard and therefore the value was adjusted to 25 000 000. The probit analysis was performed again with this adjusted value and used to plot curves of both the standards (Figure 2.13). The two curves overlie each other, thus confirming that the 25 000 000 IU/ml value assigned to the in house standard was correct.

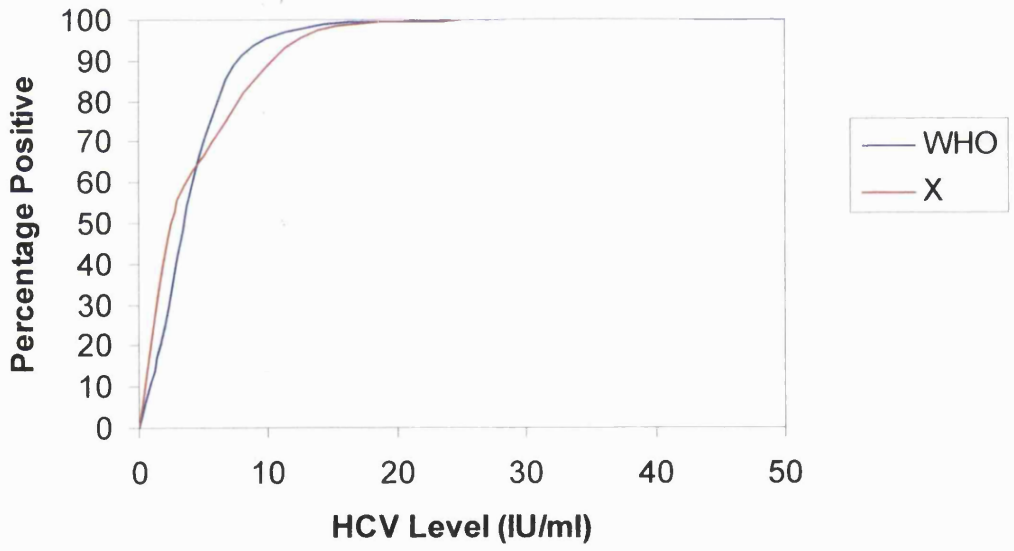

Figure 2.13: Calibration of in house standard 'X' with WHO HCV standard

2.2.5 Calculation of HCV level by analysis of HCV/BMV ratio

An HCV standard curve was run in each assay, and the ratio between the HCV and BMV signals was used to calculate the HCV level in unknown samples by comparison with the ratio obtained from the HCV standard curve.

A Microsoft Excel 2000 spreadsheet was developed to plot the $\log_{10}$ transformed HCV and BMV signals from the standard curve, which form a straight line (Figure 2.14). The lowest HCV standard does not always lie on the straight line as at this level stochastic events occur and the HCV will not always give a positive signal. A line of best fit through the standard curve data was calculated using linear regression. The lowest point on the standard curve was not included in the line of best fit analysis because this will not always fall in line with the other points. Log_{10} HCV/BMV ratios from samples of unknown HCV level can then be compared with this line of best fit to calculate the HCV level.

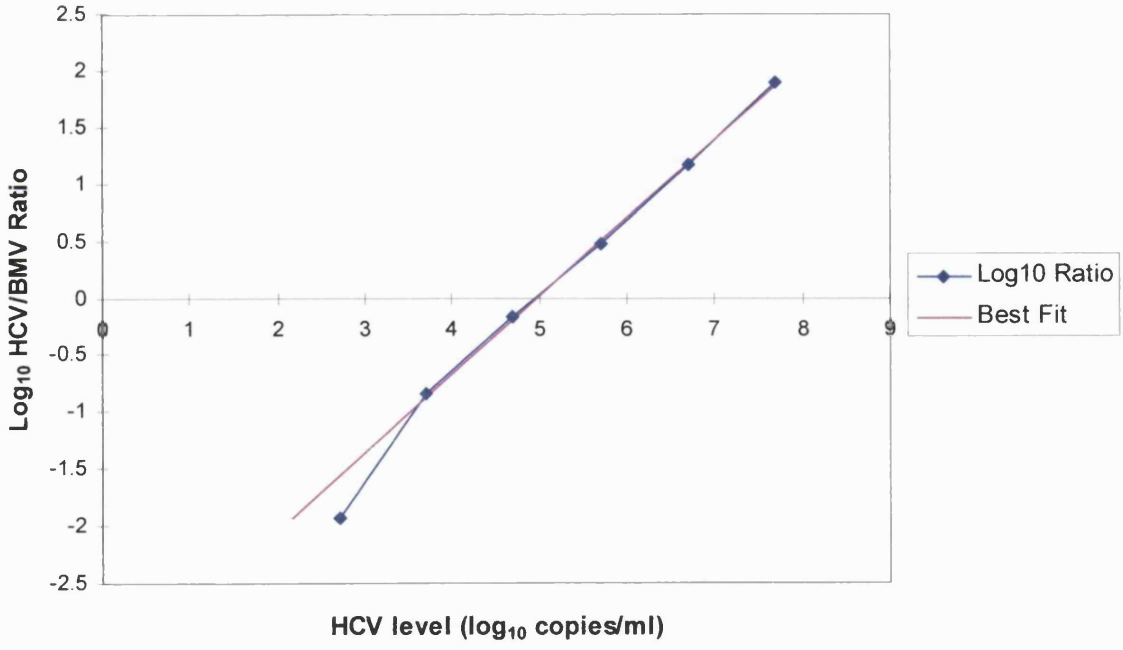

Figure 2.14: Log_{10} HCV BMV ratio for HCV standard curve.

Data from the Topcount was imported into the Excel spreadsheet using a macro (Appendix B; macro 1), the spreadsheet contained formulae that analysed the

data. The Trend worksheet function was used to generate the straight line through the standard curve data, and to calculate the HCV level in the unknown samples.

Various checks were also performed automatically on the results. An HCV cut off was calculated as five times the mean of the two negative controls. A BMV cut off was calculated as two times the standard deviation of the BMV signals obtained from the lower three HCV levels in the standard curve and the two negative controls. The HCV/BMV ratio was checked against the HCV signal and compared to the ratios obtained from the standard curve, the limits were typically set to within the 90% confidence intervals of the data from the standard curve. A flow diagram showing the decisions made before displaying the result or comment is shown in Figure 2.15. This was entirely automatic and achieved using the IF worksheet function. If the conditions were appropriate for displaying the result it was rounded to the first two digits.

The duplicate HCV and BMV results from the ELONA were also checked to ensure they were in agreement to within 25% of each other, if they were not the comment "bad duplicates" was displayed next to the result.

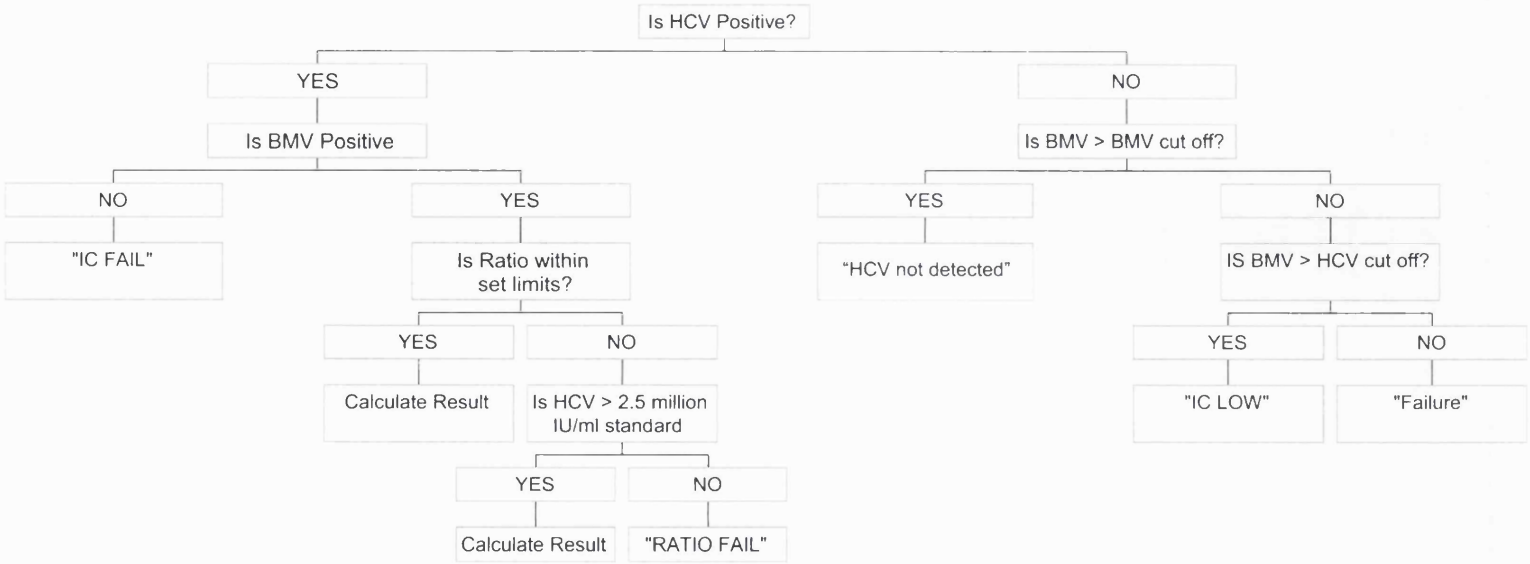

Figure 2.15: Factors considered before result or comment is displayed.

The reproducibility of the final method was determined by testing several replicates of a single sample. Seven replicates of an HCV positive sample were quantified, the results averaged 620 000 with a minimum of 430 000 and a maximum of 810 000 this represents 1.9 fold variation. The intra assay CV was 17.8%.

The accuracy of the final method was determined by testing 20 clinical samples that were also quantified using the Roche HCV Monitor assay (Section 2.1.5). The results were in agreement with the line of best fit being close to and parallel with the line of equivalence between the two assays (Figure 2.16).

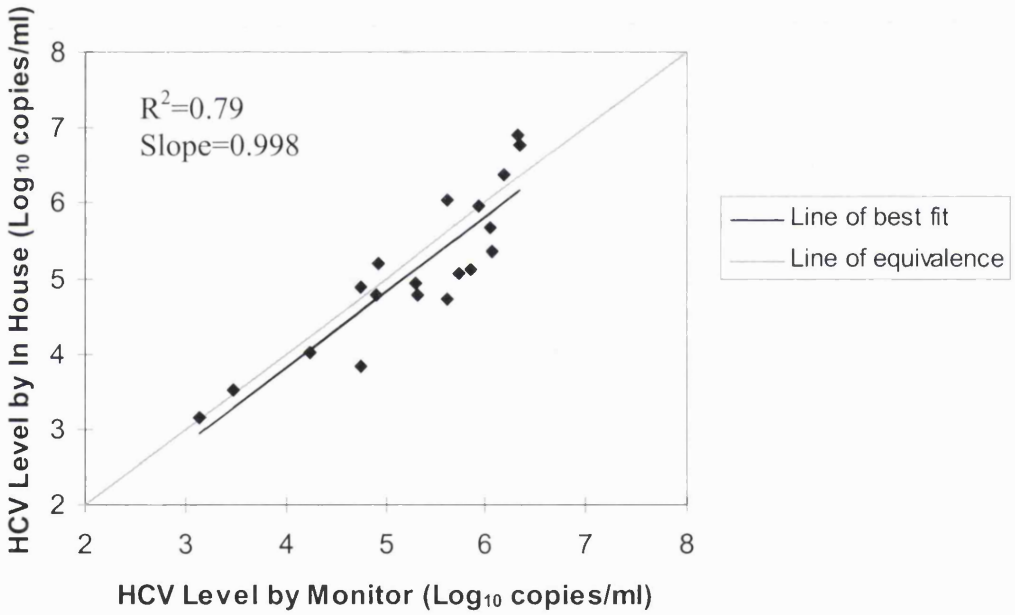

Figure 2.16: Comparison of in house assay with Roche Monitor assay.

2.2.6 Discussion

Previously in quantitative PCR assays of this type the HCV level in the test samples had been calculated using an external standard curve. The standard curve was run in each assay and covered a range of HCV levels likely to be found in the samples being tested. A curve was fitted to the output signal data obtained from the standards using a specialist software package, and the unknown samples assigned a value based on their output signal.

This earlier method gave accurate results if all the samples and controls were extracted, amplified and detected with equal efficiency. However if the extraction or amplification efficiency of any of the samples differed from the standards an inaccurate result may have been reported. For example if a sample was inhibitory to PCR it may have given no signal or only a low signal despite containing a high level of HCV.

By adding an internal control to the assay the efficiency of extraction, amplification and detection could be monitored. An inhibitory sample would not only give no (or reduced) HCV signal but also no (or reduced) internal control signal, alerting the user to a problem with the sample. One advantage of using the ratio of HCV to BMV for quantification was that the standards formed a straight line and so the specialist curve fitting software was no longer necessary. A spreadsheet containing macros (Appendix B; macro 1) was developed using Microsoft Excel 2000 for automatic calculation of the results. Another advantage of the HCV to BMV ratio analysis was that it still gave accurate quantification at high levels of HCV which in a standard PCR system would have reached a plateau.

In the system described here the quantification was calculated from the ratio between the HCV and internal control signals. In this case any difference in efficiency would not only affect the HCV but also the internal control, for example if the efficiency of extraction were to be reduced 10 fold, the signals for

both HCV and BMV would be reduced by 10 fold but the ratio would remain the same and so an accurate result would still be calculated.

In practice, however, this reduction in both HCV and BMV signals may be true for small differences in efficiency but large changes may still introduce inaccuracies in the result. For this reason the spreadsheet was developed to alert the user to these cases, for example if both the HCV and BMV give signals just above the cut off the ratio may still be 1:1, which usually occurs at about the 1 x 10^6 IU/ml level on the standard curve, however this result is not reported because the spreadsheet will check that the ratio is at the correct level for the HCV signal and in this case an error would be reported.

BMV was chosen as an easily available source of viral RNA however it does have the one disadvantage of being naked RNA. As there is no viral protein coat the BMV does not control for the viral lysis step of the procedure.

2.3 Development of HCV genotyping by 5′-NCR sequence analysis

2.3.1 Introduction

This method was developed to allow the amplification and sequencing of the 5′ non coding region (5′ NCR) of HCV. The sequence was then aligned and compared to published sequences of known genotype using the multiple sequence alignment program ClustalX (Thompson *et al.*, 1997). Phylogenetic analysis was performed to allow the sequence of interest to be placed in a cladogram and its genotype determined.

2.3.2 Sequence extraction and amplification

HCV RNA was extracted using the Qiagen QIAamp spin column method (Section 2.1.1). The RNA was reverse transcribed and amplified using the Qiagen One Step RT-PCR system (Qiagen, Hilden, Germany) containing primers to the 5′ non-coding region of HCV; NCRSEQ1 and NCRSEQ2 (Table 2.5). A portion (10 µl) of the Qiagen eluate was added to 40 µl of One Step RT-PCR mix to give a final reaction volume of 50 µl. The amounts of the components for RT-PCR in the final reaction mix were as follows: 10 µl 5x RT-PCR buffer (as supplied) giving 2.0 mM $MgCl_2$ and 0.4 mM of each deoxynucleotide triphosphate; 10 µl 5x 'Q solution' (as supplied); 0.4 µM of the 5′ non-coding region primers NCRSEQ1 and NCRSEQ2 (Table 2.5), 10 units recombinant RNAsin (Promega Corporation, Madison, USA), and 1 µl Qiagen enzyme mix containing Sensiscript and Omniscript RT enzymes.

Table 2.5: Primers used for HCV 5′NCR sequence analysis

Name	Sequence	Position[a]
NCRSEQ1	5′ CTAGCCATGGCGTTAGTATTG	-263 to -244
NCRSEQ2	5′ CTATCAGGCAGTACCACAAG	-43 to -62

[a]Primer positions are shown with the numbering system of (Choo *et al.*, 1991); Genbank accession No. M62321. Primers were synthesised by Oswel Research products Ltd. Southampton, UK.

The reactions were cycled in a GeneAmp PCR System 9600 thermal cycler in the post PCR laboratory using the following parameters: 50°C for 30 minutes and 94°C for 2 minutes followed by 40 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 68°C for 2 seconds. After a final extension at 68°C for 7 min the reactions were held at 4°C.

PCR products were visualised by gel electrophoresis (Section 2.1.11).

2.3.3 Sequencing and phylogenetic analysis

Sequence reactions were performed on the amplicons using the Big Dye terminator cycle sequence kit (Applied Biosystems, Foster City, CA, USA), with the following reaction conditions: 5 µl template amplicon; 6 µl dye terminator mix; 3.2 pmol primer (diluted to 1 µl); 8 µl RNase free water (Promega). All amplicons were sequenced using both NCRSEQ1 and NCRSEQ2 primers i.e. forward and backward. Sequence reactions were run in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following parameters: 96°C for 10 seconds; 50°C for 5 seconds and 60°C for 4 minutes, repeated for 25 cycles followed by a hold at 4°C.

After the cycle sequencing was complete any excess dye terminators were removed by ethanol precipitation with sodium acetate. The reactions were

dissolved in 5 µl loading dye/formamide (Applied Biosystems) at a ratio of 1:5 and denatured for 2min at 95°C. 0.7 µl were then loaded on the gel and run in the ABI Prism 377 sequencer (Applied Biosystems).

Sequence files were exported as *.abi files and opened in the Sequencher 3.1 program (Gene Codes Corp. Ann Arbor, MI). The Sequencher 3.1 program was used to check the chromatograms by eye for both forward and reverse sequences and generate a consensus sequence. The consensus sequence was saved as a text file for further analysis.

HCV sequences of known genotype were downloaded from the Genbank nucleotide sequence database using the Entrez search and retrieval system (http://www3.ncbi.nlm.nih.gov/Entrez/). The following sequences were used: Type 1a; HPC5NRD, HPC5NR3, HCV1A5GT, HPC5NR1, HPC5NRC, HPC5NRA. Type 1b; HCV6335, HCV6334, HCV6336, HCV6330, HCV6328, HPCHC263D, HPC5NR2, HPCHC14A, HPCHN50F, HPCHN47I, HPCHN31C, HPCHC211B, HPCHN36H, HPCHNC357G, HPCHN49J, HPCHN68E, HCV6321. Type 2a; HPC5NR5, HPCHNC404L, HPC5NR4, HPCHN18K. Type 2b; HPCHN65M, HPCHN44N, HPCHNC448O, HPC5NR6, HPC5NRB, AF057149, AF057148, AF057147. Type 3a; AF057153, AF057152, AF057150, AF057151, HCV6318, HCV6323, HPC5NRET. Type 4; HCV6325, HCV6324, HCU33432, HC33432. Type 4a; AF057155, AF057154. Type 5: HCU33430, HC33430. Type 6; HCU33431, HC33431. Type 7; HCUCORE1.

Multiple alignment of the known and unknown sequences was performed using the ClustalX v1.81 program (Thompson *et al.*, 1997) available for download from ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/.

Phylogenetic trees were generated using the neighbour joining (NJ) method in ClustalX, bootstrapping was performed with 1000 replicates. Phylogenetic trees were viewed and printed using the TreeView v1.5 program (available from http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The genotypes of the test samples were estimated by their position in the tree.

Chapter 3

Effects of Handling and Storage of Blood on the Stability of HCV RNA

3.1 Storage of Whole Blood

3.1.1 Introduction

In order to put the NAT screening program into place, the sample handling had to be carefully considered. The NBS had plenty of experience of testing blood samples by serological methods but no experience of testing by NAT methods. Therefore the type of sample required, how it was taken, how it was handled and how it was stored before testing were all considered. It was decided that a dedicated sample would be taken at the time of donation for NAT testing rather than using the serology or blood grouping samples. Having a dedicated sample for NAT testing allowed the type of sample (serum or plasma) and its handling and storage before testing to be defined without impacting on the conditions required for the other tests.

The first decision to be made was the type of sample to be taken, either serum or plasma. Previous studies had shown that plasma was preferable to serum as an analyte for HCV RNA (Busch *et al.*, 1992; Wang *et al.*, 1992), but that heparin anticoagulant would inhibit PCR. HCV stored in serum at room temperature had been shown to be very unstable. One study had found a complete loss of detectable HCV RNA in seven serum samples after five days of storage at room temperature, although only a small decline of 4% found at 4°C storage of the same samples for the same time (Halfon *et al.*, 1996). Another study showed that HCV RNA was stable in serum for 14 days at 4°C but there was a 3 to 4 $\log_{10}$ loss after 14 days at room temperature (Cuypers *et al.*, 1992). In light of these findings an EDTA plasma collection tube was chosen for use as the NAT sample in the NBS.

The stability of the HCV RNA in the EDTA plasma required investigation to define the handling and storage of these samples prior to pooling and NAT testing. It was thought to be totally impractical to separate the blood sample and remove the plasma aliquot at the donor session, but it was not known how long the blood sample could remain un-separated. The use of a plasma preparation tube (PPT; Becton Dickinson, New Jersey, USA) was investigated as this tube had a gel plug which separated the plasma from the cells after centrifugation, and could be used if the time to separation was critical. However the PPT tubes could not be centrifuged at the donor session because of issues of staff training and equipment requirements. Therefore in the studies described here, the minimum time between sample collection and separation was set to be eight hours, to allow transportation time between the donor session and the nearest pooling laboratory.

An initial study was undertaken to determine the stability of HCV RNA when stored for up to 72 hours at 4°C and 25°C as either EDTA whole blood in the standard tube or after centrifugation at eight hours in the PPT tube.

In the second study the effect of CPDA-1 anticoagulant on stability was investigated. This study was performed because the method of collection of the EDTA sample had not been decided. Before the commissioning of blood bags containing side sampling ports, the EDTA sample for NAT could be collected after the donation had been made by either cutting the line from the needle to the blood bag and allowing fresh blood from the donor to be collected into the EDTA tube or alternatively a sample from the blood donation bag could be squeezed back out into the EDTA tube after the needle was removed from the donor. The latter case was the preferred option however it would mean that the CPDA-1 anticoagulant used in blood bags would be mixed with the EDTA in the collection tube, and therefore its effect on stability needed investigation.

The second study also looked at an extension of the transport time from eight hours to twelve hours, and an investigation of the transport temperature, as providing chilled transportation had financial implications. The total time of

storage was increased to 120 hours to provide flexibility in situations such as bank holidays that may require extra time before plasma separation. Also, storage at 37°C was investigated to allow for uncontrolled storage temperatures during the summer.

The studies described here were carried out in collaboration with NLBTC where Dr. Alan Kitchen performed blood storage and sampling. These studies were undertaken to investigate the most appropriate methods for sampling, transportation and processing of blood samples from donors for the National Blood Service in England, and have since been published in the journal Vox Sanguinis (Grant *et al.*, 2000).

3.1.2 Materials and Methods

Donor Selection

All blood samples were taken from voluntary, non-remunerated blood donors in the UK, whose plasma had previously been found to contain HCV RNA, and who were not receiving any antiviral treatment.

Storage in EDTA and PPT tubes at 4°C and 25°C

Each of three HCV infected donors was bled into 11 dry K_3EDTA tubes (Greiner, Gloucester, UK) and 11 Plasma Preparation Tubes (PPT; Becton Dickinson, New Jersey, USA). Within 30 minutes of venesection one EDTA and one PPT tube per donor were centrifuged according to the manufacturer's instructions, and an aliquot of plasma withdrawn and stored at -70°C; these samples were designated 'time zero'. The remaining tubes were stored at either 4°C or 25°C. After eight hours all the PPT tubes were centrifuged. EDTA tubes were centrifuged immediately before sampling. At 8, 16, 24, 48 and 72 hours after venesection an aliquot of plasma was withdrawn from each type of tube at

both storage temperatures, frozen and stored at -70°C until tested by quantitative RT-PCR. In this study and those described below all tubes of both types were inverted at each sampling time to simulate movement during transit and handling.

Extended storage of whole blood containing CPDA-1 in EDTA tubes at 4°C, 25°C and 37°C

Five HCV infected blood donors were selected for the study as described above. Each donor was bled into the stabiliser solution CPDA-1 (citrate-phosphate-dextrose solution with adenine; Baxter) at a concentration of 1 part CPDA-1 to 7 parts whole blood (equivalent to the concentration used in blood bags). This citrated blood was transferred into dry K_3EDTA tubes and stored at 4°C, 25°C or 37°C. Plasma samples were taken at 0, 24, 48, 72, 96 and 120 hours after venesection and stored at -70°C until tested by quantitative RT-PCR.

To determine the effect of transportation temperature on the stability of HCV RNA, the same five donors were bled into CPDA-1 and the blood was transferred into dry K_3EDTA tubes as before. A time zero sample was taken and the remaining tubes were stored at 25°C and 37°C for 12 hours before being transferred to 4°C storage for the rest of the study. Plasma samples were taken at 24, 48, 72, 96 and 120 hours after venesection as before and stored at -70°C until tested by quantitative RT-PCR.

In addition each donor was also bled directly into EDTA tubes which were then held at 25°C for 12 hours before either transfer to storage at 4°C or retention at 25°C storage. These were sampled at multiple time points up to 120 hours as described above.

RNA isolation and quantitative RT-PCR

Individual donor sample sets were processed together from start to finish. The plasma samples were thawed rapidly in a 37°C waterbath and RNA was extracted using the Qiagen BioRobot 9604 and QIAamp viral RNA kits (Qiagen GmbH, Hilden, Germany), as described in Section 2.1.2, except the amount of carrier RNA in the lysis buffer was reduced to 0.825 μg/ml and the elution volume was increased to 70 μl.

The RNA was reverse transcribed and amplified using the Titan RT-PCR system (Boehringer Mannheim, Mannheim, Germany) following the method described in Section 2.2. PCR products were quantified by chemiluminescent enzyme linked oligo-nucleotide assay (ELONA) described in Section 2.1.10 using an external calibration curve based on an 'in-house' standard. The 'in-house' standard had been calibrated against an HCV RNA reference standard (96/586) provided by the National Institute for Biological Standards and Control (NIBSC), (Saldanha and Minor, 1996).

Statistics

All HCV RNA levels are expressed as geometric means. Linear regression was calculated by the method of least squares.

3.1.3 Results

The variability of the quantification system (i.e. RNA extraction, amplification and detection) was defined by testing 24 replicates of a single plasma sample containing HCV RNA. The range in viral quantification estimates was 2.2 fold or 0.34 $\log_{10}$, with a coefficient of variance (CV) of 22.9%. Thus any changes in HCV RNA level of less than 0.34 $\log_{10}$ (2.2 fold) cannot be reliably resolved using this assay. For the purpose of these studies, only changes in HCV RNA level, which exceed 0.34 $\log_{10}$ genomes per ml (2.2 fold), are considered

significant. The HCV RNA levels in PPT and EDTA tubes after storage at 4°C and 25°C did not change over a 72 hour period by more than 0.34 log_{10} (2.2 fold); (Figure 3.1, Table 3.1). To calculate the rate of any decline in HCV RNA level, linear regression was performed. The estimated mean changes (based on linear regression analysis) in HCV RNA level under various conditions after 72 hours are also shown in Table 3.1. No decline was observed in either type of tube at 4°C, while insignificant declines of 0.2 log_{10} and 0.25 log_{10} occurred at 25°C in EDTA and PPT tubes respectively.

Table 3.1: Effect of tube type and storage temperature on stability of HCV RNA in blood

Tube	Time (Hours)	Mean [a] change in log_{10} HCV RNA copies/ml	
		4°C	25°C
EDTA	0	0.00	0.00
	8	-0.05	-0.10
	16	-0.11	-0.06
	24	-0.12	-0.18
	48	0.02	-0.17
	72	-0.05	-0.23
Change over 72 hrs [b]		0.02	-0.20
PPT	0	0.00	0.00
	8	0.07	-0.01
	16	0.11	0.05
	24	0.09	-0.07
	48	0.05	-0.18
	72	0.08	-0.20
Change over 72 hrs [b]		0.03	-0.25

[a] Geometric mean of three donors
[b] Total change over 72 hours as calculated by linear regression

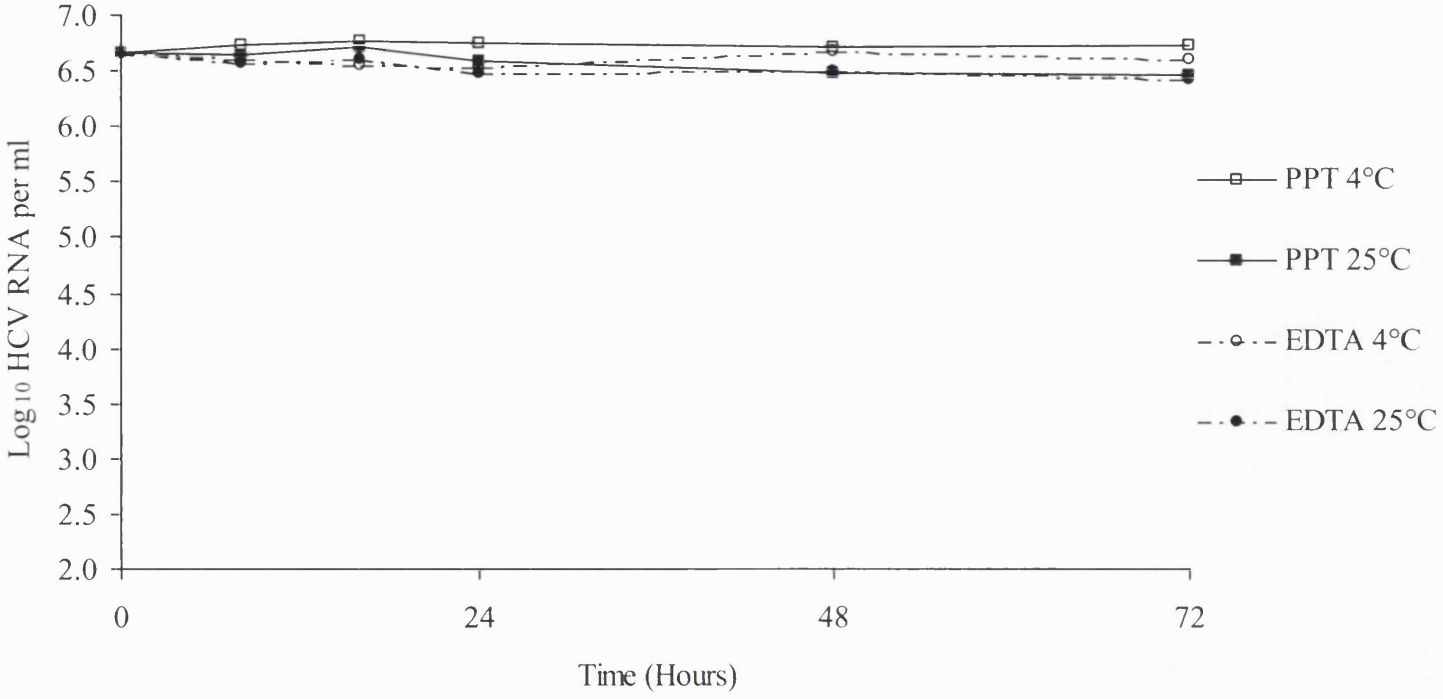

Figure 3.1: Comparison of HCV storage in PPT and EDTA tubes at 4°C and 25°C.

Blood was stored whole in EDTA tubes or separated in PPT tubes at 4°C and 25°C for the indicated number of hours. The geometric mean titre of three donors is shown for each storage condition at each time point. The data is normalised so that all time zero points are 6.7 $\log_{10}$ (mean of time zero for all storage conditions).

The effect of storage on HCV RNA level, at various temperatures, in whole blood containing a mix of CPDA-1 and EDTA anticoagulants (citrate/EDTA whole blood) is shown in Table 3.2 and Figure 3.2. Rates of change in HCV RNA level were calculated by linear regression as before. The estimated mean changes in HCV RNA level after 120 hours of storage in citrate/EDTA whole blood are also shown in Table 3.2. Only 120 hours of storage at 37°C resulted in a significant decline of 0.37 $\log_{10}$ genomes per ml. Table 3.3 shows the effect of storage temperature for the first 12 hours, representing the transportation time, before storage at 4°C for the remaining 108 hours in citrate/EDTA whole blood. The elevated temperatures during the first 12 hours did not result in any decline in HCV RNA.

Table 3.2: Effect of storage temperature on stability of HCV RNA in citrate/EDTA whole blood

Time	Mean [a] change in $\log_{10}$ HCV RNA copies per ml		
(Hours)	4°C	25°C	37°C
0	0.00	0.00	0.00
24	0.29	0.05	0.20
48	0.10	0.25	0.25
72	0.13	0.16	-0.11
96	0.26	0.20	-0.13
120	0.32	0.38	-0.25
Total Change [b]	0.23	0.33	-0.37

[a] Geometric mean of five donors
[b] Total change over 120 hours as calculated by linear regression

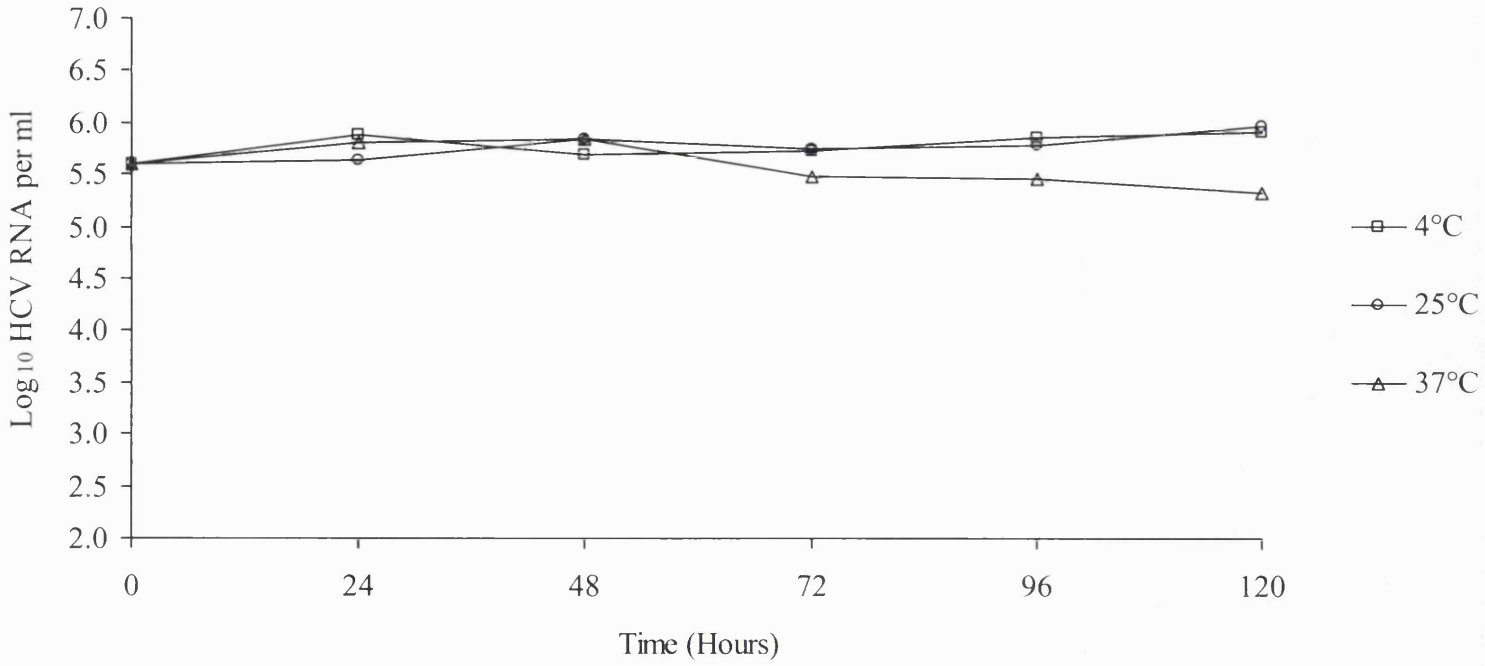

Figure 3.2: HCV storage in citrate/EDTA whole blood at 4°C, 25°C and 37°C.

Whole blood was stored in citrate/EDTA at 4°C, 25°C and 37°C for the indicated number of hours. The geometric mean titre of five donors is shown for each time point. The data is normalised so that all time zero points are 5.6 $\log_{10}$ (mean of time zero for all storage conditions).

Table 3.3: Effect of first 12 hours storage temperature on citrate/EDTA whole blood stored at 4°C thereafter

Time	Mean [a] change in log_{10} HCV RNA copies per ml		
(Hours)	4°C for 12 hours	25°C for 12 hours	37°C for 12 hours
0	0.00	0.00	0.00
24	0.29	0.20	0.34
48	0.10	0.14	0.26
72	0.13	0.10	0.27
96	0.26	0.03	0.31
120	0.32	0.24	0.36
Total Change [b]	0.23	0.09	0.25

[a] Geometric mean of five donors
[b] Total change over 120 hours as calculated by linear regression

The effects on HCV RNA level of storage for up to 120 hours in EDTA whole blood are shown in Table 3.4. An insignificant decline in HCV RNA level of 0.15 log_{10} occurred after 120 hours of storage at 25°C. No decline was noted at 4°C storage in either anticoagulant.

Table 3.4: Effect of temperature on stability of HCV RNA in EDTA whole blood

Time	Mean [a] change in log_{10} HCV RNA copies per ml	
(Hours)	4°C	25°C
0	0.00	0.00
24	0.11	0.01
48	-0.03	-0.38
72	-0.03	-0.19
96	0.12	-0.16
120	0.20	-0.14
Total Change [b]	0.15	-0.15

[a] Geometric mean of five donors
[b] Total change over 120 hours as calculated by linear regression

3.1.4 Discussion

The ability to detect HCV by NAT can depend upon the stability of the HCV RNA target itself and upon the suitability of the analyte for whichever NAT method is used. In target amplification assays inhibitors of reverse transcription or PCR may build up over time although they will have no effect upon signal amplification assays such as the branched DNA assay. However, in the latter assay, serum and stored plasma may generate materials in the analyte that inhibit nucleic acid binding to the solid phase.

The type of RNA extraction method used will affect the variety of inhibitor co-extracted and introduced into the detection assay. It is therefore important to investigate effects of signal degradation employing assays of the same principle as those which will be used to actually test these samples in practice. In England it was anticipated that HCV RNA would be detected using Qiagen RNA extraction and Roche AMPLICOR amplification and detection. This investigation used Qiagen extraction technology, but substituted the Roche qualitative assay with an in house quantitative RT-PCR assay. Although the assay used in this study utilises different RT-PCR enzymes to the AMPLICOR assay it was nevertheless felt that signal degradation due to generation of or failure to remove RT-PCR inhibitors would be likely to affect both assays in the same way.

The suitability of various sample types and storage conditions for use in a protocol employing RT-PCR for detection of HCV RNA were assessed. These data were required prior to the introduction of genome testing in transfusion practice in England and had an impact upon the operative procedures used.

It should be pointed out that the HCV samples used in this study were all post-seroconversion and therefore contained anti-HCV antibodies. NAT screening is intended to detect antibody negative window phase donations, and the stability of these samples may be different to those described here. However for the purpose of this study HCV positive carriers were bled directly rather than spiking HCV

negative blood with HCV containing samples. At the time donors in the window phase of HCV infection were not available, but the issue of antibody negative versus antibody positive stability will be addressed in section 3.2.

It had been decided that a dedicated sample tube would be taken for NAT testing at the time of blood donation. However the method by which the sample was to be taken (direct from the donor or from the donation pack) and the most appropriate kind of sample to use, was unclear from a study of the literature and a review of current practices. Previous studies have shown that plasma is preferable to serum as an analyte for HCV RNA (Busch *et al.*, 1992; Wang *et al.*, 1992) and HIV-1 RNA (Holodniy *et al.*, 1995; Bruisten *et al.*, 1997). These findings were considered to confirm the unsuitability of serum and therefore plasma was chosen as the analyte. The anticoagulant heparin was considered unsuitable because it is known to be a potent inhibitor of PCR (Wang *et al.*, 1992). These studies therefore investigated the stability of HCV RNA in EDTA and citrate anticoagulated blood specimens.

The method of sample collection required investigation. One possible method of collecting the sample for NAT would be to fill an EDTA tube directly by reverse flow from the blood donation pack line, a procedure likely to give a mixture of fresh venous blood and increasing amounts of citrated blood. Therefore, the suitability of a mixed EDTA and citrate sample was investigated by adding citrated whole blood to EDTA tubes. This was judged to represent the highest level of citrate mixed with EDTA anticoagulant likely to occur in practice. Direct sampling into EDTA or citrate tubes was also considered to be a possibility.

It was unknown whether release of RT-PCR inhibitors and RNAses from the cells would affect the ability to detect HCV RNA in the sample, so the effect of separation of cells from plasma was also investigated. PPT tubes were evaluated because they contain EDTA anticoagulant and a gel plug which forms a barrier between the cells and the plasma after centrifugation of the tube, thus allowing effective separation of the cells from plasma without the need to withdraw the plasma to a separate tube. After removal of the plasma for testing, the whole tube

can be frozen as an archive. The disadvantage of PPT tubes is one of considerable additional cost and the need for additional storage space.

In the first part of this study it was found that the separation of cells from plasma, prior to storage, using PPT tubes, did not offer any advantage over storage of whole blood in EDTA tubes in terms of the HCV signal detected after various lengths of time. Similar results have previously been shown for HIV-1 using the branched DNA assay (Holodniy *et al.*, 1995). Subsequent studies have confirmed that there is no difference between PPT and EDTA tube for storage of HCV (Dockter *et al.*, 1998; Cardoso *et al.*, 1999b; Jarvis and Simmonds, 2002).

All blood samples for NAT testing were intended to be transported from the donor session to one of the three pooling laboratories. Eight hours was considered likely to be the maximum travelling time from some of the more remote donor sessions to the pooling centre. It was considered inappropriate to centrifuge sample tubes to separate cells from plasma at the donation centre and thus the blood would remain un-separated until it reached the pooling site. This time schedule was mimicked in the first study in order to investigate influence of the time to separation upon HCV RNA stability.

Storage of EDTA plasma or whole blood at 25°C resulted in a slight decline in the HCV RNA level after 72 hours of storage but this was within the variability of the quantitative assay used and can therefore be discounted. At 4°C there was no decline in the HCV RNA level in either EDTA plasma or whole blood after 72 hours. Similar findings have been reported for citrated plasma (Wang *et al.*, 1992). These findings contrast with a previous report that suggested that HCV RNA was more stable when stored at 25°C than 4°C in EDTA whole blood (Cuypers *et al.*, 1992).

In further studies, following the initial stability data, the "transportation time" was increased to 12 hours, allowing a less stringent time table, and the effects of temperature during this period were also investigated. This included 37°C storage to mimic temperatures that may be reached in non-refrigerated vehicles during the summer, and an extended storage time of 120 hours to cover

situations, such as public holidays; these conditions represent the worst likely to be encountered including the upper permissible limit for elapsed transit time.

The addition of CPDA-1 anticoagulant to the EDTA and extension of the storage time up to 120 hours did not reduce HCV stability, in fact slight increases in the HCV levels were seen at 4°C and 25°C, although these were within the limits of variability of the assay. This phenomenon has been noted in other published studies (Krajden *et al.*, 1999b; Kessler *et al.*, 2001). It may be postulated that this increase in HCV levels upon storage is due to the gradual release of HCV upon lysis of the PBMCs which are known to harbour HCV (Bouffard *et al.*, 1992).

The only significant loss of HCV found in this study was observed in citrate/EDTA whole blood stored at 37°C, this gave a mean change of -0.37 $\log_{10}$ after 120 hours. This decline was greater than the 2.2 fold difference that the quantification assay was able to resolve and was therefore likely to represent a true loss of signal. Storage of citrate/EDTA whole blood at 37°C for periods longer than 120 hours would probably result in a progressively greater loss of HCV RNA. However, storage at 37°C for a short period of time (12 hours) before refrigeration did not cause any decline in HCV RNA. Interestingly after 48 hours of storage at 37°C there was a slight increase in HCV levels as was observed at 4°C and 25°C storage but by 72 hours levels had declined and continued to decline for the rest of the study.

Many others have recently reported similar HCV stability for storage at temperatures less than 37°C. No significant decline was found at 4°C storage for up to seven days in serum, EDTA and PPT tubes (Cardoso *et al.*, 1999b). Kessler and colleagues did not note any decline in HCV RNA after 96 hours storage at room temperature in EDTA whole blood (Kessler *et al.*, 2001). One study found that greater than 75% HCV RNA was retained after five days of storage at room temperature in acid citrate dextrose (ACD), EDTA or PPT tubes (Dockter *et al.*, 1998). Another study showed that whole EDTA blood was stable for up to 72 hours at room temperature or refrigerated temperatures and the authors estimated that a 25% reduction would require more than 200 hours storage at room

temperature (Stramer *et al.*, 1998). Miskovsky and colleagues noticed slight decline of 0.1 log over 48 hours at room temp with no significant difference between EDTA, ACD or serum (Miskovsky *et al.*, 1996).

However, some conflicting reports have been published showing a decline in HCV RNA. One study showed a decline in HCV RNA level in whole EDTA blood of 0.38 $\log_{10}$ after 48 hours of storage at 4°C (Damen *et al.*, 1998). Gessoni and colleagues studied the storage of whole blood in PPT tubes from six donors and found a small 0.5 log decline in HCV RNA after 168 hours at 4°C, this was within the limit of variation of the assay used. However a significant drop of 0.7 log was found in one donor (Gessoni *et al.*, 2000).

The assumption that one may extrapolate a decline over a period of time longer than that of the actual study may not be justified, because rates of decline may change. For example once haemolysis begins to occur the rate of decline in RNA levels may increase. One extended storage study showed HCV RNA losses of 3 to 4 $\log_{10}$ in whole EDTA blood stored for 14 days at room temperature (Cuypers *et al.*, 1992). Equal losses were found in serum at 25 °C but no losses at 4°C.

The results of the studies reported here show that it is not necessary to separate cells from plasma before storage, that refrigerated transport between the donor session and the pooling laboratory is not necessary and that HCV RNA remains stable for considerable periods of time. It also shows that the mixing of EDTA and citrate whole blood does not affect the stability of HCV RNA compared to EDTA alone. These findings have allowed the implementation of greater flexibility in sample collection and transport and have had considerable implications for the costs incurred in handling the large numbers of blood samples involved in blood donor screening by NAT in England.

3.2 Storage in Minipools

3.2.1 Introduction

As part of the NAT testing process in the NBS, the blood samples are sent to the pooling laboratory, separated and the plasma pooled using a Tecan Genesis robotic sample processor (RSP) to generate minipools of initially 96 donations each, now reduced to 48 donations. The RSP also adds an aliquot of each plasma sample to an 8 by 12 format deep well plate, which is kept as an archive. The minipools are tested by NAT and if any positive mini-pools are detected, a cross pooling system is used to identify the individual positive donation within the pool (Mortimer, 1997).

The stability of the HCV RNA in these minipools required investigation to define appropriate handling and storage conditions of the minipools before the NAT testing was performed, and to ensure that minipooling itself did not affect the detection of HCV RNA. This study was performed in collaboration with BPL where Dr. Cate Sims carried out the storage and sampling.

3.2.2 Materials and methods

Minipools containing 96 donations were prepared in duplicate according to standard protocols at the Leeds pooling laboratory. An antibody positive and antibody negative HCV RNA positive donation was spiked into each of four minipools in duplicate to give a concentration of approximately 10^5 geq/ml in the pool. The minipools were divided into 16 aliquots of 300 µl and one of the duplicates stored at chill and the other at ambient temperature. The HCV RNA positive spike samples were also added to an EDTA blood tube, mixed and divided into 300 µl aliquots and stored as above. A sample was removed immediately after set-up (time 0) and at days 1, 2, 3, 4, 5, 7, 9, 11 and 14 and stored at -70°C. Duplicate samples were also taken at time 0 and days 1, 2, 3, 7 and 14 in case of extraction or amplification failure. Dataloggers were placed

immediately adjacent to tubes to monitor temperatures for the duration of the study.

When all the time course samples had been collected the frozen samples were thawed rapidly in a 37°C waterbath before RNA was extracted using the Qiagen BioRobot 9604 and QIAamp viral RNA kits (Qiagen GmbH, Hilden, Germany), as described in Section 2.1.2, except the amount of carrier RNA in the lysis buffer was reduced to 0.825 μg/ml and the elution volume was raised to 70 μl.

The RNA was reverse transcribed and amplified using the Titan RT-PCR system (Boehringer Mannheim, Mannheim, Germany) following the method described in Section 2.2. PCR products were quantified by a chemiluminescent enzyme linked oligo-nucleotide assay (ELONA) described in Section 2.1.10 using an external calibration curve based on an 'in-house' standard. The 'in-house' standard has been calibrated against an HCV RNA reference standard (96/586) provided by the National Institute for Biological Standards and Control (NIBSC), (Saldanha and Minor, 1996).

3.2.3 Results

The mean difference and range of HCV RNA levels for all spiked minipools is shown in Table 3.5. The mean differences are also represented in Figure 3.3. The undiluted antibody positive and antibody negative material showed no decrease in RNA levels when stored at 4°C. There was no decline in the antibody positive-spiked minipool samples when stored at 4°C and a slight decline of -0.09 $\log_{10}$ in the antibody negative minipool sample. The quantitative assay used, however, was unable accurately to resolve any changes less than 0.34 $\log_{10}$ copies/ml and so the decrease of -0.09 $\log_{10}$ cannot be considered to be significant. Any differences in rates of decline in the different minipools used were also within the 0.34 $\log_{10}$ limit of variability.

All samples stored at 25°C showed significant decreases in HCV RNA copy number with the exception of the antibody positive donation. The considerable

decline seen for other samples may have been exacerbated by a temperature spike (up to approximately 35°C) for about 45 minutes which was seen during the first few hours of the study. The reasons for this apparent temperature fault in the incubator are not clear. The antibody negative-spiked minipools showed a decrease of over 1 log more than the antibody positive-spiked minipool. It should be emphasised, however, that this may be idiosyncratic and associated in part with patient-to-patient differences rather than antibody status. As was seen for the minipools stored at 4°C there was no significant inter-minipool difference.

Table 3.5: Effect of storage temperature and antibody status on stability of HCV RNA in individual samples and spiked into 96 donation minipools

	Mean change in log_{10} HCV RNA copies/ml after 14 days of storage (range)	
Sample	**4°C**	**25°C**
Ab Positive minipool	0.01 (-0.31 to 0.24)	-0.81 (-0.86 to -0.74)
Ab Positive donation	0.02	0.03
Ab Negative minipool	-0.09 (-0.34 to 0.25)	-1.84 (-2.05 to -1.64)
Ab Negative donation	0.06	-0.92

Significant decreases in log_{10} HCV RNA copies/ml are noted in Ab positive and negative minipools and in the Ab negative donation at 25°C. No significant changes are seen in any sample at 4°C.

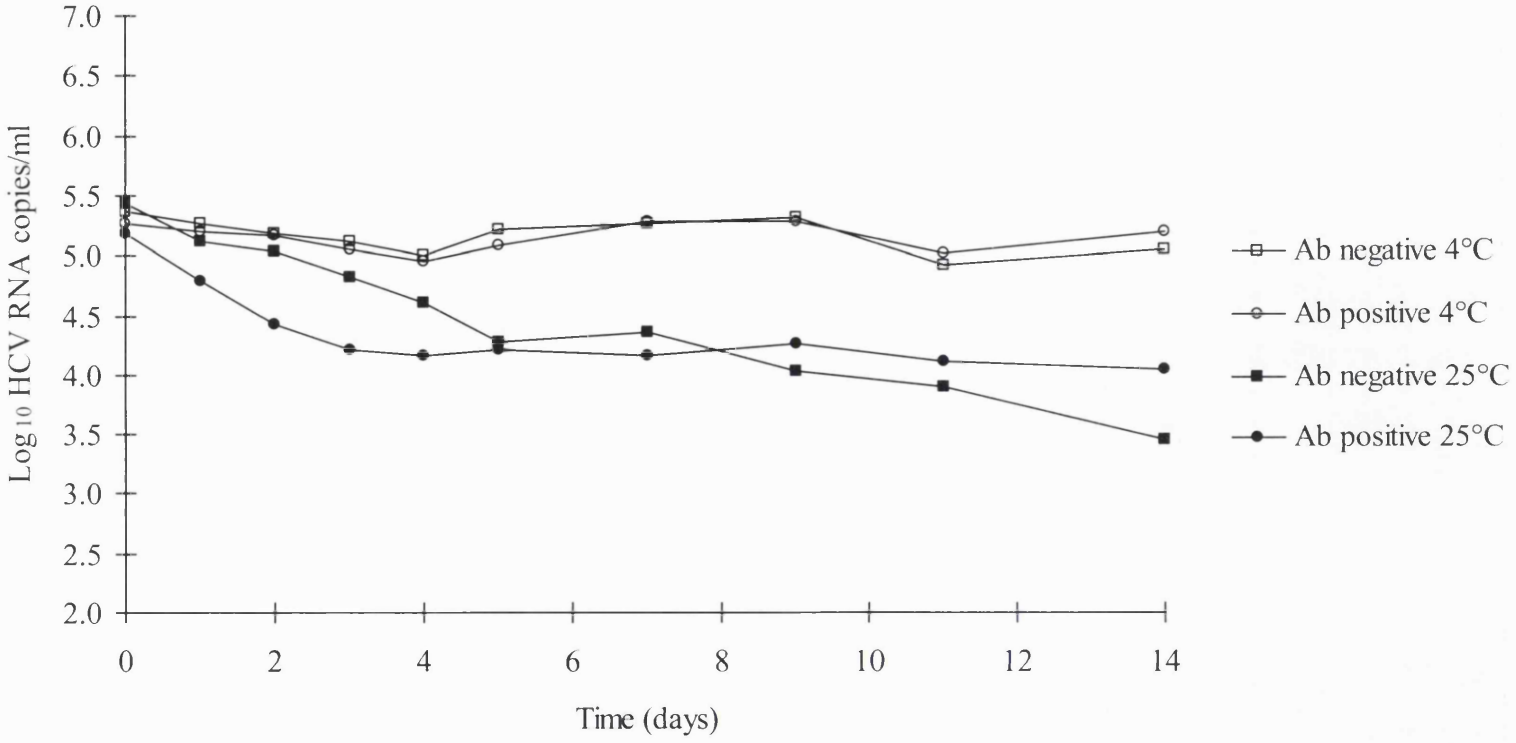

Figure 3.3: Comparison of stability of HCV antibody positive and antibody negative minipools stored at 4°C and 25°C

3.2.4 Discussion

The minipools and undiluted samples stored at 4°C showed no significant decrease over 14 days. Current NBS protocols specify that minipools should be maintained at 4°C for no longer than three days; consideration could be given to extending the storage time of the minipools if this were felt to be logistically advantageous. The HCV RNA decreases found in minipools stored at 25°C do underline the necessity for all plasma to be refrigerated whenever possible rather than being left at ambient temperature.

The results described here are in accordance with another published study in which a pool was spiked with an HCV positive sample showed that HCV RNA remained detectable by qualitative assay even after 21 days of storage at 4°C (Cardoso *et al.*, 1998b).

The minimal inter-pool variability (shown in Table 3.5) and concordance in pooling protocols at the three pooling laboratories suggest that these stability data are representative of all minipools made by the NBS.

The difference in the stability of antibody positive and antibody negative material stored at 25°C may be significant. However, the results of the one donation of each type tested cannot be taken to be representative of all antibody positive or antibody negative donations as the difference in stability may be due to donor-to-donor differences rather than antibody status.

In theory the antibody status of the sample may affect the stability of the HCV, for example in an antibody positive sample the anti-HCV coating the virus particles binding them together into a complex may promote greater stability upon storage. In order to determine the effect of antibody status a much larger study would be required involving many samples both antibody positive and negative.

3.3 Freeze-Thaw Studies

3.3.1 Introduction

Although the NAT samples are not frozen before testing they are archived in 96 deep well microplates and stored at -40°C. Samples in the archive plate will be subjected to freeze/thaw cycles during the removal of adjacent samples for confirmatory tests. The effect of this and the potential for partitioning of viral particles in the wells during the initial freezing raised concerns about the integrity of the archive after one or more freeze/thaw cycles. The partitioning will occur after initial thawing due to the formation of a protein gradient within the well. The automated sample retrieval will take an aliquot from the top of the well without mixing the contents first and depending upon where the virus has partitioned within the protein gradient the aliquot may or may not contain an accurate sample of the virus. The studies described were carried out in collaboration with the Brentwood blood centre where Steve Tassen performed the thawing and sampling of deep well archive plates according to the normal NBS procedures.

3.3.2 Materials and methods

A plasma sample containing a known level of HCV was added to wells at the centre (D7) and the edge (H12) of a 96 deep well archive plate in aliquots of 1 ml. The surrounding wells were filled with HCV negative normal human plasma. The 96 deep well plate and a separate aliquot of the HCV positive sample were frozen at -40°C. The plate was then thawed at 37°C on a heating block in a fan assisted warm air incubator, when the plate was fully thawed 250 µl of each of the HCV samples were withdrawn and frozen at -40°C in a separate tube. The deepwell plate was refrozen as before. The thawing, sampling and refreezing was repeated three times to give a total of four freeze thaw samples. The HCV levels in the samples were quantified using an in house quantitative RT-PCR assay as

described in Section 2.2. PCR products were quantified by a chemiluminescent enzyme linked oligo-nucleotide assay (ELONA) described in Section 2.1.10 using an external calibration curve based on an 'in-house' standard. The 'in-house' standard has been calibrated against the WHO HCV RNA international standard (96/790) obtained from the National Institute for Biological Standards and Control (NIBSC), (Saldanha *et al.*, 1999) as described in section 2.2.4.

3.3.3 Results

The mean HCV RNA level after each cycle of freezing and thawing for both the wells in the 96 well plate are compared with the HCV positive starting material (cycle 0) in Table 3.6. The HCV RNA level in both the inner and outer well positions declined by a similar amount, with a total reduction after four freeze thaw cycles of 0.79 $\log_{10}$ HCV RNA IU/ml in well H12 and 0.97 $\log_{10}$ HCV RNA IU/ml in well D7. This represents declines of 0.20 and 0.24 $\log_{10}$ HCV RNA IU/ml per freeze thaw cycle respectively or 0.22 $\log_{10}$ HCV RNA IU/ml per cycle overall.

Table 3.6: HCV RNA level in each well and decline after each freeze thaw cycle.

	Well H12		Well D7	
Freeze Thaw Cycles	**$\log_{10}$ HCV RNA IU/ml**	**Change from Cycle 0**	**$\log_{10}$ HCV RNA IU/ml**	**Change from Cycle 0**
0	6.35	0.00	6.35	0.00
1	5.93	-0.42	6.04	-0.31
2	5.80	-0.55	5.92	-0.43
3	6.10	-0.25	6.06	-0.29
4	5.56	-0.79	5.38	-0.97

3.3.4 Discussion

There is a mean reduction in HCV RNA level of 0.22 $\log_{10}$ IU/ml per freeze thaw cycle, this is equivalent to a loss of approximately one third of the titre. Both inner and outer wells of the 96 well plate are affected in the same way. This means that a sample containing 100 IU/ml of HCV which is the level in the NBS assay go / no go control would drop below the 95 % limit of detection (12.8 IU/ml defined in section 4.8.3) after only five freeze thaw cycles, therefore any unnecessary freeze thawing should be avoided.

The decline in the level of HCV RNA after freezing and thawing could be due to disruption of the viral genome during freezing and thawing or due to 'settling' of the virus to the lower level of the well. The latter case would appear to cause a decline in the virus because the Tecan pooling robot would sample from the top layer of the archive without mixing the sample in the well.

It is thought that rapid thawing of samples, for example with the use of a 37°C water bath, will result in the least loss of virus. However this is not possible with a 96 deep well archive plate. Even using a 37°C air incubator the time taken to completely thaw a 96 deep well archive plate from -40°C storage would be slow, which could make the reduction in virus levels more pronounced. In this case there was no difference in virus reduction between the outer and inner wells of the plate, although it might be expected that the outer well would thaw first and the inner wells would remain frozen for longer.

Published studies have shown conflicting results, one study found increasing RNA levels after up to eight freeze thaw cycles (Krajden *et al.*, 1999a). Another found a slight decline of 16% after five freeze thaw cycles (Halfon *et al.*, 1996). However another study found average decreases of one log after five freeze thaw cycles (Gessoni *et al.*, 2000).

There are some differences in the methods used which may explain the difference between the studies of Krajden and Halfon finding no or slight

decreases and that of the study reported here and Gessoni finding significant declines. The first is the method used for quantification of HCV RNA, as the two studies finding little or no decline used bDNA technology and those finding significant declines used RT-PCR. This may suggest that RT-PCR assays are more sensitive to viral degradation by freeze thawing than bDNA assays. However the second difference is the speed of the freezing and thawing. Krajden and Halfon used -70°C for rapid freezing and room temperature water baths for rapid thawing of aliquots of plasma or serum. The study by Gessoni used PPT tubes containing 5 ml of sample which would take longer to freeze and thaw. The study described in this chapter used a 96 deep well block containing a total of approximately 100 ml plasma which would considerably increase the time taken to freeze and thaw samples.

Whatever the reasons for the discrepancies, the method used to test the stability of HCV RNA upon freeze thaw cycling should be as close as possible to the actual conditions that would be used in practice. The study described here has used the freezing, thawing and sampling conditions that are used in routine practice within the NBS.

Chapter 4

Development and characterisation of NBS automated HCV NAT screening assays

4.1 Introduction

The main aim of the work leading to this thesis was the development of an automated NAT assay for the NBS utilising commercial components. At the time the most sensitive commercially available assay for the detection of HCV RNA in plasma was the Roche HCV Amplicor. There was an automated amplification and detection available for this assay, the COBAS Amplicor (DiDomenico *et al.*, 1996), but no automated sample extraction. The only high throughput automated sample extraction available was from Qiagen, the BioRobot 9604.

To ensure that the different technologies were compatible a feasibility study was performed using the respective manual versions of the BioRobot and COBAS instruments. In the first instance the QIAamp spin columns were used for extraction of HCV RNA which was then amplified and detected using the microwell plate (MWP) version of the Amplicor v1.0 assay. Soon after this initial study was complete the manufacturer of the Amplicor assay released a new version (v2.0). Therefore the QIAamp spin column extraction was tested with the v2.0 Amplicor assay. These limited initial studies were intended to assess the compatibility of the two components and ensure that adequate detection sensitivity could be achieved.

After the initial studies had shown that the two components could be successfully combined, the automation of the RNA extraction was investigated with the BioRobot 9604. As the BioRobot was still new technology but the COBAS was already established the automated QIAamp extraction was combined with the MWP version of the Amplicor assay. This also meant that the BioRobot performance could be directly compared to the previous spin column performance using the same amplification and detection assay.

The combined assay was assessed for its ability to detect a range of HCV genotypes, and for the potential for sample cross contamination from the BioRobot. Finally after modification to the BioRobot protocol the automated extraction was linked to automated amplification and detection using the COBAS Amplicor and HCV detection sensitivity was assessed.

The combination of the modified BioRobot 9604 viral RNA protocol with the COBAS HCV Amplicor v2.0 was the assay that was introduced at BPL for HCV NAT screening in 1999. However, after the introduction of this assay into the NBS, Qiagen released an upgraded protocol for the BioRobot, the 'Virus' protocol, which was claimed to be more sensitive and robust for RNA extraction and also capable of DNA and RNA co-extraction. The Virus protocol was compared to the viral RNA protocol for extraction of HCV RNA with the COBAS Amplicor v2.0 assay used for amplification and detection. The Virus protocol was also assessed for the extraction of HBV DNA using an in house amplification and detection assay.

Roche then released the AmpliScreen assay for the COBAS, intended solely for blood screening purposes. The old and new methods for extraction and amplification plus detection were assessed in a four-way study to see which would result in the best combination.

As a result of this study the original NBS NAT assay was replaced by the BioRobot 9604 Virus protocol and COBAS HCV AmpliScreen v2.0 assay in 2000.

These studies have since been published in part in the journal Vox Sanguinis (Grant *et al.*, 2002a).

4.1.1 HCV RNA extraction using QIAamp spin columns

The QIAamp spin column viral RNA mini kit uses a silica gel membrane in a spin column for capture of RNA. The plasma sample is mixed with lysis buffer (AVL) which contains the chaotropic salt guanidinium thiocyanate. This chaotrope acts upon the virus coat destroying the protein and releasing the viral nucleic acid, and also destroys any RNAses that may be present. Carrier RNA (poly-A) added to the lysis buffer improves the binding of the viral RNA to the membrane, especially when the viral RNA levels are low. Carrier RNA also limits the action of any residual RNAses upon viral RNA by increasing the total amount of RNA present and therefore reducing the percentage of viral RNA that is attacked.

The conditions are made favourable for binding of the viral RNA to the silica matrix by the addition of ethanol. The mixture is passed through the column by centrifugation or by vacuum during which RNA of greater than around 200 bases in length is bound to the silica membrane. Once the RNA has been bound to the membrane any contaminants are removed by two washes. The wash buffer AW contains a high level of salt to maintain the binding of the RNA to the silica membrane. After the two washes the purified RNA is released from the membrane by the addition of RNAse free water.

4.1.2 Roche Amplicor v1.0

In the Amplicor HCV version 1.0 test (Young *et al.*, 1993) 100 µl of plasma sample are added to a lysis solution containing the chaotropic salt guanidinium thiocyanate to release the viral RNA by disruption of the protein coat. The lysis solution also contains glycogen, which acts as a carrier and allows the nucleic acid pellet to be visible after centrifugation. Once the nucleic acid is released it is precipitated with isopropanol and centrifuged to form a pellet, this pellet is washed in 70% ethanol, and then re-suspended in 1ml of specimen diluent. The

diluent is a bicine buffered solution containing manganese to which the IC is added before use.

The internal control is an *in vitro* RNA transcript with the same primer binding sites as the HCV sequence, generating a product of similar length but with a randomised internal region which is recognised by the internal control probe in the detection assay (Rosenstraus *et al.*, 1998b).

RT-PCR reactions containing 50 µl of the RNA re-suspension and 50 µl of the Amplicor master mix are placed in a GeneAmp PCR system 9600 for thermal cycling. The HCV and IC sequences are amplified by RT-PCR using recombinant *Thermus thermophilus* polymerase (r*Tth* pol), this enzyme has enhanced RT activity in the presence of manganese (Myers and Gelfand, 1991) allowing both the reverse transcription and PCR steps to occur within a single tube.

An antisense biotinylated primer KY78 (5′-biotinyl-CTCGCAAGCACCCT ATCAGGCAGT) binds to the HCV and IC sequences and the r*Tth* pol carries out the reverse transcription to form cDNA. Once cDNA synthesis is completed the mixture is thermocycled to allow PCR amplification using the primers KY78 and KY80 (5′-GCAGAAAGCGTCTAGCCATGGCGT) located in the 5′ untranslated region of the HCV genome to form a 244 base pair amplicon.

The Amplicor system will not re-amplify amplicons from previous reactions due to the presence of uracil-N-glycosylase (UNG); (Longo *et al.*, 1990). UNG will destroy DNA strands containing deoxyuridine, which is incorporated during amplification in place of deoxythymidine. Thus UNG catalyses the cleavage of amplicons from previous reactions so that they do not cause false positive reactions, however at temperatures above 55°C UNG is inactive and therefore it will not affect the newly amplified DNA. After amplification is complete the reactions are denatured by the addition of sodium hydroxide, this acts to denature the UNG as well as the HCV and IC amplicons.

During the detection assay, the biotinylated strands of the HCV and IC amplicons bind to probe sequences coated onto the wells of a MWP. After washing to remove any unbound material and NaOH, an avidin–horseradish peroxidase conjugate is added which binds to the biotin on the amplicon. After washing to remove any unbound conjugate the remaining horseradish peroxidase is detected by the addition of hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine (TMB). The horseradish peroxidase catalyses the oxidation of TMB in the presence of hydrogen peroxide to form a coloured complex. The reaction is stopped by the addition of sulphuric acid and the optical density is measured at 450 nm in a spectrophotometer.

4.1.3 Roche Amplicor v2.0

The Amplicor HCV version 2.0 assay contains several changes from the version 1.0 which were designed to improve the sensitivity and specificity of the assay (section 2.1.4).

The plasma sample volume used was increased from 100 µl to 200 µl and the RNA pellet is re-suspended in 200 µl specimen diluent rather than 1000 µl as in version 1.0. These changes in volume give a ten fold increase in sensitivity (Lee *et al.*, 2000). The internal control, rather than being added at the PCR stage, is added to the lysis buffer in order to control for extraction as well as amplification and detection. In the version 2.0 assay the Mn^{2+} is added directly to the master mix rather than via the diluent. The version 1.0 HCV Amplicor assay produced unequal amplification of the different HCV genotypes, types 1 and 2 were favoured and types 3 and 4 were amplified less efficiently with type 4 giving only 6% of the type 1b level (Rosenstraus *et al.*, 1998a). In version 2.0 the composition of the master mix has been changed such that secondary structure of the RNA is reduced thus increasing reverse transcription efficiency, this change give more equivalent amplification of the different HCV genotypes (Lee *et al.*, 2000).

4.1.4 Extraction and amplification methods

Initially, an estimation of the sensitivity of the Amplicor v2.0 assay was performed using the manufacturer's instructions described in section 2.1.4 i.e. including the Amplicor RNA extraction.

The compatibility of the QIAamp extraction with the Amplicor amplification and detection was assessed. HCV RNA was extracted using the spin column method with the modifications described in section 4.3.1, except that Roche IC was added to the lysis buffer AVL at a concentration of 10.9 µl per ml AVL, or 50 µl IC in 4.6 ml of AVL (enough for eight extractions).

The RT-PCR master mix was activated by adding 100 µl Mn^{2+} to 700 µl master mix. The Qiagen eluate was added to the working master mix (50 µl to 50 µl) in a PCR plate and sealed with adhesive foil seal. The amplification and detection steps were performed according to the manufacturer's instructions described in section 2.1.4.

4.1.5 The BioRobot 9604

The BioRobot 9604 (Figure 4.1) is a robotic sample processor controlled by a PC. It has a robotic arm which carries four probes that can be fitted with disposable filtered tips for sample handling. The tips are coated with carbon making them conductive and thereby allowing liquid level detection so that sample tubes with random volumes may be used. Barcodes on the sample tube are automatically scanned before the samples are processed. The barcode information is saved as a text file and can be passed on to other applications.

Large volumes such as wash buffers are dispensed via the probes without tips using a peristaltic pump connected to external buffer bottles. Four Hamilton syringes are used for more accurate dispensing of small volumes such as the plasma samples and the eluate. After lysis and addition of ethanol the nucleic

acid is bound to the QIAamp 96 plate, a microtitre format plate with 96 columns containing the silica matrix. The lysate and wash buffers are passed through the QIAamp 96 plate using a vacuum. A plate centrifuge is also provided to spin the QIAamp 96 plate dry after the final wash and to spin the eluate into collection microtubes.

The BioRobot 9604 is produced with two configurations for the work top, A and B. Configuration A has the basic worktop. Configuration B includes a thermoblock that can be temperature controlled by the protocol running on the PC, used for heated lysis for DNA extraction from viruses.

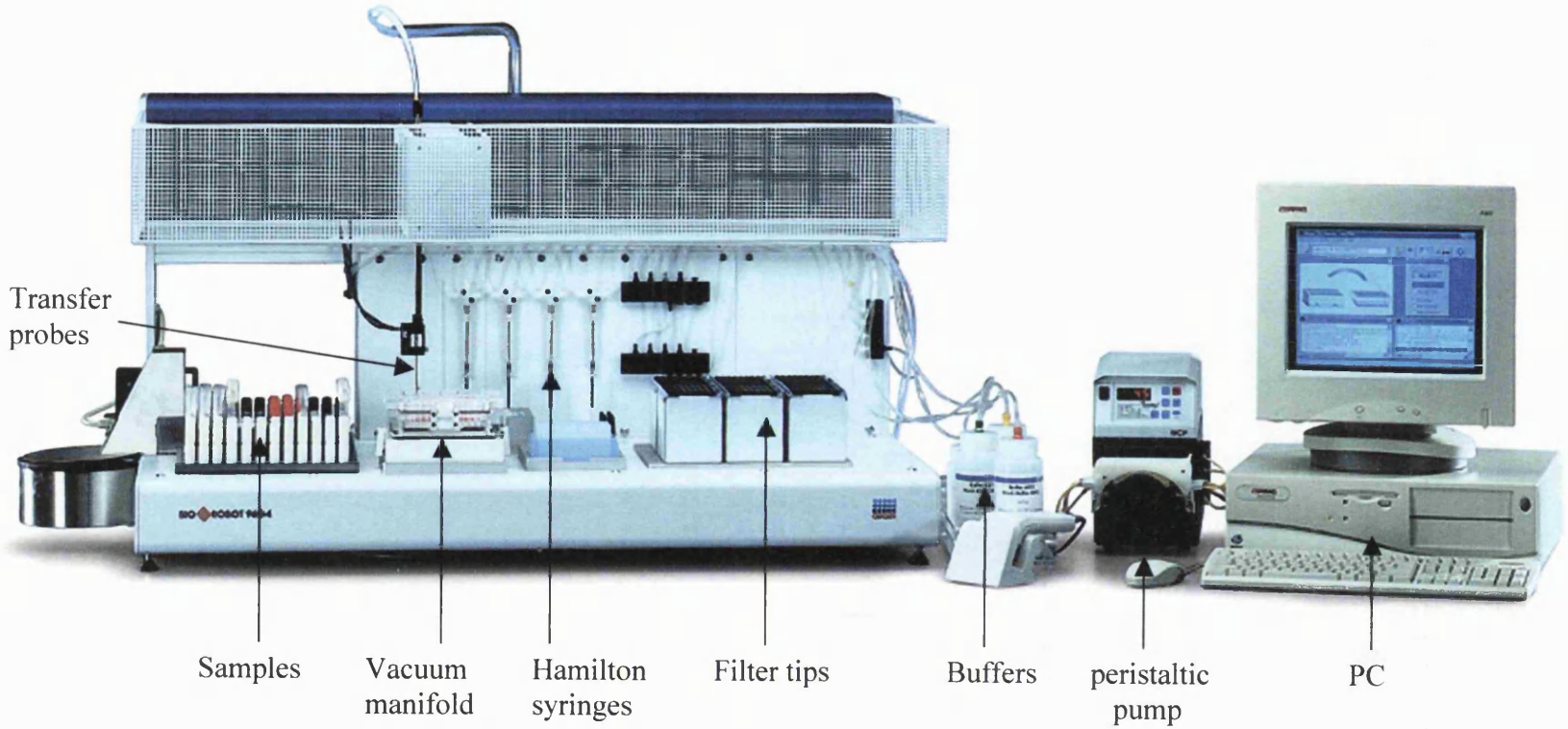

Figure 4.1: The BioRobot 9604

Photo reproduced with kind permission of Qiagen GmbH.

4.1.6 The COBAS Amplicor Analyser

The COBAS Amplicor analyser (Figure 4.2) automates the amplification and detection steps of the Amplicor assay (DiDomenico *et al.*, 1996). The instrument contains two thermalcycler blocks, each with a capacity of twelve reaction tubes, a fixed temperature heating block, a wash station and a spectrophotometer (Figure 4.3). RNA and master mix are prepared in the usual way but are added to 12 tube A-rings that are then placed on the COBAS. The reagents are held in bar-coded reagent cassettes. A single probe is used to transfer amplicons and reagents, the detections are carried out in separate tubes which can be moved around the instrument with the robotic arm. The capture probe is coated onto magnetic beads so that they can be immobilised by a magnet while the tube is washed.

After the amplification has finished the amplicons are denatured, aliquots are added to the magnetic beads coated with either HCV or IC specific capture probes in separate tubes. The capture probe hybridises to the target sequence which is biotinylated. The avidin-horseradish peroxidase conjugate is added and binds to the biotin. The horseradish peroxidase catalyses a colour change in the TMB substrate. This colour change is read in the spectrophotometer at a wavelength of 660 nm.

Figure 4.2: The COBAS Amplicor™ analyser

Photo reproduced with kind permission of Roche Diagnostics UK.

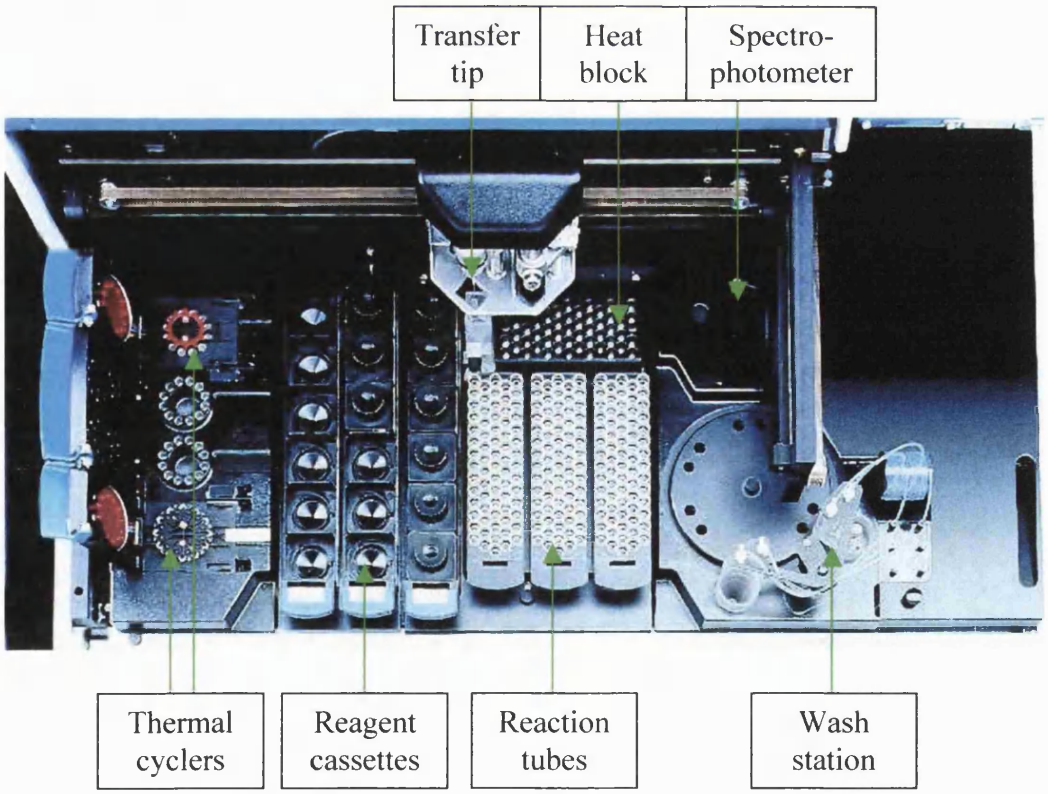

Figure 4.3: The COBAS Amplicor™ analyser components

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4.2 Methods

4.2.1 QIAamp extraction with Amplicor v1.0

The Amplicor v1.0 specimen preparation was substituted with QIAamp spin column extraction following the standard protocol (April 1997) described in section 2.1.1 and including a 50 µl re-elution step. To allow compatibility with the Amplicor assay 25 µl of the HCV RNA eluate from the spin columns was mixed with 25 µl of the Roche sample diluent (containing 5 µl Roche internal control per 700 µl) and added to 50 µl of the Roche master mix in a PCR plate and sealed with adhesive foil. PCR reactions were performed in a PE Applied Biosystems GeneAmp PCR system 9600 thermal cycler according to the manufacturer's instructions described in section 2.1.4. Detection was performed using the microwell plate assay according to the manufacturer's instructions described in section 2.1.4.

The QIAamp spin column HCV RNA extraction and Roche Amplicor v1.0 amplification and detection was assessed using the NIBSC HCV RNA working reagent (96/586) diluted in K_2 EDTA normal human plasma.

4.2.2 QIAamp extraction with Amplicor v2.0

The sensitivity of the version 2.0 Amplicor assay used in accordance with the manufacturer's instructions was assessed using a dilution series of the NIBSC HCV RNA working reagent (96/586 containing 710 IU/ml).

The sensitivity of the modified QIAamp Viral RNA extraction and the Amplicor v2.0 assay was assessed using a dilution series of the NIBSC and CLB PELISPY HCV RNA working standards.

4.2.3 HCV RNA extraction using Qiagen BioRobot 9604 QIAamp 96 viral RNA protocol

The automated QIAamp procedure using the BioRobot 9604 is similar to the spin column manual method however there are some differences to allow the protocol to be used on a vacuum manifold. A different wash buffer, AW1 is used for the first wash containing the chaotropic salt guanidinium chloride, the second wash uses AW2 which is the same formulation as AW in the spin column protocol. The elution is performed at room temperature.

The manufacturer's protocol for HCV RNA extraction from plasma supplied with the BioRobot (the QIAamp 96 viral RNA protocol) is described in section 2.1.2. The supplied protocol was modified to allow compatibility with the Roche Amplicor v2.0 assay as follows; the concentration of the carrier RNA in the viral lysis buffer was decreased from 7.1 µg per ml to 0.825 µg per ml as this had improved sensitivity with the spin column assay (section 4.3.1). The Roche Amplicor v2.0 internal control was added to the viral lysis buffer (AVL) at a concentration of 4.44 µl per ml AVL to give the equivalent input of IC to the PCR as in the Amplicor method. The elution volume was increased from 60 µl to 70 µl to allow adequate eluate for addition to the Amplicor assay as approximately 20 µl is retained in the silica matrix of the QIAamp plate.

For the purpose of this document these modifications will be referred to as the standard protocol. Dilutions of the NIBSC working reagent 96/586 diluted in NHP were extracted with the QIAamp 96 viral RNA standard protocol, and amplified and detected using the Amplicor v2.0 protocol.

After this initial assessment several modifications were made to the QIAamp 96 viral RNA standard protocol as described in section 4.3.4. The sensitivity of the modified protocol was assessed as before using the Amplicor v2.0 protocol.

4.2.4 Detection of different HCV genotypes

In order to assess the ability of the hybrid assay to detect the various genotypes of HCV, samples of genotypes 1 to 4 previously genotyped by InnoLiPA (Innogenetics, Ghent, Belgium), (Stuyver *et al.*, 1996), that had been quantified by the Chiron Quantiplex v2.0 (bDNA) assay (Detmer *et al.*, 1996), were tested at end-point dilution (between 59 and 75 copies/ml). A 1:100 dilution of the NIBSC HCV working reagent (40 copies/ml or 7.1 IU/ml, genotype 3) was run at the same time. The HCV RNA concentration of the four genotyped samples was calculated using the Poisson distribution method (Simmonds *et al.*, 1990). In this method the HCV concentration in the sample is calculated using an assumed reverse transcription (RT) efficiency, which is usually considered to be 10%. The NIBSC standard was assayed by the same method at the same time to allow a check of the calculations involved.

4.2.5 Sensitivity of the BioRobot QIAamp 96 viral RNA protocol with Amplicor HCV v2.0 using the COBAS Amplicor analyser

Although good sensitivity had been found with the modified BioRobot extraction protocol with the MWP version of the Amplicor v2.0 assay (section 4.3.4), the sensitivity using the COBAS amplification and detection required investigation, as the automated assay was the version required in the NBS. Therefore the sensitivity of HCV detection using the COBAS Amplicor system was compared to the MWP version with the modified BioRobot QIAamp 96 viral RNA extraction described in section 4.3.4. Also, the Air Pore tape was omitted during the 10 minute drying spin to allow better evaporation of residual ethanol, which had been shown to contaminate the eluate at levels that could lead to PCR inhibition (C. Sims, personal communication).

The Paul Ehrlich Institute (PEI) HCV standard (75/98) was used in this study as it is made from an HCV RNA containing sample in the window phase of infection. As the reason for the development of NAT tests was to detect window

phase samples, the PEI standard was used in this final study to ensure that the NAT system described was able to detect these samples with adequate sensitivity.

Dilutions of the PEI HCV standard (75/98) were extracted in duplicate on each BioRobot run then half of the RNA from each run was amplified and detected using COBAS and the other half using the MWP version of the Amplicor v2.0 assay.

4.3 Results

4.3.1 Sensitivity of Amplicor v1.0 using the QIAamp system for extraction of HCV RNA

The performance of the combination of QIAamp spin column HCV RNA extraction and Roche Amplicor v1.0 amplification and detection was assessed using the NIBSC HCV RNA working reagent (96/586) diluted in K_2 EDTA NHP. Initially inadequate performance was achieved using the standard protocol with 3 of 4 positives for the neat NIBSC (710 IU/ml) and 0 of 10 at a 1 in 10 dilution of NIBSC (71 IU/ml). Some modifications were then made to the QIAamp protocol (detailed below).

The plasma input volume was increased from the standard 140 µl to 280 µl or 560 µl with an equivalent increase in the AVL and ethanol volumes, but this did not increase the sensitivity. An RNA to diluent to master mix ratio of 1:1:1 decreased sensitivity and made detection of IC unreliable.

It was noted that elution at 80°C caused the eluate to evaporate and re-condense around the top of the column, therefore the temperature of elution was reduced from 80°C to 45°C, this resulted in a slight increase in sensitivity with 5 of 14 positive at a 1 in 10 dilution of NIBSC (71 IU/ml). At a 1 in 100 dilution of NIBSC (7.1 IU/ml) elution at 80°C gave 2 of 8 positive whereas elution at 45°C gave 4 of 8 positive.

The greatest increase in sensitivity was gained by reducing the concentration of the carrier RNA in the Qiagen lysis buffer from 21 µg/ml to 0.825 µg/ml. This resulted in a change from 37.5% to 100% detection of a 71 IU/ml control (Table 4.1).

Table 4.1: Effect of carrier RNA concentration on HCV detection sensitivity with the QIAamp spin column protocol and Roche Amplicor v1.0

Carrier Concentration (µg/ml of AVL)	Number Positive/Tested [a]	Percentage Positive
21.0	3 / 8	37.5%
7.0	7 / 8	87.5%
0.825	8 / 8	100%

[a] A 1:10 dilution of the NIBSC working reagent 96/586 (equivalent to 71 IU/ml)

Variability in the eluate volume was reduced by adding an additional spin with a clean collection tube after the second wash step. This ensured that any residual wash buffer around the sides of the spin column was removed, thus eliminating contamination of the eluate with ethanol or salt from the wash buffer, which could cause inhibition of the PCR.

As the manufacturer stated that the centrifugation speed may be increased without affecting RNA yield, the centrifugation speed was increased to 21 000 *g* so that spin times for the lysis mixture and washes could be reduced to 30 seconds each. The original spin times of 2.5 minutes for the drying spin (after second wash) and one minute for the elution spin were maintained. Using the final modified protocol improved sensitivity was achieved with 25% detection at a 1:100 dilution of the NIBSC working reagent 96/586 equivalent to 7.1 IU/ml (Table 4.2). This compares to the results of the original protocol of 75% detection at 710 IU/ml and 0% detection at 71 IU/ml.

Table 4.2: Sensitivity of QIAamp spin column protocol with Amplicor v1.0

HCV Concentration [a] (IU/ml)	Number Positive/Tested	Percentage Positive
71.0	8 / 8	100%
7.1	6 / 24	25%

[a] Using the NIBSC working reagent 96/586

4.3.2 HCV sensitivity of Roche Amplicor v2.0

The sensitivity of the version 2.0 Amplicor assay used in accordance with the manufacturer's instructions was assessed using a dilution series of the NIBSC HCV RNA working reagent (96/586 containing 710 IU/ml); (Table 4.3). The 95% limit of detection using this assay with the NIBSC working standard, calculated by probit analysis, was 22.4 IU/ml (95% CI = 14.2 to 105.9 IU/ml).

Table 4.3: Sensitivity of Roche Amplicor v2.0 performed to manufacturer's instructions

HCV level [a] IU/ml	Number Positive/Tested	Percentage Positive
35.5	8 / 8	100%
17.8	7 / 8	87.5%
7.1	5 / 8	62.5%
1.8	4 / 10	40%
0	0 / 2	0%

[a] NIBSC HCV RNA 96/586 diluted in NHP.

4.3.3 Sensitivity of Amplicor v2.0 using the QIAamp system for extraction of HCV RNA

The sensitivity of the modified QIAamp Viral RNA extraction and the Amplicor v2.0 assay was assessed using a dilution series of the NIBSC and CLB PELISPY HCV RNA working standards and is shown in Table 4.4. The 95% limits of detection calculated by probit analysis were 23.8 IU/ml (95% CI = 16.8 to 52.1 IU/ml) with the NIBSC standard was and 22.4 IU/ml (95% CI = 12.1 to 87.7 IU/ml) with the Pelispy standard. There was no significant difference between the spin column and Amplicor extractions at any dilution of the NIBSC standard.

Table 4.4: Sensitivity Amplicor v2.0 with QIAamp spin column RNA extraction

NIBSC Concentration [a] IU/ml	Number Positive/Tested	Percentage Positive
71.0	8 / 8	100%
35.5	16 / 16	100%
17.8	14 / 16	87.5%
7.1	56 / 83	67.5%
1.8	22 / 40	55.0%
0	0 / 8	0%
Pelispy Concentration [b] IU/ml	**Number Positive/Tested**	**Percentage Positive**
312.5	12 / 12	100%
100	12 / 12	100%
31.3	12 / 12	100%
10	9 / 12	75%
3.1	4 / 12	33.3%
1	1 / 12	8.3%
0	0 / 8	0%

[a] NIBSC HCV RNA 96/586 diluted in NHP.
[b] CLB PELISPY HCV RNA standard diluted in NHP.

4.3.4 Sensitivity of the BioRobot 9604 QIAamp 96 viral RNA protocol with Amplicor HCV v2.0 MWP

The use of the standard QIAamp 96 viral RNA protocol on the BioRobot 9604 with modifications to allow compatibility with HCV Amplicor v2.0 resulted in reduced sensitivity compared to the spin column protocol as shown in Table 4.5. The 95% limit of detection using the BioRobot viral RNA protocol was 27.1 IU/ml (CI = 18.1 to 54.1 IU/ml) as compared to 23.8 IU/ml using the spin column protocol.

Table 4.5: Sensitivity of the standard BioRobot QIAamp 96 viral RNA protocol compared to the spin column protocol

HCV Level [a]	BioRobot standard protocol		Spin Column protocol	
IU/ml	Number Positive/Tested	Percentage	Number Positive/Tested	Percentage
35.5	24 / 24	100%	16 / 16	100%
17.8	21 / 24	87.5%	14 / 16	87.5%
7.1	12 / 24	50.0%	56 / 83	67.5%
1.8 [b]	3 / 24	12.5%	22 / 40	55.0%
0	0 / 8	0%	0 / 8	0%

[a] NIBSC HCV RNA 96/586 diluted in NHP.
[b] At 1.8 IU/ml there was a significant difference between the BioRobot and spin column methods using Fisher's exact test P = 0.001212.

Several modifications were made to the BioRobot standard protocol, including double elution and substituting BioRobot elution buffer with Amplicor diluent (Table 4.6). None of these modifications significantly improved the sensitivity.

Table 4.6: Modifications to BioRobot QIAamp 96 viral RNA elution

HCV level [a] (IU/ml)	Number Positive/Tested (Percentage)			
	70µl Elution [b]	50 + 30µl Elution	40 + 40µl Elution	40 + 40µl Elution [c]
17.8	21 / 24 (87.5%)	4 / 8 (50.0%)	5 / 8 (62.5%)	-
7.1	12 / 24 (50.0%)	3 / 8 (37.5%)	8 / 16 (50.0%)	8 / 16 (50.0%)
1.8	3 / 24 (12.5%)	1 / 8 (12.5%)	2 / 8 (25.0%)	-

[a] NIBSC HCV RNA 96/586 diluted in NHP.
[b] Standard protocol elution.
[c] Roche diluent used in place of BioRobot elution buffer.

The efficiency of the vacuum steps was investigated using spin columns with the Qiagen vacuum manifold. Passing buffers through the spin columns by vacuum instead of centrifugation produced a significant decrease in sensitivity (Table 4.7). Using the BioRobot buffer volumes and elution, the addition of the BioRobot drying spin and substitution of wash buffer AW2 with 70% ethanol did not increase the sensitivity. Comparison of vacuum versus centrifugation of the wash steps produced interesting results, and when investigated further it was shown that centrifuging the last wash was all that was required to match the sensitivity of the spin column centrifuge method (Table 4.7).

Table 4.7: Investigation of the vacuum steps of the QIAamp protocol using spin columns on a vacuum manifold.

Parameters used	Number positive/tested 7.1 IU/ml [a]	Percentage Positive	Significant change from spin column?
Spin column method	56 / 83	67.5%	NA
Vacuum method	3 / 24	12.5%	P=0.000003
BioRobot volumes	1 / 7	12.5%	P=0.009
BioRobot volumes & drying spin	0 / 8	0%	P=0.0003
BioRobot volumes & 70% Ethanol 2nd wash	0 / 8	0%	P=0.0003
Vacuum method except AW2 spun at 21000 x *g*	10 / 16	62.5%	NS
Vacuum method except AW1 & 2 spun at 21000 x *g*	7 / 8	87.5%	NS

[a] A dilution of NIBSC HCV RNA working standard 96/586 giving 7.1 IU/ml.
NA = Not applicable
NS = Not significant P>0.05 by Fisher's exact test.

Paradoxically however, when the centrifugation of the last wash step was performed using the BioRobot and plate centrifuge the sensitivity was decreased rather than increased (Table 4.8). Other modifications to the wash steps were investigated, including increasing the AW2 wash volume and number of washes, extending the drying spin time and introducing a short spin between washes (Table 4.8).

Table 4.8: Modification of the BioRobot QIAamp 96 viral RNA standard protocol wash steps

Method	Number positive/tested 7.1 IU/ml [a]	Percentage Positive	Significant change from standard protocol?
Single 900 µl AW2 wash 70 µl elution. Standard protocol	18 / 40	45.0%	NA
Single 900µl AW2 wash by centrifugation. 70 µl elution	0 / 32	0.0%	P=0.000003
Double 1250 µl AW2 wash 70 µl elution	4 / 16	25.0%	NS
Single 1250 µl AW2 wash 80 µl elution	32 / 48	66.6%	P=0.053
Double 1250 µl AW2 wash 80 µl elution	24 / 48	50.0%	NS
Single 1250 µl AW2 wash 20 minutes dry, 80 µl elution	19 / 48	39.6%	NS
Double 1250 µl AW2 wash 1 min spin between, 80 µl elute	21 / 48	43.8%	NS

[a] At a dilution of NIBSC HCV RNA working standard 96/586 giving 7.1 IU/ml.
NA = Not applicable
NS = Not significant P>0.05 by Fisher's exact test.

The greatest improvement in sensitivity was achieved by increasing the volume of the AW2 wash from 900 µl to 1250 µl, and increasing the volume of the elution from 70 µl to 80 µl. This modified protocol was then used to determine the HCV sensitivity by testing a dilution series of the NIBSC HCV RNA working standard; this is shown in Table 4.9.

Although the 95% limit of detection using this modified protocol was only slightly improved to 26.7 IU/ml (95% CI = 19.7 to 47.2 IU/ml), the 50% limit of

detection was more than twice as sensitive, at 2.25 IU/ml (95% CI = 0.0 to 6.29 IU/ml) vs. 6.04 IU/ml (95% CI = 4.22 to 8.16 IU/ml) for the standard protocol. There was no significant difference between the modified BioRobot protocol and the spin column protocol at any dilution of the NIBSC working reagent.

Table 4.9: Comparison between the sensitivities of the modified and standard BioRobot QIAamp 96 viral RNA protocols

HCV Level [a]	BioRobot modified protocol		BioRobot standard protocol	
IU/ml	Number Positive/Tested	Percentage	Number Positive/Tested	Percentage
71.0	24 / 24	100%	-	-
35.5	24 / 24	100%	24 / 24	100%
17.8	19 / 24	79.2%	21 / 24	87.5%
7.1	32 / 48	66.6%	12 / 24	50.0%
1.8	11 / 24	45.8%	3 / 24	12.5%
0	0 / 8	0.0%	0 / 8	0.0%

[a] NIBSC HCV RNA 96/586 diluted in NHP.

4.3.5 Sensitivity of the Amplicor v2.0 assay with BioRobot 9604 RNA extraction to HCV genotypes 1 to 4

The copy number in the original sample was calculated as in Table 4.10. Using this method the estimation of copy number in the NIBSC standard was 3750 copies/ml, this agrees with the actual value for this standard of 4000 copies /ml.

Table 4.10: Calculation of copy number by Poisson distribution

Calculation	Explanation
–ln(freq negs)	DNA copies /PCR from the Poisson distribution
x10	RNA copies/PCR assuming 10% RT efficiency
x1.6	RNA copies/extract as only 50µl of 80 µl eluate is added to the PCR, also assumes 100% extraction efficiency.
x5	RNA copies/ml as only 200 µl plasma into the system
x dilution factor	to get copies/ml in the original sample.

None of the estimations of viral concentration differ from the bDNA derived concentrations by more than one half $\log_{10}$ indicating reliable detection of HCV genotypes 1 to 4. The estimated HCV concentrations are shown in Table 4.11.

Table 4.11: Sensitivity of detection of HCV genotypes 1 to 4

Genotype	Quantiplex bDNA	Qiagen Roche
	Viral load (Meq/ml) [a]	Estimated Viral load [b] (Meq/ml)
Type 1	6.5	2.3
Type 2	5.9	5.6
Type 3	7.4	11.1
Type 4	7.5	11.1

[a] 1 Meq = 1 million genomes per ml.
[b] Estimated by Poisson distribution method.

4.3.6 Investigation of sample cross contamination using the BioRobot

To address any concerns that contamination of HCV negative samples, from those containing HCV, might occur during the robotic viral RNA extraction, a grid of HCV positive samples with high viral concentration interspersed with negative samples was extracted by the BioRobot 9604 and tested by the Amplicor v2.0 assay. Sixteen plasma samples containing HCV at a concentration of 2×10^6 copies/ml were arranged in a grid surrounded by 48 negative samples. After testing with the Amplicor v2.0 assay all 48 of the negative samples were found to be negative for HCV RNA, and all 16 HCV samples were found positive for HCV RNA.

4.3.7 Sensitivity of the BioRobot QIAamp 96 viral RNA protocol with Amplicor HCV v2.0 using the COBAS Amplicor analyser

The sensitivity of the modified BioRobot viral RNA protocol with both the MWP and COBAS methods of Amplicor v2.0 amplification and detection are shown in Table 4.12. The 95% limit of detection was 22.6 IU/ml (95% CI = 15.8 to 45.9 IU/ml) for the MWP assay and 25.5 IU/ml (95% CI = 17.7 to 51.6 IU/ml) for the COBAS assay. No significant difference was found in the detection sensitivity between the two assays at any dilution using Fisher's exact test.

Table 4.12: Sensitivity of the modified BioRobot viral RNA protocol with COBAS and MWP Amplicor HCV v2.0

HCV Level [a]	MWP		COBAS	
IU/ml	Number Positive/Tested	Percentage	Number Positive/Tested	Percentage
150	24 / 24	100%	24 / 24	100%
50	24 / 24	100%	24 / 24	100%
37.5	23 / 24	95.8%	24 / 24	100%
20	23 / 24	95.8%	22 / 24	91.7%
10	19 / 24	79.2%	15 / 24	62.5%
4	4 / 12	33.3%	6 / 12	50.0%
0	0 / 12	0.0%	0 / 12	0.0%

[a] Values are based on the dilution of the PEI 75/98 working standard in NHP.

This sensitivity compared well with the data using the spin column extraction and MWP Amplicor v2.0 which gave a 95% sensitivity of 23.4 IU/ml. The sensitivities at end point dilution for the BioRobot with MWP and COBAS and the spin column with MWP were all very similar and are shown in Figure 4.4.

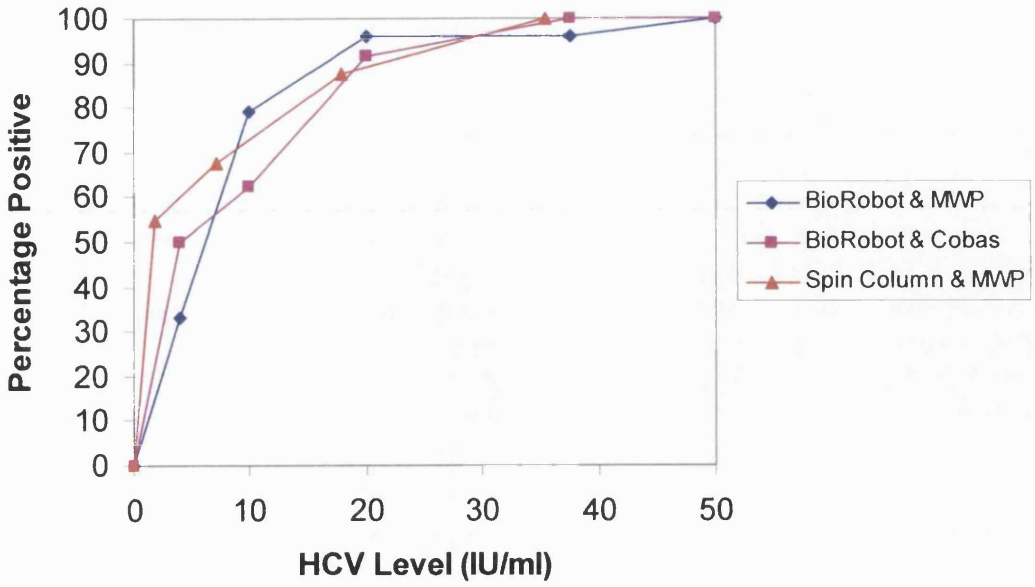


Figure 4.4: Comparison of end point sensitivity between the BioRobot and spin column methods.

It was noted that some of the ODs from the COBAS were lower than those of the MWP, when these were plotted it became clear that there was a difference between the ODs generated by the two methods (Figure 4.5). The absorbance readings from the COBAS version of the assay are lower compared to those of the MWP, even taking into account the fact that the reader in the COBAS has an upper limit of 4 units whereas the reader used for the MWP version has an upper limit of 3 units. Although these reduced OD readings still represent positive results, the goal of a qualitative assay is for a digital response i.e. all or nothing for easy interpretation of positive and negative results. With the COBAS system although only apparent at very low concentrations there is more likelihood of grey zone results.

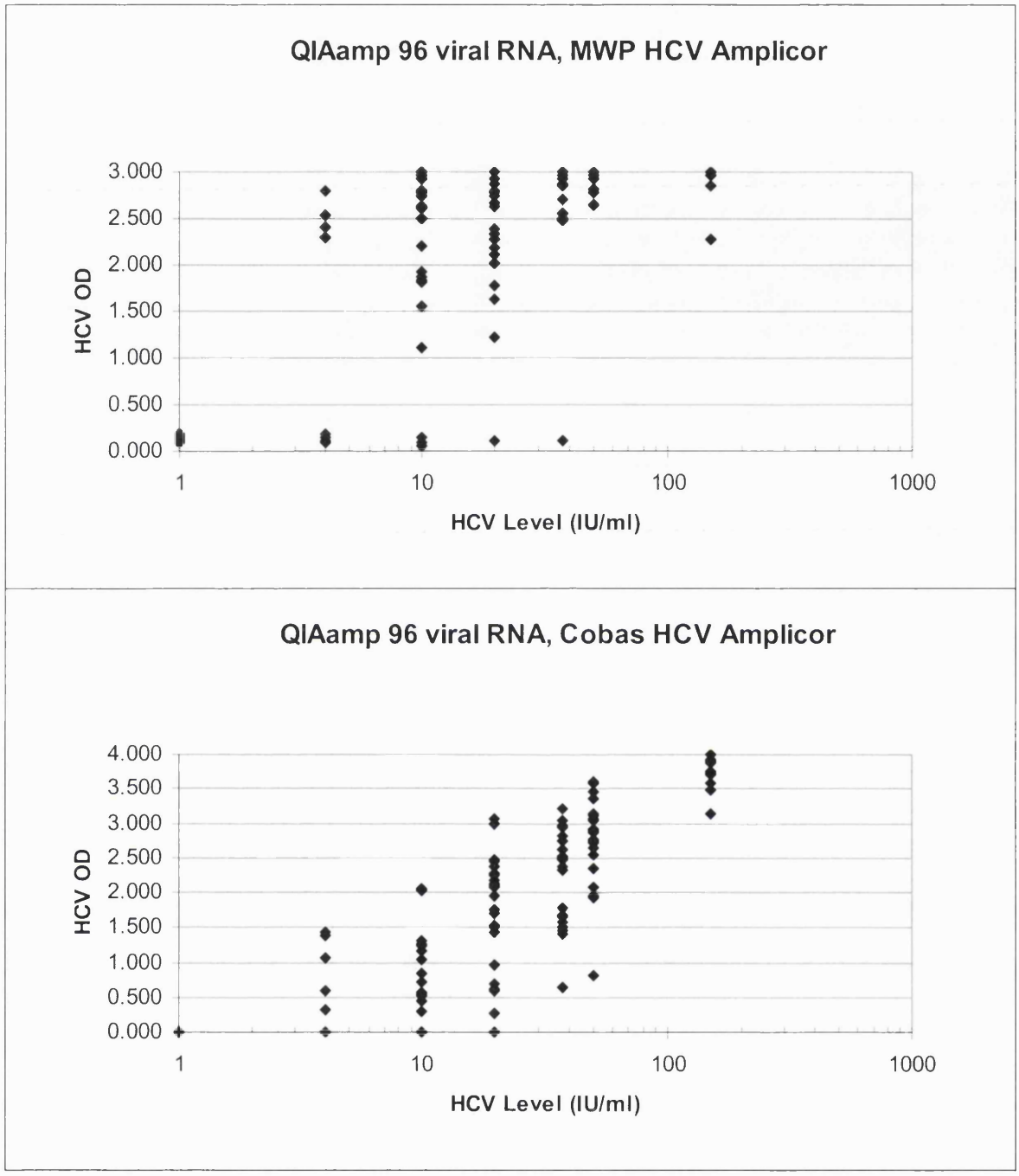


Figure 4.5: Comparison of the HCV optical densities (OD) at the limit of detection between the MWP and COBAS versions of HCV Amplicor v2.0

4.4 Discussion

Spin column extraction with Amplicor v1.0

The initial studies showed that the Amplicor v1.0 could be successfully combined with the QIAamp spin column extraction. In fact the combination of the QIAamp extraction with the Amplicor v1.0 amplification and detection was able to achieve a greater sensitivity than other published studies that used Amplicor v1.0 assay in full and found an end point sensitivity of around 1000 copies/ml (equivalent to 250 IU/ml); (Lee *et al.*, 2000).

The Roche Amplicor assay uses rTth polymerase which requires manganese ions (Mn^{2+}) to function correctly and these are introduced into the system via the Roche sample diluent. This is also the stage at which the internal control is added. In order to substitute the QIAamp spin column extraction for the normal Roche extraction the QIAamp eluate had to be mixed with the Roche sample diluent before being added to the master mix, to allow introduction of Mn^{2+} and internal control.

This had two disadvantages, primarily only 25 µl RNA eluate could be added to the PCR mix rather than 50 µl, also the amount of Mn^{2+} introduced into the PCR was half the normal level. The internal control concentration could be doubled in the sample diluent used so as to give the same final concentration in the PCR as in the standard Roche assay. A 1:1:1 ratio of eluate, sample diluent and PCR mix made the PCR reaction unreliable, presumably due to lowering the PCR reagent concentrations in the final mix. Addition of extra Mn^{2+} to the sample diluent to bring the final concentration in the PCR to the correct level did not increase sensitivity.

Lowering elution temperature from 80°C to 45°C caused a slight increase in sensitivity. Interestingly, since this work was carried out Qiagen have updated the protocol and in the new version (dated January 1999) suggest that the elution be performed at room temperature not 80°C. Elution volumes were made more

consistent by introducing a drying spin with a clean collection tube after the second wash step. The shape of the collection tube and the angle within the centrifuge caused wash buffer that had been spun through the spin column to collect between the spin column and the wall of the collection tube. This small amount of wash buffer on the column would then be spun into the eluate, where the ethanol and salt in the buffer could cause inhibition of the PCR. By introducing another spin with a clean collection tube after the final wash, any residual wash buffer was removed before the elution step.

The greatest increase in sensitivity was obtained after reducing the concentration of carrier RNA in the AVL lysis buffer. In the standard protocol this was 21 μg per ml AVL, but it was noted that in the BioRobot extraction the carrier concentration was 7 μg per ml in the AVL. Too much carrier RNA may inhibit the reverse transcription or the PCR reaction by reducing the level of free cations available that are required to allow primer to target binding as well as to maintain the functionality of the polymerase enzyme. Reducing the carrier concentration to the level used in the BioRobot increased the sensitivity. The level was then decreased further to 0.825 μg per ml AVL which gave a further slight increase in sensitivity. The figure 0.825 μg per ml was chosen as an easy dilution (1:1000) of the 825 μg of lyophilised carrier provided in the kit. Using the final modified protocol improved sensitivity was achieved with 25% detection at a 1:100 dilution of the NIBSC working reagent (Table 4.2).

Spin column extraction with Amplicor v2.0

When the v2.0 Amplicor assay was introduced there were several changes from the v1.0 assay which affected the compatibility of the QIAamp extraction with the Amplicor amplification and detection. The change in the introduction of the internal control, from the PCR stage to the lysis buffer was mirrored in the QIAamp assay by adding internal control directly to the Qiagen lysis buffer AVL. In the v2.0 assay the Mn^{2+} is added directly to the master mix rather than via the diluent. This allows a full 50 μl QIAamp eluate to be added to the PCR as there is no longer any need to add Roche diluent. The sensitivity of the assay run

according to the manufacturer's instructions is shown in Table 4.3 with a 95% detection of 22.4 HCV IU/ml.

Using the combination of QIAamp spin column extraction and Amplicor v2.0 amplification and detection resulted in the sensitivity shown in Table 4.4 with a 95% limit of detection calculated by probit analysis of 23.8 HCV IU/ml using the NIBSC 96/586 working reagent.

This sensitivity represents quite a large improvement over the spin columns with Amplicor v1.0, a probit analysis cannot be performed on the v1.0 data as there were not enough HCV concentrations tested, however just comparing the 1:100 dilution of the NIBSC showed that 25% detection (6 of 24) for the v1.0 assay was increased to 67.5% (56 of 83) with the v2.0 assay. Although the increase in sensitivity between the two versions of the Amplicor assay was probably due to the doubling of the volume of RNA added to the PCR reaction, it may also in some part be related to the reformed master mix and improved detection of genotype 3 samples, claimed by the manufacturer (Lee *et al.*, 2000), as the NIBSC standard is of genotype 3.

BioRobot extraction with Amplicor v2.0

After a number of modifications to the manufacturer's original BioRobot QIAamp 96 viral RNA protocol to allow it to be linked to the Amplicor assay in either COBAS or MWP form, an assay with equivalent sensitivity to the spin column was achieved (Figure 4.4).

The wash steps appeared to be a critical factor in the sensitivity achieved with the BioRobot because wash buffer is passed through the QIAamp plate by vacuum rather than by centrifugation as in the spin column protocol. In the original BioRobot protocol the wash volumes were the same as the lysate volume so any splashing of lysate or wash buffer around the top of the wells would remain present and may even be dried on to the QIAamp plate due to air flow caused by the vacuum. The lysate in particular, having a high ethanol content, will very quickly evaporate leaving a salt deposit. These salt deposits could be forced onto

the membrane during the drying centrifugation and then dissolved in the eluate causing inhibition of the PCR and alteration of the stringency of primer binding.

To avoid salt contamination the wash steps were modified so that the final wash fills the wells to a higher level than the lysate or AW1 first wash, thus removing any 'tide mark' deposits before the elution. The volume of the elution was also increased to ensure that at least 50 µl eluate were available for addition to the PCR master mix. A proportion of the eluate will inevitably remain in the silica membrane after the centrifugation as the *g*-force applied is not as high as that used in the spin column centrifugation. To account for this dead volume the amount of eluate added to the QIAamp plate was increased to 80 µl.

These modifications resulted in increased sensitivity of the robotic sample preparation method equivalent to that of the spin column extraction method.

The use of the COBAS in place of the MWP assay is necessary for use in the NBS, where complete automation is needed. Using the COBAS did not significantly decrease the sensitivity of the assay but the absorbances generated were generally lower than those of the MWP assay at the very low HCV input levels. This was probably because the COBAS assay uses only 36 cycles of PCR (R. Sun - Roche Molecular Systems, personal comment) compared to 37 in the MWP assay. The extra cycle of PCR in the MWP assay would make the amplicon levels higher and thus generate higher absorbance levels in the detection assay. The reduction in absorbance using the COBAS was not a significant problem for the NBS as any positive results were retested for confirmation even if they were equivocal.

The sensitivity of the NAT screening assay was of paramount importance to the NBS, and this was shown to be quite sufficient with the BioRobot QIAamp 96 viral RNA protocol with COBAS Amplicor v2.0 assay. The final protocol described here (section 4.3.7) was introduced into routine use within the NBS in 1999, to screen all donations from England and Wales for HCV RNA for the release of long shelf life (over 35 days) blood components.

4.5 BioRobot 9604 QIAamp 96 viral RNA extraction with Amplicor HIV Monitor v1.5

4.5.1 Introduction

This small feasibility study was performed to determine if the automated QIAamp extraction method could be used for HIV-1 RNA detection if this became a requirement in the NBS. At the time this work was done there was no qualitative HIV-1 RNA assay from Roche. The only HIV-1 RNA assay available from Roche was HIV-1 Monitor v1.5 quantitative assay, however this was not suitable for use as a screening assay. It was important for the NBS to use a Roche assay rather than another manufacturer or an in house assay to remain compatible with the HCV screening. The advantage of the Monitor assay was that it was available for use with the COBAS instrument allowing complete automation. It was therefore decided to modify the monitor assay so that it would perform in a similar way to the HCV Amplicor assay.

4.5.2 Methods

The HIV-1 standard PWS-1 from NIBSC (99/634) was diluted in HIV-1 negative EDTA plasma to various concentrations. These were extracted using the BioRobot 9604 viral RNA modified protocol as described in section 4.3.4. The Monitor quantitative standard (QS) control was added to the lysis buffer at a concentration of 1.04 µl per ml AVL.

After extraction using the QIAamp 96 viral RNA modified protocol, 50 µl of the RNA was immediately transferred to 50 µl of working HIV-1 master mix in a 0.2 ml tube PCR tray. This was sealed with adhesive foil and cycled using the manufacturer's or modified cycling parameters shown in Table 4.13.

Table 4.13: Modification to manufacturer's HIV-1 Monitor v1.5 RT-PCR cycling conditions

RT-PCR stage	Manufacturer's method	Modified method
Activation of UNG	2 min at 50°C;	2 min at 50°C;
Reverse transcription	30 min at 60°C	30 min at 60°C
Initial PCR cycling	10 seconds at 95°C 10 seconds at 52°C 10 seconds at 72°C for 8 cycles	10 seconds at 95°C 10 seconds at 52°C 10 seconds at 72°C for 8 cycles
Main PCR cycling	10 seconds at 90 °C 10 seconds at 55°C 10 seconds at 72°C **for 23 cycles**	10 seconds at 90 °C 10 seconds at 55°C 10 seconds at 72°C **for 32 cycles**
Final extension	15 min at 72°C	15 min at 72°C
Total PCR cycles	31	40

The amplified products were removed from the machine and immediately denatured with the addition of 100µl NaOH solution.

At this point the reactions were held for up to a week at 4°C or continued into the detection reaction as follows: 100 µl hybridisation buffer were added to the appropriate number of wells in the MWP. The MWP was coated with HIV capture probes in rows A to F, and QS capture probes in rows G and H. An Amplicor electronic pipettor was used to add 25 µl of the denatured PCR amplicons to row A of the MWP and to mix by pipetting up and down 10 times. In the standard Monitor protocol the amplicons should be diluted by continuing the transfers from row A to row F, however for this study the rest of the rows were left blank. For the detection of the QS 25 µl of denatured amplicon were

added to row G of the MWP mixed as before. Row H was left blank. The MWP was covered and incubated at 37°C for one hour to allow the amplicons to bind to the MWP. The rest of the detection procedure was performed as for the other Amplicor tests described in section 2.1.4.

4.5.3 Results

Using the manufacturer's thermal cycling parameters (31 cycles of PCR) the optical densities at 450 nm (OD_{450}) for dilutions of PWS-1 testing positive, shown in Figure 4.6, were only just above the stated cut off of the assay. In light of these results it was decided to modify the cycling parameters by increasing the cycle number to 40 cycles so as to make the assay qualitative rather than quantitative. Using these modified parameters (40 cycles) OD_{450} values of up to 3.0 (the upper limit of the microwell plate reader) were obtained for positive samples.

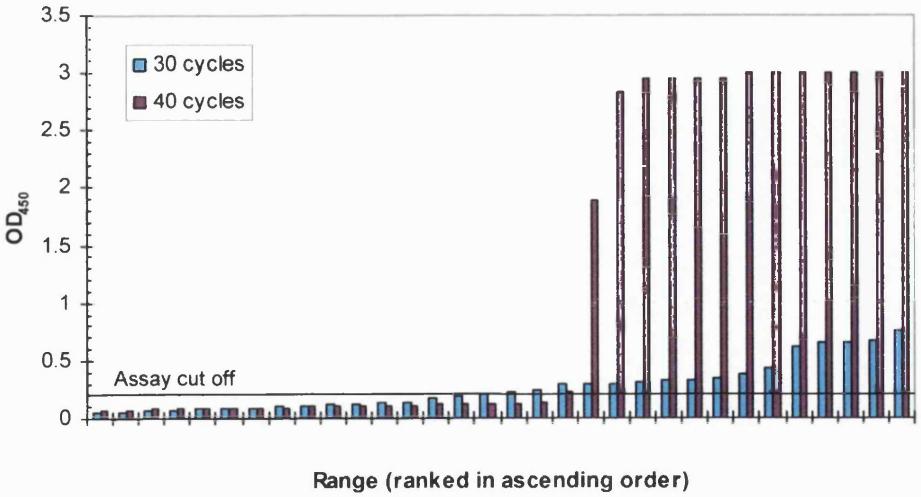


Figure 4.6: Effect of standard and modified thermal cycling parameters on OD_{450} in the Roche HIV-1 Monitor v1.5 assay.

The ODs for each protocol are individually ranked in ascending order.

The sensitivity of this modified version of the HIV-1 Monitor v1.5 assay was assessed using a dilution series of the NIBSC HIV-1 working standard PWS-1 (99/634) (Davis *et al.*, 2003) in EDTA plasma (Table 4.14, Figure 4.7). The PWS-1 standard has been assigned an HIV-1 level of 3.6 $\log_{10}$ IU/ml in an international study (Davis *et al.*, 2003).The 95% limit of detection for the modified Monitor assay, calculated by probit analysis, was 482.7 IU/ml (95% CI = 377.9 to 709.6 IU/ml).

Table 4.14: Sensitivity of Qiagen BioRobot 9604 with a modified Roche HIV-1 Monitor v1.5 assay.

Dilution of PWS-1	HIV-1 level IU/ml	Number Positive/Tested	Percentage Positive
1:5	796	24 / 24	100%
1:10	398	20 / 24	83.3%
1:20	199	16 / 24	66.7%
1:40	100	13 / 24	54.2%
1:80	50	7 / 24	29.2%
1:160	25	3 / 24	12.5%

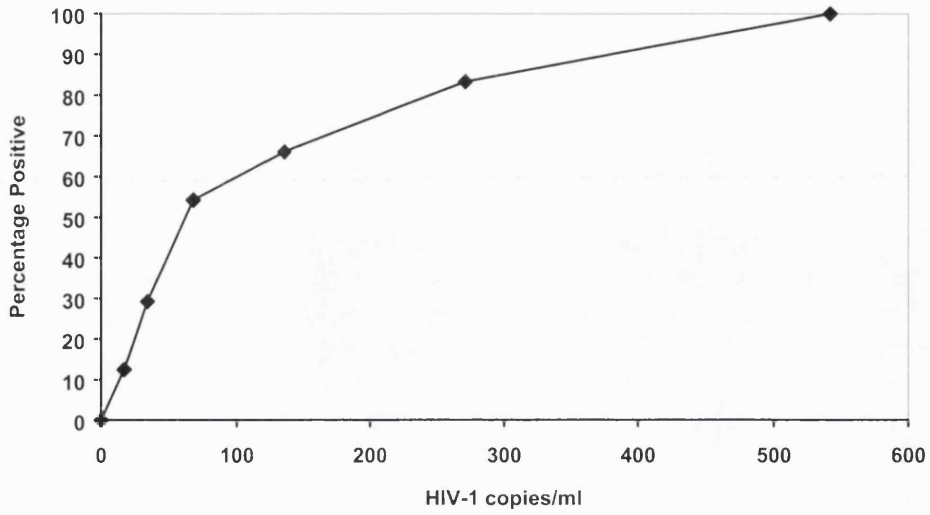


Figure 4.7: Sensitivity of a modified version of the Roche HIV-1 Monitor v1.5 assay using a Qiagen BioRobot 9604 extraction.

4.5.4 Discussion

With data to show that a Qiagen extraction was compatible with the Roche Amplicor assay for detection of HCV, it was decided to determine whether the same was true for HIV-1. At the time this study was performed Roche only supplied a quantitative test for HIV-1 RNA (HIV-1 Monitor v1.5) this was before the development of the AmpliScreen HIV-1 assay. Initial data showed that in this form the test was not suitable for blood screening where good sensitivity is required with an unambiguous positive or negative result. By increasing the number of cycles of PCR it was possible to increase the OD of the positive results so that there was a digital response at the Poisson end point of the assay. Clear positive or negative results are required for a screening assay rather than a gradual decline in OD to the assay cut off and below.

Using these modifications the assay has a 95% limit of detection of 482.7 IU/ml calculated by probit analysis.

All the results in this study were based on testing plasma samples without pre-centrifugation, the sensitivity of the methods described may be increased by centrifugation of a larger sample volume at high speed for an hour before testing, although this could be impractical for screening purposes.

Since this study was performed Roche have released a qualitative assay for HIV-1 RNA, the Roche AmpliScreen HIV-1 test v1.5 (Yang *et al.*, 1999; Yang *et al.*, 2001). It is likely that when used with Qiagen BioRobot 9604 extraction this new assay will have similar characteristics to the modified Monitor method described here, as the cycling parameters are the same as those used in this study.

The NBS has so far chosen not to introduce HIV-1 NAT screening, as the residual risk of HIV-1 window phase donations is much lower than for HCV. This has been confirmed by the experience of other countries that NAT screen for HIV-1, in which the number of window phase donations detected has been very low compared to HCV. In North America the rates of window phase donations found dutring NAT screening have been 62 out of 16.3 million donations screened (1 in 263 000) for HCV compared to 4 out of 12.6 million (1 in 3 150 000) for HIV-1 (Stramer *et al.*, 2000). In Germany the rates have been 1 in 600 000 for HCV and 1 in 1 800 000 for HIV-1 (Drosten *et al.*, 2001)

4.6 BioRobot 9604 QIAamp 96 Virus protocol

4.6.1 Introduction

The QIAamp 96 Virus protocol was designed for the co-purification of both RNA and DNA using the BioRobot 9604. The manufacturer claimed that the QIAamp 96 Virus protocol was more sensitive and robust than the QIAamp 96 Viral RNA protocol.

The QIAamp 96 Virus protocol differs quite considerably from the viral RNA protocol. Several methods are employed for the viral uncoating and release of nucleic acid. Firstly the plasma sample is mixed with a protease so that the protein virus coat is digested. Then a lysis buffer (AL) is added and mixed, this contains the chaotropic salt guanidinium chloride, which will disrupt viral proteins as well as inactivating any RNAses or DNAses. The lysis buffer also contains a high amount of detergent to disrupt any lipids in the sample. These processes are aided by incubation at 56°C.

The heated lysis step (56°C for 10 minutes) included in this protocol requires the addition of a heating block to the BioRobot and therefore the work table configuration was altered. This altered worktop is referred to as BioRobot configuration B.

After lysis has occurred ethanol is added to adjust the binding conditions to those favourable for binding of the nucleic acid to the silica matrix of the QIAamp plate. A cap of wash buffer AW1 is placed on top of the lysate and ethanol mixture after it has been transferred to the QIAamp plate, to reduce foaming due to the detergent in the lysis buffer. A metal channelling block is fitted inside the vacuum manifold to stop foam building up below the plate.

There are three wash steps in the QIAamp 96 Virus protocol (1 x AW1 and 2 x AW2) which have increasing volumes so that any salt deposits are washed away and cannot contaminate the eluate. The incremental wash steps were included in

this assay as the manufacturer was aware of the beneficial effect of incremental wash volumes that had been demonstrated with the QIAamp 96 Viral RNA protocol described in section 4.3.4.

The sensitivity, cross contamination potential and robustness of the QIAamp 96 Virus protocol were assessed for HCV using the Amplicor HCV v2.0 assay. The ability to co-extract DNA was assessed using an in house PCR assay for HBV.

4.6.2 Methods

The QIAamp 96 Virus protocol was used for extraction of HCV RNA as described in section 2.1.3. Carrier RNA was used at 5 μg per ml in the buffer AL giving a final concentration of 1 μg per prep or 0.625 μg per PCR. To allow compatibility with the Amplicor assay, internal control was added to the buffer AL at a concentration of 11.5 μl per ml giving 2.3 μl per extraction.

The sensitivity was assessed by extracting the PEI HCV RNA standard #75/98, calibrated against the WHO international HCV RNA standard and assigned 25 000 IU/ml, diluted in NHP. The RNA was amplified and detected by either the MWP or COBAS versions of the Amplicor HCV v2.0 assay.

The potential for sample cross contamination was assessed in two extraction runs each containing 48 HCV NAT negative samples and 48 HCV NAT positive samples, of approximately 8 x 10^6 IU/ml, arranged in a grid. The RNA was amplified and detected using the COBAS Amplicor assay. This experiment also served as an indication of robustness in the extraction and amplification of the 96 HCV positive samples.

As the manufacturer claimed that the QIAamp 96 Virus protocol could be used for extraction of either RNA or DNA viruses, extracted material was also used to test for HBV. The sensitivity of HBV detection was determined using dilutions of the Eurohep HBV standard which contains 2.7 x 10^9 copies /ml of HBV (Heermann *et al.*, 1999).. The extracted HBV DNA was amplified using an in-

house PCR with *Taq* Gold enzyme and primers located in the surface gene. The PCR master mix was made up as follows (per reaction): 5 µl 10x PCR buffer (supplied with enzyme) giving 1.5 mM $MgCl_2$; 0.2 mM of each deoxynucleotide triphosphate (Pharmacia); 1 unit of *Taq* Gold enzyme (Applied Biosytems); 0.4 µM of each of the HBV surface gene primers (Designed by Steve Kaye at UCL):
Qsense: TCTAGACTCGTGGTGGACTTCTCT
Qantisense: ATAAAACGCCGCAGACACATCCA

The master mix was made up to 30 µl with nuclease free water (Promega). The 30 µl master mix per reaction was added to a 0.2 ml thin walled PCR plate (ABgene) and made up to 50 µl with the addition of 20 µl extracted HBV DNA. The plate was sealed with adhesive foil sealer (ABgene) and cycled in a GeneAmp PCR System 9600 thermal cycler in the post PCR laboratory using the following parameters: 95°C for 10 minutes followed by 40 cycles of 94°C for 20 seconds, 53°C for 20 seconds and 72°C for 20 seconds. The amplicons were detected using gel electrophoresis as described in Section 2.1.11.

4.6.3 Results

The HCV sensitivity achieved using these methods is shown in Table 4.15. The 95% detection sensitivity calculated by probit analysis was 24.9 IU/ml (95% CI = 16.2 to 61.7) for the MWP method and 21.5 IU/ml (95% CI = 15.4 to 41.0) for the COBAS method.

Table 4.15: Sensitivity of BioRobot QIAamp 96 Virus protocol with COBAS and MWP Amplicor HCV v2.0

HCV Level[a]	MWP		COBAS	
IU/ml	Number Positive/Tested	Percentage	Number Positive/Tested	Percentage
150	24 / 24	100%	24 / 24	100%
50	24 / 24	100%	23 / 23 [b]	100%
37.5	23 / 24	95.8%	24 / 24	100%
20	22 / 24	91.7%	22 / 24	91.2%
10	20 / 24	83.3%	18 / 24	75.0%
4	6 / 12	50.0%	4 / 12	33.3%
0	0 / 12	0.0%	0 / 12	0.0%

[a] Values are based on the dilution of the PEI 75/98 working standard in NHP.
[b] 23 replicates were used because one test failed i.e. the internal control was negative and therefore the result cannot be counted.

The sensitivity of the QIAamp 96 Virus protocol was similar to the sensitivity found with the QIAamp 96 Viral RNA protocol described in section 4.3.7 (Figure 4.8). The 95% sensitivities for the QIAamp 96 Virus and Viral RNA protocols with Amplicor HCV v2.0 MWP and COBAS assays are shown in Table 4.16

Table 4.16: Probit analysis

Probit	QIAamp 96 Virus protocol		QIAamp 96 Viral RNA protocol	
	MWP	COBAS	MWP	COBAS
95% IU/ml	24.9	21.5	22.6	25.5

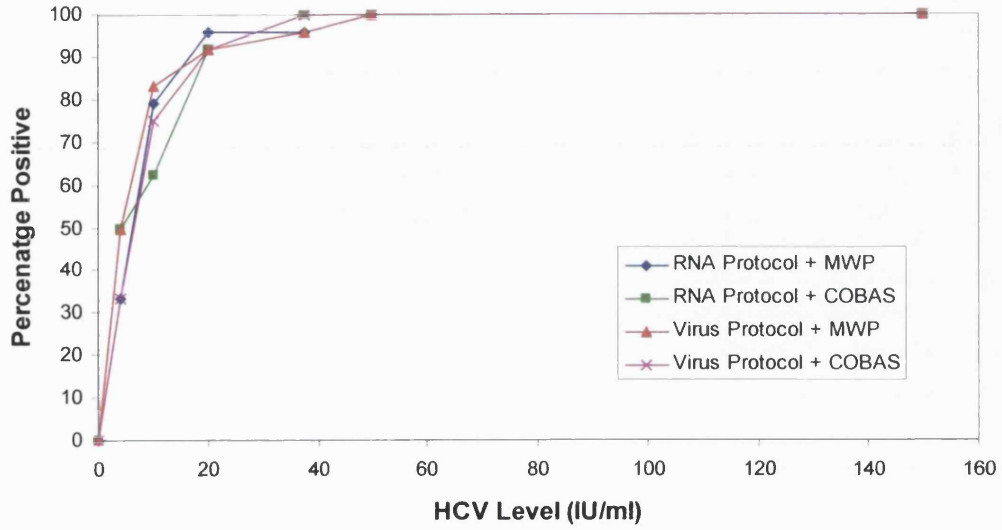


Figure 4.8: Comparison of Qiagen BioRobot extraction methods with Manual and Automated Roche Amplicor

The results of the cross contamination study showed several contamination events had occurred. In the first run 5 of the 48 negative samples had positive HCV ODs. In the second run 3 of 48 negative samples had positive HCV ODs. The contaminated samples had low ODs averaging 1.227 (range = 0.397 to 3.721) as compared to the positive spiked samples, which were all found to be positive with an average OD of 3.859 (range = 3.426 to 4.000).

The HBV sensitivity achieved using these methods is shown in Table 4.17. The 95% detection sensitivity calculated by probit analysis was 49.0 copies/ml (95% CI =36.8 to 84.6 IU/ml).

Table 4.17: Sensitivity of BioRobot Virus protocol with HBV PCR

HBV Concentration [a] copies/ml	Number Positive/Tested	Percentage Positive
400	16 / 16	100%
200	16 / 16	100%
100	24 / 24	100%
50	23 / 24	95.8%
25	17 / 24	70.8%
12.5	16 / 24	66.7%
6	10 / 24	41.7%
0	0 / 24	0.0%

[a] Based on dilutions of the Eurohep HBV standard in NHP.

4.6.4 Discussion

The QIAamp 96 Virus protocol differs from the QIAamp 96 Viral RNA protocol in that it has a much more stringent viral lysis stage. The QIAamp 96 Viral RNA protocol uses the chaotropic salt guanidinium thiocyanate for viral lysis, whereas the QIAamp 96 Virus protocol uses protease, the chaotropic salt guanidinium chloride, detergent and heat for viral lysis. The extra viral lysis methods are required for the tougher DNA viruses such as HBV, but should also enhance the lysis of RNA viruses such as HCV and HIV-1. The wash steps have also been improved giving the graded washes that had been shown to improve sensitivity with the QIAamp 96 Viral RNA protocol.

The manufacturer claimed that the sensitivity and robustness of the QIAamp 96 Virus protocol was better than the QIAamp 96 Viral RNA protocol. However when the HCV detection sensitivities of the COBAS and MWP forms of the Amplicor v2.0 assay with QIAamp 96 Virus protocol were compared with those

obtained using the QIAamp 96 viral RNA protocol described in section 4.3.7 it was found that the sensitivities of all combinations were very similar (Figure 4.8). The 95% limit of detection for all four combinations ranged from 21.5 to 25.5 IU/ml (Table 4.16).

The cross contamination found with the QIAamp 96 Virus protocol was the cause of great concern, as this had not been found with the QIAamp 96 Viral RNA protocol. Upon closer examination droplets of lysate and ethanol mixture could be seen on the top of the QIAamp plate after transfer. A jerking motion in the robotic arm was causing micro droplets of the lysate mixture to be sprayed onto the QIAamp plate causing the cross contamination. This had not occurred with the QIAamp 96 Viral RNA protocol and an analysis of the robotic arm movement speeds in the software protocols between the two assays showed that the ramp rates and speed of the arm movement were higher in the QIAamp 96 Virus protocol than in the QIAamp 96 Viral RNA protocol.

The ramp rates and speeds of the robotic arm movements were reduced and the cross contamination experiments were repeated as reported in section 4.8 and also as part of the validation work performed at the Brentwood NAT laboratory. With the reduced speeds the jerking motion no longer occurred and the results of the experiments at Brentwood showed that cross contamination was no longer occurring.

HBV DNA was successfully extracted using the QIAamp 96 Virus protocol proving that the same method may be used for extraction of both RNA and DNA viral nucleic acid from plasma.

The sensitivities for HCV and HBV are similar if the number of DNA copies per PCR is calculated for each (Table 4.18). Although this calculation assumes 100% extraction efficiency for both HCV and HBV and assumes that the RT efficiency for the HCV RT-PCR is 10%. The 95% detection limits were 1.72 cDNA copies per PCR for HCV and 2.29 DNA copies per PCR for HBV.

Table 4.18: Calculation of PCR copy number giving 95% detection for HCV and HBV

Factor	Calculation	HCV	HBV
95% probit		25.5 IU/ml	49 copies/ml
HCV IU to copies	x 6	153 copies/ml	49 copies/ml
200 µl extracted	÷ 5	30.6 copies	9.8 copies
Proportion of NA into PCR	[a]87 ÷ 50 for HCV 87 ÷ 20 for HBV	17.6 copies	2.25 copies
RT efficiency	10% for HCV	1.76 copies	2.25 copies

[a] Elution volume = 87 µl

4.7 Roche COBAS HCV AmpliScreen v2.0

4.7.1 Introduction

The COBAS AmpliScreen HCV v2.0, assay is a qualitative test for HCV RNA in plasma. This assay is similar to the HCV Amplicor v2.0 assay (Rosenstraus *et al.*, 1998a), but is modified specifically for blood screening purposes. During the period of these studies the manufacturer stated that for legal reasons the Amplicor assay would not be available for blood screening purposes after 2001. Both the manual Amplicor and the BioRobot and Amplicor assays were in use within the NBS at the time the AmpliScreen assay was launched. Therefore, studies were required into the use of the AmpliScreen assay as a stand-alone manual assay and in combination with the BioRobot extraction.

Roche has conducted clinical trials to establish the performance of AmpliScreen when used with plasma pools of 24 donations each. A validated pooling procedure using an automated robotic pooling device is included in the instructions. A positive 24-donation primary pool is resolved by first testing four secondary pools of six donations each, and then testing the six individual donations in a positive secondary pool. The assay has two manual sample processing procedures; the Multiprep procedure, used for testing primary and secondary plasma pools, and the Standard Prep procedure, used for testing individual donor samples. The Multiprep procedure employs a high-speed centrifugation step intended to pellet the viral particles from the sample before viral lysis.

The AmpliScreen assay has a generic sample preparation procedure that is compatible with HCV, HIV-1 and HBV (Sun *et al.*, 1999). The assay is not a multiplex, but these viruses can be amplified and detected in separate COBAS assays.

The high speed centrifugation step in the Multiprep sample preparation procedure is probably intended to pellet HBV and HIV-1, however this would be unlikely to concentrate HCV because its buoyant density is similar or lower than that of plasma (Carrick *et al.*, 1992). It would be difficult to justify using a centrifugation procedure routinely on large numbers of samples, for HCV only, and so both the Multiprep and standard extraction procedures were investigated.

This assay was intended to be used by the NBS for the detection of HCV RNA positive donations in the window phase of infection. Therefore an antibody negative HCV standard (PEI) was used in this study in case the antibody status of the sample affected the amount of concentration of the virus.

4.7.2 Methods

As this assay was only assessed for its sensitivity rather than being used to detect HCV in blood donations the pooling protocol was not used, but both the Multiprep and Standard extraction procedures were investigated.

The EDTA plasma used in this study was made by pooling 250 HCV negative EDTA minipools the resulting pool was then tested again by a NAT assay and found to be negative for HCV RNA. The PEI antibody-negative HCV RNA reference preparation #75/98 was diluted to HCV RNA levels of 100, 50, 20, 10 and 4 IU/ml in the pooled EDTA plasma. These PEI dilutions and the EDTA plasma were tested a total of 24 times each in six separate assays. The sensitivity of the Multiprep and Standard sample preparation procedures were assessed according to the kit instructions shown in brief in Table 4.19 and described fully in section 2.1.6.

Table 4.19: Comparison between Multiprep and Standard AmpliScreen sample preparation procedures.

Protocol Step	Procedure	
	Multiprep	**Standard**
Centrifuge 1ml plasma for	**1 hour at 23600g / 4°C**	**No spin**
Plasma input volume	**100 µl**	**200 µl**
Add lysis buffer	600 µl	600 µl
Incubate at RT for	10 minutes	10 minutes
Add propanol	**700 µl**	**800 µl**
Centrifuge for	15 minutes	15 minutes
Resuspend pellet in	1ml 70% ethanol	1ml 70% ethanol
Centrifuge for	5 minutes	5 minutes
Resuspend pellet in	200 µl sample diluent	200 µl sample diluent
Amplify and detect in COBAS	50 µl nucleic acid	50 µl nucleic acid

Note: Differences between sample preparation procedures are shown in **bold**.

Due to concern over compatibility of the high speed centrifugation employed in the Multiprep procedure with HCV robustness and cross contamination, studies were performed to investigate this procedure. The robustness of the Multiprep sample preparation procedure was tested using 22 different 96 donation minipool samples spiked with the PEI preparation to a final HCV level of 150 IU/ml (three times the limit of detection- see section 4.7.3). A cross contamination study was performed using alternating HCV positive and negative samples, comprising 11 samples with an HCV level of 10^6 IU/ml and 11 HCV negative samples.

4.7.3 Results

The results of the sensitivity study are shown in Table 4.20; samples that tested HCV negative and internal control negative are not included as this suggests an assay failure for that sample.

Table 4.20: Sensitivity of AmpliScreen HCV Multiprep and standard methods.

HCV Level[a]	Multiprep procedure		Standard procedure	
IU/ml	Number Positive/Tested	Percentage	Number Positive/Tested	Percentage
100	22 / 24	91.6%	24 / 24	100%
50	23 / 23[b]	100%	23 / 24	95.8%
20	21 / 24	87.5%	19 / 24	79.2%
10	22 / 24	91.6%	16 / 23[b]	69.6%
4	12 / 24	50%	8 / 22[b]	36.4%
0	0 / 17[b]	0%	0 / 17[b]	0%

[a] Values are based on the dilution of PEI #75/98 standard in pooled EDTA plasma.
[b] Reduced number due to internal control failure. 24 positives and 18 negatives.

The 95% limit of detection calculated by probit analysis for the Multiprep sample preparation procedure was 50.7 IU/ml with two internal control failures out of a total of 138 tests (1.45%). The 95% limit of detection for the standard procedure was 44.3 IU/ml with four internal control failures out of a total of 138 tests (2.90%). The results of the sensitivity study are presented graphically in Figure 4.9.

Of the 22 minipools spiked with 150 IU/ml, 21 were HCV positive; the remaining sample had an internal control failure. The HCV absorbances for the 21 positive samples were all greater than 3.5 units.

The results of the cross contamination study showed no evidence of cross contamination occurring, i.e. 11 of 11 positive spiked samples tested HCV positive and 0 of 11 negative samples tested HCV positive.

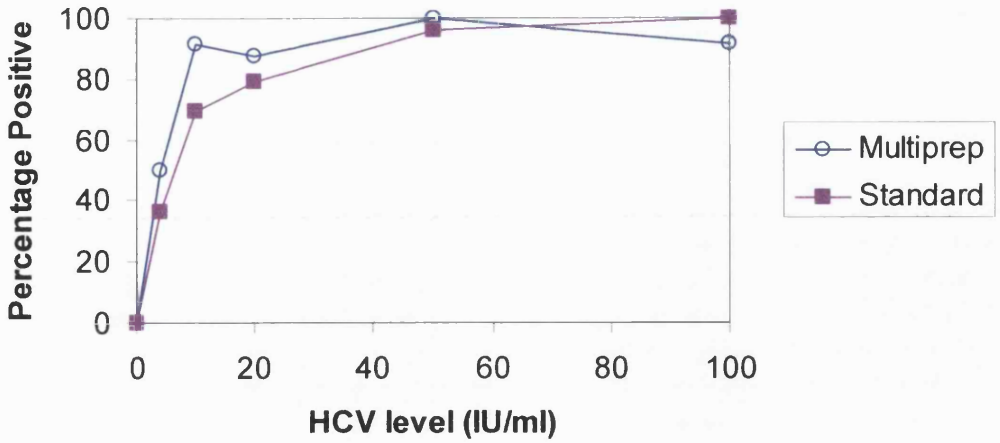


Figure 4.9: Sensitivity of AmpliScreen HCV Multiprep and standard methods

4.7.2 Discussion

In this study, the addition of a high speed centrifugation step in the Multiprep procedure did not increase the sensitivity for HCV compared to the Standard procedure. The 95% limit of detection for the Multiprep procedure was 50.7 IU/ml slightly higher than the 44.3 IU/ml using the standard protocol, however these values may have been influenced by the loss of two positives at the 100 IU/ml level in the Multiprep procedure which rendered the probit curve non significant and thereby raised the 95% limit of detection. Based on the data found in this study the centrifugation step does not seem to be warranted for HCV detection.

The manufacturer has reported the analytical sensitivity of the AmpliScreen HCV assay as 25 IU/ml (Sun *et al.*, 1999; Sun *et al.*, 2000), roughly two fold better than that reported here. Another recently published study quoted a 95% sensitivity for HCV of 126 copies/ml (Lelie *et al.*, 2002), this equates to 33 IU/ml based on the calibration of the standard used with the WHO international standard (Saldanha *et al.*, 2000).

The question as to whether HCV will be concentrated by centrifugation in the Multiprep method is an important one. As the Multiprep method centrifuges 1ml of plasma of which 100 µl is input into the sample preparation an HCV concentration of up to 10 fold is possible. A quarter (50 µl of 200 µl) of the eluate is added to the PCR so the plasma equivalent of the Multiprep method is 25 µl to 250 µl depending on the concentration efficiency of the centrifugation step. The standard method has no centrifugation step but takes 200 µl into the sample preparation and therefore the plasma equivalent to the PCR is 50 µl.

In theory the Multiprep method should display half to five fold the sensitivity of the standard method depending upon HCV concentration. This calculation assumes that in the worst case no concentration occurs, i.e. just 100 µl plasma containing HCV enters the sample preparation. However, if the HCV is associated with lipid it may be forced to the top of the tube with the lipid layer during the centrifugation, as the top 900 µl of plasma is discarded to leave only the 'pellet' and 100 µl plasma. In this case the sensitivity of the Multiprep method could be significantly reduced.

The sample preparation procedure is also intended for simultaneous use in HIV-1 and HBV extraction and these viruses can be readily pelleted by high-speed centrifugation as they have much higher buoyant densities; HIV = 1.15 g/cm^3 (Campbell *et al.*, 2002) and HBV = 1.28 to 1.36 g/cm^3 (Moritsugu *et al.*, 1975) compared to 1.08 g/cm^3 for HCV (Miyamoto *et al.*, 1992). It may be possible that the presence of antibodies to HCV may form immune complexes with the virus in the sample and that these complexes pellet more efficiently. This has yet to be shown, and so in this study an antibody negative HCV standard was used so that the reported sensitivity would be equivalent to the window phase donations screened for in the NBS.

In the robustness and cross contamination experiment all HCV positive samples of 150 IU/ml were detected and no evidence of cross contamination was found. However the failure to detect 2 of 24 of the 100 IU/ml HCV dilution using the Multiprep procedure is of concern as this is the level of the go/no-go control used

in the NBS. If the 100 IU/ml control is not detected in NBS routine screening the samples of an A-ring or an entire extraction run must be repeated (described in section 6.2) and therefore the use of the AmpliScreen assay for routine screening may result in assay runs requiring repeat testing. There were a number of internal control failures (two of 138 Multiprep = 1.45% and four of 138 standard = 2.90%) during this evaluation which would also cause repeat testing if used in the NBS.

On the basis of these results the NBS did not introduce the AmpliScreen assay in its manual form as described here.

4.8 QIAamp 96 Virus protocol with the COBAS AmpliScreen HCV v2.0 - comparison with Amplicor and QIAamp 96 Viral RNA protocols (Four way cross study)

4.8.1 Introduction

The QIAamp 96 Viral RNA and Virus protocols for the BioRobot 9604 had both been tested with the COBAS Amplicor v2.0 assay with similar results (sections 4.3.7 & 4.6). However the performance of the QIAamp 96 Viral RNA protocol with COBAS Amplicor v2.0 in the NBS NAT laboratories was not as good as had been found in the evaluations described in section 4.3.7 and in initial evaluations carried out in the NBS NAT laboratories (Chapter 6). The manufacturer of the QIAamp 96 Virus protocol claimed that this should be more sensitive and robust than the previous Viral RNA protocol and also that the Virus protocol would eventually replace the Viral RNA protocol.

At the same time the manufacturer of the COBAS AmpliScreen HCV v2.0 assay was warning that the COBAS Amplicor HCV v2.0 assay would be withdrawn from blood screening use in the future due to legal reasons.

For these reasons it was decided to evaluate the combination of the QIAamp 96 Virus protocol and the COBAS AmpliScreen HCV v2.0 assay. The other combinations of the QIAamp 96 Virus protocol and COBAS Amplicor HCV v2.0 and QIAamp 96 Viral RNA protocol with both COBAS AmpliScreen and Amplicor HCV v2.0 were also included for comparison. The combination of assays was assessed for sensitivity and robustness.

4.8.2 Methods

Sensitivity

The following HCV levels were tested by each assay combination with a total of 24 replicates from three independent runs: 100 IU/ml, 50 IU/ml, 20 IU/ml, 10 IU/ml, 4 IU/ml and diluent alone. These were dilutions of the PEI HCV RNA reference preparation #75/98 diluted in pooled HCV RNA negative EDTA plasma.

Three BioRobot extraction runs using the QIAamp 96 Virus protocol and three extraction runs using the QIAamp 96 Viral RNA protocol were performed with the extracted RNA from each run being amplified and detected in both the COBAS Amplicor and AmpliScreen HCV v2.0 assays.

As the two different amplification and detection assays used different internal controls (IC) the lysis buffer was split so that half contained the Amplicor IC and half contained the AmpliScreen IC thus enabling both COBAS protocols to be run from a single BioRobot extraction run. The BioRobot lysis buffer containing IC is normally added from four tubes located on the BioRobot worktable. In this study the Amplicor or AmpliScreen internal controls were added from two lysis buffer tubes each. The amounts of IC were calculated to give 2.3 µl per extraction for Amplicor and 1.66 µl for AmpliScreen; these are the same levels as used in the Roche manual protocols.

Each HCV dilution was extracted in 16 replicates per run, 8 with Amplicor IC and 8 with AmpliScreen IC therefore each BioRobot run produced 48 extracts for amplification and detection in the COBAS using the Amplicor protocol and 48 extracts for amplification and detection in the COBAS using the AmpliScreen protocol, giving the best comparability between the two Roche assays.

The 95% sensitivities for each assay combination were calculated using probit analysis.

Robustness

The robustness of each assay combination was assessed in terms of number of 100 IU/ml control failures and number of internal control failures, as these would impact directly upon NAT screening in the NBS. The HCV and IC absorbance values of all four combinations were also considered, as low absorbance values, particularly for the IC had been associated with reduced sensitivity in the NBS laboratories (discussed in Chapter 6).

A formal robustness study was conducted using minipools of 96 donations each previously shown to be HCV RNA negative which were spiked with the PEI standard to a final HCV concentration of 40 IU/ml, approximately three times the 95% detection limit. The QIAamp 96 Virus protocol with the COBAS AmpliScreen HCV v2.0 assay was used to test 92 different spiked minipools, with a negative control in each set of 24 extractions.

Cross contamination

A cross contamination experiment was performed using the QIAamp 96 Virus protocol with the reduced transfer speeds as discussed in section 4.6 using COBAS Amplicor for amplification and detection. The extraction run was set up with 48 HCV positive samples of 1 $x10^6$ IU/ml and 48 negative plasma samples arranged in a chessboard pattern.

4.8.3 Results

Sensitivity

The results were interpreted according to the manufacturer's instructions. The HCV absorbance cut off for Amplicor was 0.150 while that of AmpliScreen was 0.200. The performance of the each of the four assay combinations is shown in Table 4.21. Although 24 replicates were tested in all cases where a figure is

given as less than 24 this is due to internal control failures in samples testing HCV negative. The 95% and 50% probit analysis of these data are shown in Table 4.22.

Table 4.21: Sensitivity

HCV [a] IU/ml	QIAamp 96 Virus protocol		QIAamp 96 Viral RNA protocol	
	Amplicor Positives [b]	AmpliScreen Positives [b]	Amplicor Positives [b]	AmpliScreen Positives [b]
100	24 / 24 100%	24 / 24 100%	23 / 24 95.8%	21 / 24 87.5%
50	24 / 24 100%	24 / 24 100%	19 / 24 79.2%	23 / 23 100%
20	24 / 24 100%	24 / 24 100%	12 / 22 54.2%	10 / 24 41.7%
10	20 / 24 83.3%	21 / 24 87.5%	4 / 21 19.0%	2 / 19 10.5%
4	15 / 24 62.5%	15 / 24 62.5%	1 / 22 4.5%	1 / 18 5.6%
0	0 / 23 0.0%	0 / 24 0.0%	0 / 22 0.0%	0 / 19 0.0%

[a] Values are based on the dilution of PEI #75/98 standard in pooled EDTA plasma.
[b] Number positive / number tested and percentage positive are shown.

The percentage positives for the QIAamp 96 Virus protocol with both COBAS methods are shown in Figure 4.10 while those of the QIAamp 96 viral RNA protocol are shown in Figure 4.11.

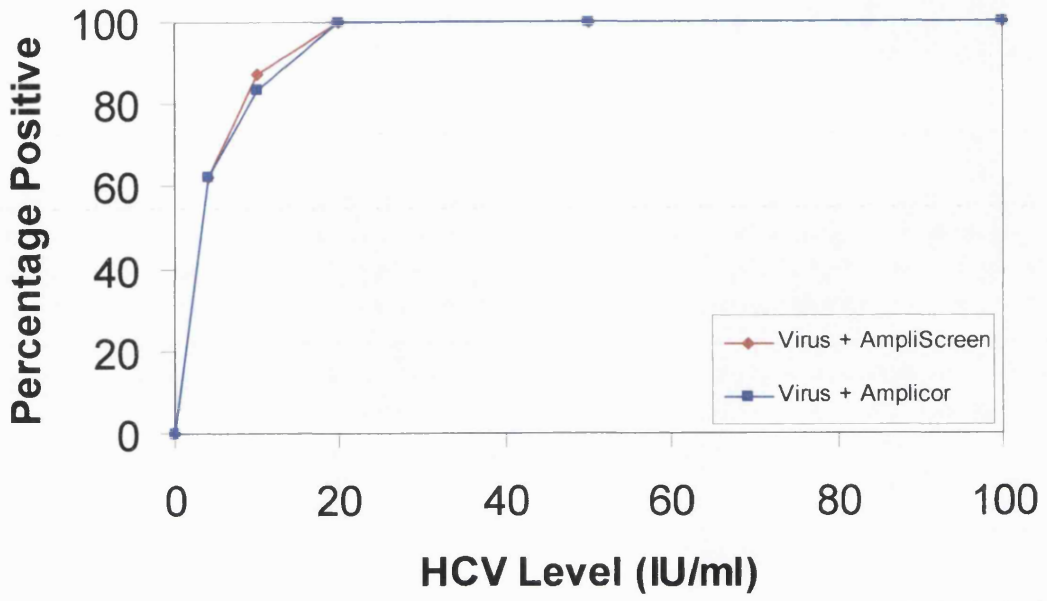


Figure 4.10: Sensitivity of BioRobot Virus protocol with Amplicor and AmpliScreen.

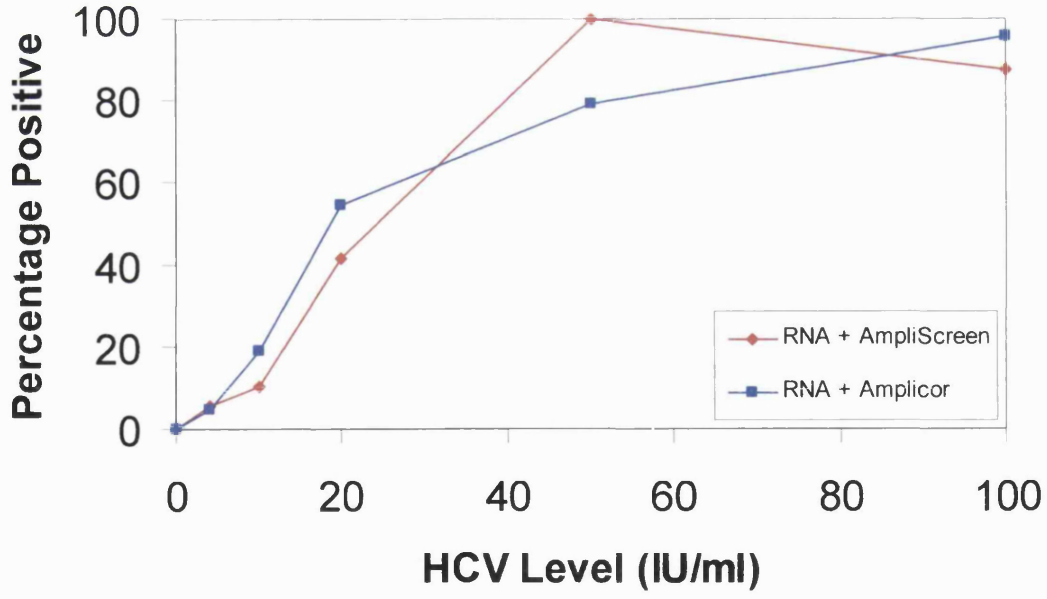


Figure 4.11: Sensitivity of BioRobot viral RNA protocol with Amplicor and AmpliScreen.

Table 4.22: Probit analysis showing 95% and 50% limits of detection for the four assay combinations

Probit	QIAamp 96 Virus protocol		QIAamp 96 Viral RNA protocol	
	Amplicor	AmpliScreen	Amplicor	AmpliScreen
95% IU/ml	14.5	12.8	99.1	95.9
50% IU/ml	3.2	3.2	20.6	22.1

Robustness

Figure 4.12 shows the HCV absorbances for all four combinations while the internal control absorbances are shown in Figure 4.13. There were no IC failures (out of 144 tests) with the Virus and AmpliScreen combination, 1 out of 144 (0.7%) with the Virus and Amplicor combination, 24 out of 144 (16.7%) with viral RNA and AmpliScreen and 14 of 144 (9.7%) with the viral RNA and Amplicor combination. Some of these IC failures were HCV reactive and therefore are not included in the results shown in Table 4.21 as these are considered valid results routinely.

The average OD of the internal controls that were detected by manufacturer's criteria, i.e. excluding failures, was calculated for each assay combination (Table 4.23).

Table 4.23: Average HCV and IC OD values for the four assay combinations

OD	QIAamp 96 Virus protocol		QIAamp 96 Viral RNA protocol	
	AmpliScreen	Amplicor	AmpliScreen	Amplicor
HCV	3.374	2.401	2.167	1.188
IC	3.923	3.836	3.424	2.224

The AmpliScreen assay gave higher ODs than the Amplicor assay for both extraction methods. The ODs from the QIAamp Virus protocol were higher than those from the QIAamp Viral RNA protocol.

In the robustness study all 92 spiked minipools were successfully detected, with a mean absorbance of 3.91 (range 2.745 to 4.000). All 96 internal controls were detected with an average OD of 3.853 (range 1.909 to 4.000). All HCV negative controls were negative.

Cross contamination

All 48 negatives in the cross contamination study were negative with an average OD of 0.004 (range 0.002 to 0.005). All 48 HCV positive samples were detected with an average OD of 3.884 (range 3.425 to 4.000). All 96 internal controls in the cross contamination study were detected. The average IC OD for the negative samples was 3.879 (range 3.074 to 4.000). The IC ODs for the positive controls were suppressed due to the high HCV level. The average IC OD for the positive controls was 0.752 (range 0.375 to 1.142).

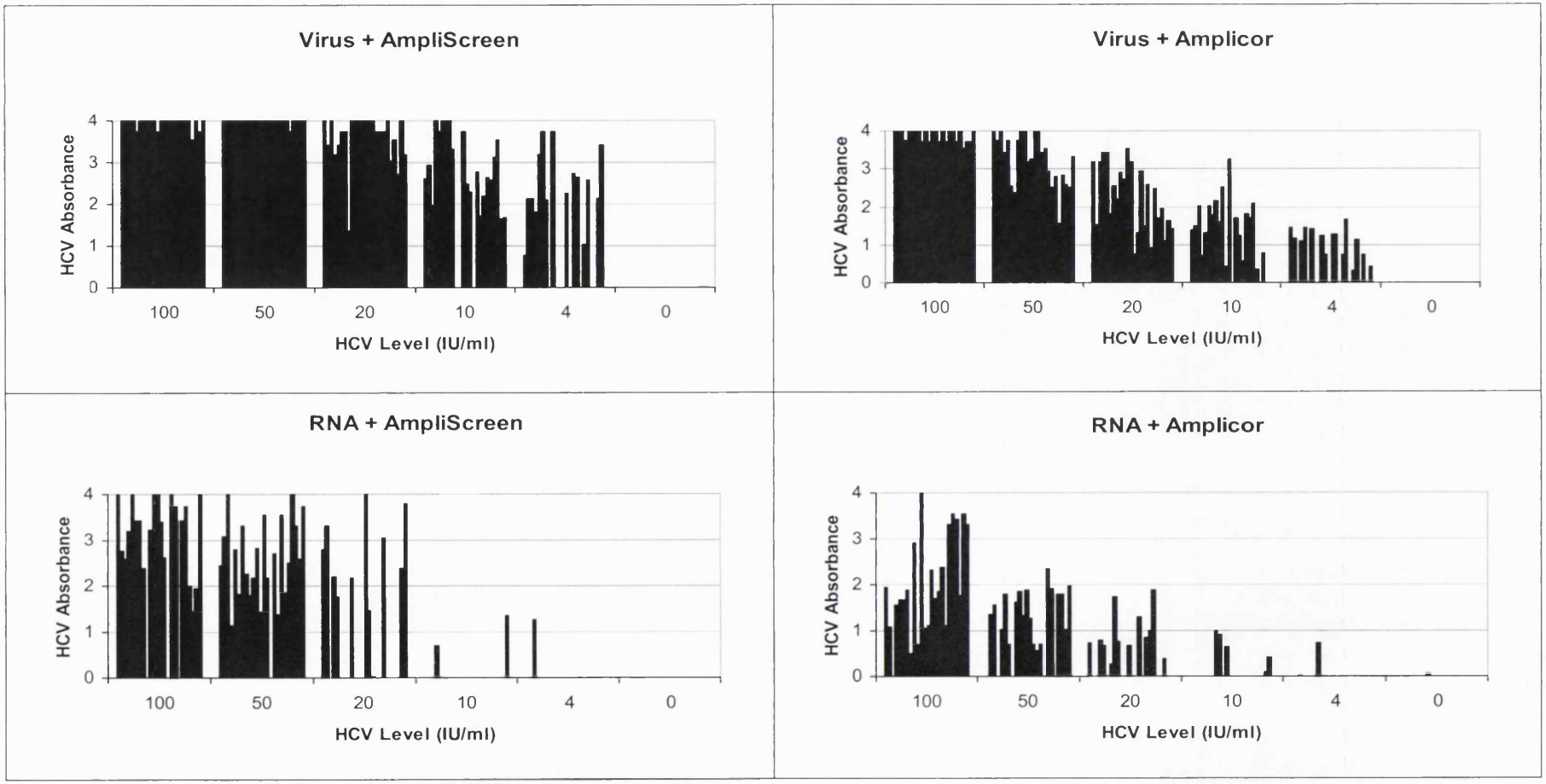


Figure 4.12: HCV absorbance values for different assay combinations.

The HCV absorbance values are shown at five HCV levels from 100 IU/ml to 4 IU/ml and HCV negative for each assay combination. Each dilution of HCV has 24 replicates.

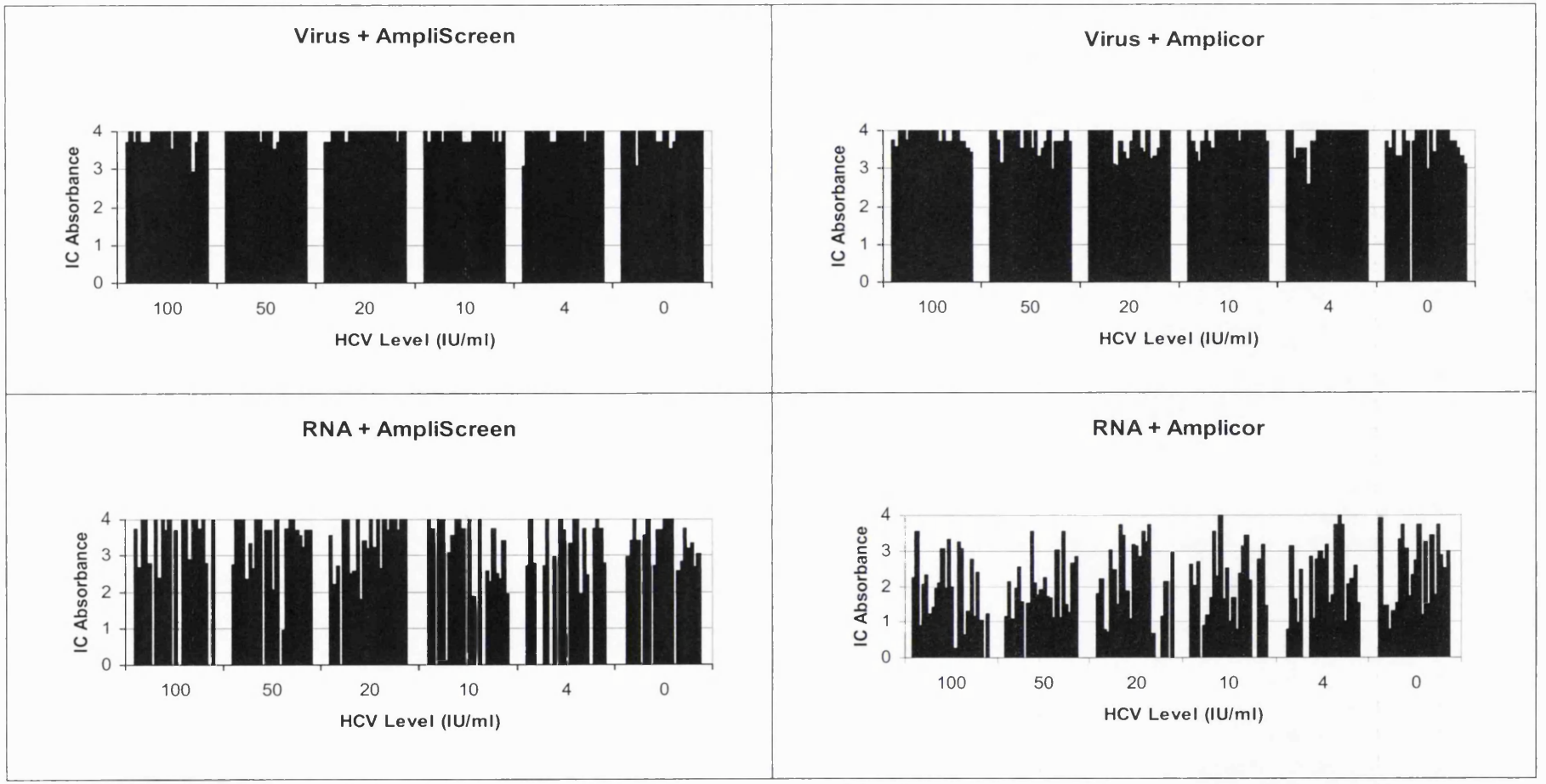


Figure 4.13: Internal Control absorbance values for different assay combinations.

The IC absorbance values are shown for each assay combination. The six HCV levels (including HCV negative) each has 24 replicates i.e. a total of 144 replicates per assay combination.

4.8.4 Discussion

The 95% sensitivity for each of the four assay combinations was determined and a difference in sensitivity was found between the QIAamp 96 Viral RNA and QIAamp 96 Virus extraction methods. The 95% sensitivities calculated from the Amplicor and AmpliScreen results using the QIAamp 96 Viral RNA extraction were approximately seven fold less than those using the QIAamp 96 Virus extraction. No significant difference was found in sensitivity between the COBAS Amplicor and AmpliScreen HCV v2.0 assays for either extraction method.

It is not possible to say why there was such a deterioration in the performance of the viral RNA protocol with the COBAS Amplicor assay in this study compared to that shown in section 4.3.7 which gave a 95% HCV detection sensitivity of 25.5 IU/ml, when the two studies were less than a year apart and used the same HCV standard. However a similar deterioration in sensitivity was noticed in the assay at the NBS Brentwood NAT laboratory during routine testing. Whatever factor had affected the viral RNA protocol did not seem to affect the Virus protocol.

The QIAamp 96 Virus protocol should in theory be more sensitive and robust than the QIAamp 96 Viral RNA protocol for the reasons discussed in section 4.6. In the study described here a better sensitivity was achieved than in section 4.6.3 using the QIAamp 96 Virus extraction and COBAS Amplicor HCV v2.0 assay 14.5 IU/ml vs. 21.5 IU/ml. The 95% sensitivity of 12.8 IU/ml found with the QIAamp 96 Virus extraction and COBAS AmpliScreen HCV v2.0 assay represents the greatest sensitivity of any of the methods evaluated so far.

As well as the reduction in sensitivity, the QIAamp 96 Viral RNA extraction protocol resulted in reduced robustness, with both internal control failures and 100 IU control failures. An internal control failure rate of 13% overall compared to 0.35% for the QIAamp 96 Virus protocol. A 100 IU control failure rate of 8.3% compared to 0 %.

An analysis of the absorbance values for the four assay combinations showed that not only was there a reduction in sensitivity using the QIAamp Viral RNA protocol but the ODs of the positive samples for HCV and IC in both the COBAS assays were lower than those of the QIAamp Virus protocol. The reduction in ODs with the QIAamp Viral RNA protocol compared to the QIAamp Virus protocol could be due to less efficient amplification and detection of the RNA due to co-extraction of an inhibitor for example, or a reduction in the amount of RNA extracted.

The AmpliScreen assay did not significantly enhance the sensitivity of either extraction protocol over Amplicor, however an analysis of the absorbance values of both the HCV and internal control showed higher ODs with AmpliScreen than with Amplicor. This is to be expected as the COBAS AmpliScreen assay has four more rounds of PCR than the COBAS Amplicor assay, and would therefore generate a higher level of amplicon. The absorbance levels in a qualitative assay should in theory be all or none, giving a clear indication of a positive or negative result. In practice however, low-level positives may give intermediate absorbances.

Consideration of the absorbance values can give important information on the performance of the assay. The absorbance values of the internal control should be assessed not only for whether they meet the manufacture's criteria but also for the OD level they reach. A failure of an IC can indicate a technical failure in a particular sample such as a blocked well in the QIAamp plate during extraction, or inhibition of the RT-PCR. This is acceptable as long as the other samples give the expected values. As the amount of the IC added is the same in all extracted samples so the level of the OD should be the same with the exception of technical failures. However, if the IC OD levels show considerable variation this may indicate that there is a problem with the assay. Analysis of the data generated at the Brentwood NBS NAT laboratory showed that assay runs with a lower average IC OD had a lower detection rate of the low level HCV positive monitoring control. Therefore the IC was not just assessed by manufacturer's

criteria but additional criteria were imposed (see Chapter 6 for full discussion of this).

The best combination in terms of absolute OD values and 95% sensitivity was the QIAamp Virus and COBAS AmpliScreen combination.

In the formal robustness study using the QIAamp 96 Virus protocol and COBAS AmpliScreen all 92 minipools spiked at 40 IU/ml (three times the 95% detection level) were detected, showing that this assay would be well suited to use in the NBS for minipool screening. The fact that a level of 40 IU/ml gave 100% detection using this assay combination is important because the 'go or no go' control used in routine NAT runs in the NBS is 100 IU/ml, 2.5 times the level used here. This indicates that the number of repeat runs due to the failure of the go or no go control would be low using this assay.

The BioRobot Virus and HCV AmpliScreen method has now replaced the BioRobot viral RNA and HCV Amplicor method within the NBS for HCV RNA screening of 48 donation minipools. The increased robustness of the assay and the reduction in minipool size has allowed the HCV NAT test to be used for the release of all blood components with a shelf life of 24 hours or more (discussed in Chapter 6).

4.9 General Discussion of development of automated assays

The combination of QIAamp spin column extraction and Amplicor amplification and detection has shown that it is possible to combine the two procedures from different manufacturers and, with some modifications, achieve a good HCV RNA sensitivity whilst providing robotic sample processing and positive sample identification. The sensitivity achieved was actually approximately 10 fold better than using the manufacturer's protocol for Amplicor v1.0 assay. One of the major criticisms of the Amplicor v1.0 assay was that the extraction procedure had no purification step, thus extraneous proteins were included in the extract as well as the nucleic acid. In order to decrease the potential for inhibition of the RT-PCR step the elution was performed with 1 ml of diluent of which 50 µl (i.e. one 20^{th}) were added to the RT-PCR. As 200 µl of plasma were extracted the equivalent plasma input into the RT-PCR was only 10 µl. In comparison, using the QIAamp spin column extraction with Amplicor v1.0 gave an equivalent plasma input of 70 µl because 140 µl plasma were extracted and half of the 50 µl volume of eluate was added to the RT-PCR. This demonstrates the advantage of the QIAamp method, as RNA is purified by binding to the silica membrane allowing any contaminants to be washed away.

Shortly after the work with Amplicor v1.0 was carried out, Roche released version 2.0 of the assay which incorporated several changes. When the assay was run according to the manufacturer's instructions the sensitivity was increased compared to v1.0, because the elution volume was decreased to 200 µl thus increasing the equivalent RT-PCR plasma input volume to 50 µl. The amplification mix had been changed to give equivalent amplification of all genotypes of HCV. Perhaps the most important change to affect its compatibility with the QIAamp spin column extraction was that the Mn^{2+} ions were provided in a separate tube and added to the master mix immediately prior to amplification, and the internal control was introduced at the lysis buffer stage. These changes allowed the QIAamp eluate to be added directly to the PCR master mix without the need to mix with Roche diluent, this doubled the amount

of RNA added to the amplification and resulted in a PCR plasma equivalent volume of 140 µl.

The combination of QIAamp spin column extraction and Amplicor v2.0 amplification and detection resulted in a 95% HCV detection sensitivity of 23.8 IU/ml. This sensitivity was comparable to the 95% HCV detection sensitivity of 22.4 IU/ml using the Amplicor v2.0 assay according to the manufacturer's instructions, even though the equivalent plasma volume of the QIAamp method was almost three times greater than the Amplicor method. This may be due to less than 100% recovery of the nucleic acid in the QIAamp method, as not all the nucleic acid will bind to the silica matrix, and not all will be released during elution, whereas using the Amplicor extraction most of the nucleic acid is extracted, but inhibitors may also be co-extracted.

Replacing the manual Amplicor extraction with a manual QIAamp extraction was a model for QIAamp automated extraction. However, when the BioRobot 9604 was used to automate the QIAamp assay the resulting HCV sensitivity was decreased, suggesting initially that the automated QIAamp assay could not be used as a direct replacement of the manual assay. A series of experiments were performed to address the differences between the BioRobot assay and the spin column assay and to determine what was causing the lack of sensitivity in the BioRobot assay.

The BioRobot elution was quite different to the spin column elution for practical reasons as neither re-elution of the eluate or the use of a heated eluate with the same temperature across the plate were possible. Various modifications to the BioRobot elution step were performed as outlined in section 4.3.4, none of which resulted in any significant improvement in sensitivity.

Another obvious difference between the two extraction methods was the use of vacuum to draw reagents through the QIAamp plate in the BioRobot method compared to centrifugation in the spin column assay. When the QIAamp spin column assay was performed using a vacuum manifold in place of the

centrifugation steps the sensitivity was reduced similar to that seen with the BioRobot.

More detailed experiments were then performed to determine precisely which steps were being adversely affected by the use of vacuum. This drew attention to the wash steps of the protocol. In direct comparisons with one set of spin columns using vacuum wash steps and another set being centrifuged at the wash steps only, it was concluded that the vacuum wash steps were causing the loss of sensitivity. It was thought that this could be due to "tide marks" of the wash buffers on the side of the column. As the volume of the washes was the same as the volume of the lysate addition, any splashing around the sides of the tube would not be washed away, and may be forced into the eluate at the final centrifugation during elution. The lysis buffer and buffer AW1 both contain guanidinium salts which are a known inhibitors of PCR. The "tide mark" effect would not be seen in the protocol using centrifuged wash steps because any drops on the side of the tube would be forced out by the centrifugation.

With these findings in mind the BioRobot wash steps were modified so that the second wash step had a greater volume than the first thus reducing the effect of the "tide marks". These modifications resulted in improved sensitivity of 26.7 IU/ml.

In order for the NBS to have positive sample ID and electronic sample tracking throughout the procedure, the amplification and detection stages had to be automated using the COBAS analyser. When the MWP assay was replaced by the COBAS assay with the viral RNA extraction protocol, a 95% detection sensitivity of 22.6 IU/ml was achieved. This was actually slightly better than the sensitivity achieved with the MWP assay and even the spin column protocol.

This assay combination resulted in a fully automated, high throughput assay with electronic sample tracking and confirmation of negative tests with an internal control. Its sensitivity allowed compliance with the CPMP 100 IU/ml assay sensitivity and the PEI 5000 IU/ml per donation recommendations. This was the protocol combination used by the NBS when it started testing minipools in 1999.

Qiagen subsequently released the new BioRobot extraction protocol called the QIAamp 96 Virus protocol which was able to extract both RNA and DNA virus nucleic acid. When HCV RNA extracted using this protocol was amplified and detected using either the MWP or COBAS form of the Amplicor v2.0 assay, very similar sensitivities to the Viral RNA protocol were achieved.

At around the same time Roche released the AmpliScreen assay, based on the Amplicor assay but intended for blood screening use only. The QIAamp 96 Virus protocol and AmpliScreen assay were tested in combination with the Viral RNA protocol and Amplicor, with the best 95% sensitivity of 12.8 IU/ml achieved with the new assays. The AmpliScreen assay gives the advantage of higher HCV absorbance values due to the extra four cycles of PCR, this is important in qualitative assays when an all or nothing response is required and not the ambiguity of a grey zone result.

The QIAamp Virus protocol appears to be much more robust, possibly due to the introduction of protease and heat to the lysis step. The combination of the QIAamp Virus protocol with COBAS AmpliScreen HCV v2.0 was introduced into NBS NAT screening from 2000 and has produced better results in routine use than the previous combination (section 6.3). This final version of the assay is now used for the release of all blood components in the NBS with a shelf life of greater than 24 hours.

Chapter 5

Alternative HCV detection methods

Although the RT-PCR method is used for blood donor screening in many countries around the world, there are alternatives available for the detection of window phase HCV donations. The first of these is an alternative NAT method using transcription-mediated amplification (TMA); (McDonough *et al.*, 1998). This technology was developed by Gen-Probe Inc and is discussed in detail in section 1.4.2. A multiplex HCV / HIV-1 assay developed by Gen-Probe Inc. is marketed by Chiron and has recently been granted a licence for blood screening by the US FDA (FDA, 2002).

To avoid discrepancies in the NAT screening used in different parts of the country it was important that any assay introduced only had HCV detection capability, and avoided introducing HIV-1 testing in only one part of the country. The version of the TMA assay investigated here was the HCV discriminatory part of the multiplex. This HCV assay is marketed by Bayer and was investigated on behalf of the NBS as a possible back-up assay for the Qiagen and Roche combination already in routine use within the NBS. This was considered because it is NBS practice to have more than one screening system available in case one test fails or can no longer be provided. Also in the case of HCV NAT screening, Chiron are the patent holders for the HCV sequence and as such there were fears that Roche would no longer be allowed to provide an HCV NAT test. In this situation the only commercial alternative would be the TMA assay.

The other proposed alternative donor screening method is not NAT but a serological method. The recently developed Ortho HCV core antigen assay was investigated in both its free antigen first generation form and the quantitative total antigen second generation form. During this study important information was gained on the level of HCV found in the window phase of infection using a panel of US donors.

5.1 The Bayer HCV TMA assay

5.1.1 Introduction

Transcription-mediated amplification uses two enzymes, reverse transcriptase and RNA polymerase to achieve greater than 10^9 fold amplification without thermal cycling. A primer with an RNA polymerase promoter sequence attached is used to anneal to the RNA template and prime cDNA synthesis with reverse transcriptase, the RNA is then degraded with RNAse H activity to leave a single strand of DNA which includes the promoter sequence. Another primer is then used to generate the complimentary strand. The RNA polymerase enzyme generates 100 to 1000 RNA copies from the DNA template which go through the process again (Figure 1.7 section 1.4.2).

The Bayer TMA assay is the HCV detection version of the HIV/HCV assay developed by Gen-Probe Incorporated (McDonough *et al.*, 1998). This assay uses magnetic microparticle assisted oligo-capture to extract HCV RNA from 500µl of plasma, this is then amplified by TMA followed by detection with a hybridisation protection capture assay and automatic results processing with a PC attached to the detection luminometer.

Each sample is processed from start to finish in a single tube thereby reducing the risk of sample to sample cross contamination. The manufacturer claims that the extraction, amplification and detection procedures may be performed within a single laboratory i.e. there is no requirement for amplicon containment in a separate laboratory. An internal control is included which is added at the RNA isolation stage. The TMA assay is performed manually, including both the RNA extraction step and the amplification and detection steps. The manual assay will process up to 100 samples and controls in one run at a time and takes one operator approximately four hours to complete. A fully automated version of this assay, known as Tigris, is under development.

The TMA assay was developed by Genprobe as an HCV/HIV-1 multiplex assay; the Bayer version, licensed from Chiron, is for the detection of HCV only. The Bayer assay is intended for use in a diagnostic rather than blood screening setting, and has been successfully used in the monitoring of HCV during antiviral drug therapy (Sarrazin *et al.*, 2001).

5.1.2 Methods

Four genotypes of HCV were used to test the TMA system: two of these were internationally recognised standards; a Type 1 from the PEI [#75/98] and a Type 3 from NIBSC [98/576]. Type 2 and 4 were in house standards genotyped using the InnoLipa line probe assay (Innogenetics, Ghent, Belgium); (Stuyver *et al.*, 1996) and calibrated using the Chiron Quantiplex 2.0 assay Chiron Corporation (Emeryville, CA, USA); (Detmer *et al.*, 1996). These standards were tested in two fold dilutions from 100 copies per ml to 6.25 copies per ml in the TMA assay using the manufacturer's protocol described in section 2.1.9.

To assess the suitability of the test for blood screening purposes, 100 minipools of 96 donations that had been found to contain HCV RNA through routine PCR screening were tested by the TMA assay. These positive minipools were tested by quantitative RT-PCR as described in section 2.2. In addition, 100 HCV RNA negative anonymous minipools were also tested by the TMA assay.

5.1.3 Results

The results for the four different genotype standards are shown in Table 5.1, the HCV level in IU/ml is shown for the standards that have been calibrated against the WHO HCV RNA International Standard (Saldanha *et al.*, 2000). The data are also plotted in Figure 5.1. Probit analysis was performed on these data for types 1 to 3 to calculate the 95% limit of detection for each genotype (Table 5.2). Probit analysis could not be performed on the Type 4 standard as the percentage positive did not drop below 80% and a curve could not be generated.

Table 5.1: **Sensitivity of the TMA assay with HCV genotypes 1 to 4.**

Genotype	HCV Level IU/ml	HCV Level Copies/ml	Number Tested	Number Positive	Percentage Positive
Type 1	31.3	100	24	23	95.8
	15.6	50	24	24	100.0
	7.8	25	23	20	87.0
	3.9	12.5	23	18	78.3
	1.9	6.25	24	16	66.7
	0	0	12	0	0.0
Type 2		100	16	16	100.0
		50	16	14	87.5
		25	16	7	43.8
		12.5	16	6	37.5
		6.25	16	4	25.0
		0	8	0	0.0
Type 3	17.8	100	25	23	92.0
	8.9	50	25	23	92.0
	4.4	25	24	15	62.5
	2.2	12.5	29	10	34.5
	1.1	6.25	25	6	24.0
	0	0	16	0	0.0
Type 4		100	15	15	100.0
		50	14	12	85.7
		25	16	15	93.8
		12.5	16	13	81.2
		6.25	16	13	81.2
		0	8	0	0.0

Table 5.2: 95% limit of detection by probit analysis

Genotype	95% detection (Copies/ml)	95% detection (IU/ml)
Type 1	49.6	15.5
Type 2	100.8	
Type 3	103.3	18.3
Type 4	ND	

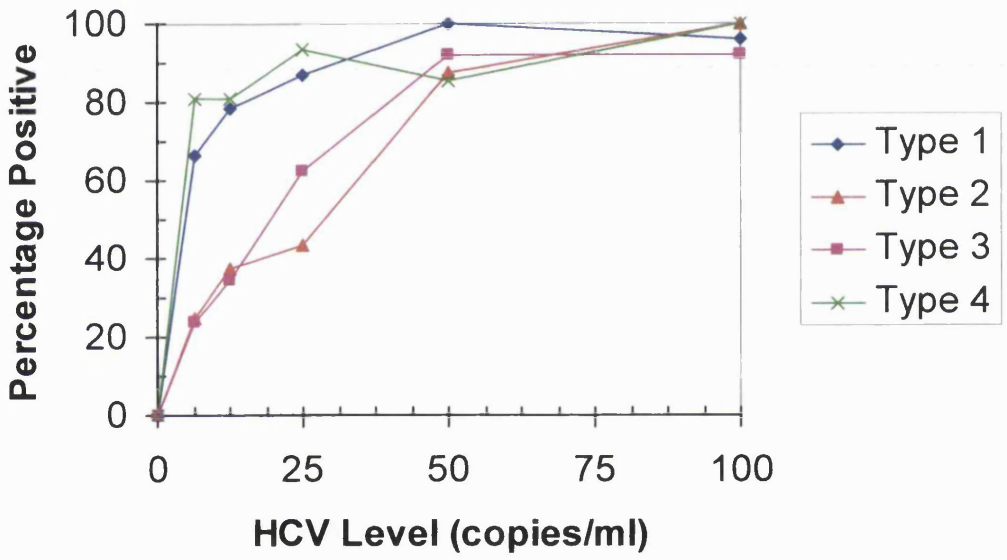


Figure 5.1: Comparison of detection of HCV genotypes 1 to 4 (copies/ml)

Percentage positives are shown for dilutions of HCV genotypes 1 to 4, each dilution was tested between 16 and 29 times.

From the data shown in Figure 5.1 it appears that the genotype 1 and 4 standards are detected more efficiently than the genotype 2 and 3 standards. The probit analysis in Table 5.2 gives a two-fold difference in the 95% end points between genotypes 1 and 3. However the standards of genotype 1 and 3 had also been quantified against the WHO international standard and therefore had HCV levels assigned in IU/ml. When the same data was plotted in IU/ml for the type 1 and 3 standards the sensitivity curves were more equal (Figure 5.2). The probit analysis

shown in Table 5.2 also shows that the 95% detection limits for these two standards are more similar, 15.5 IU/ml for type 1 vs. 18.3 IU/ml for type 3.

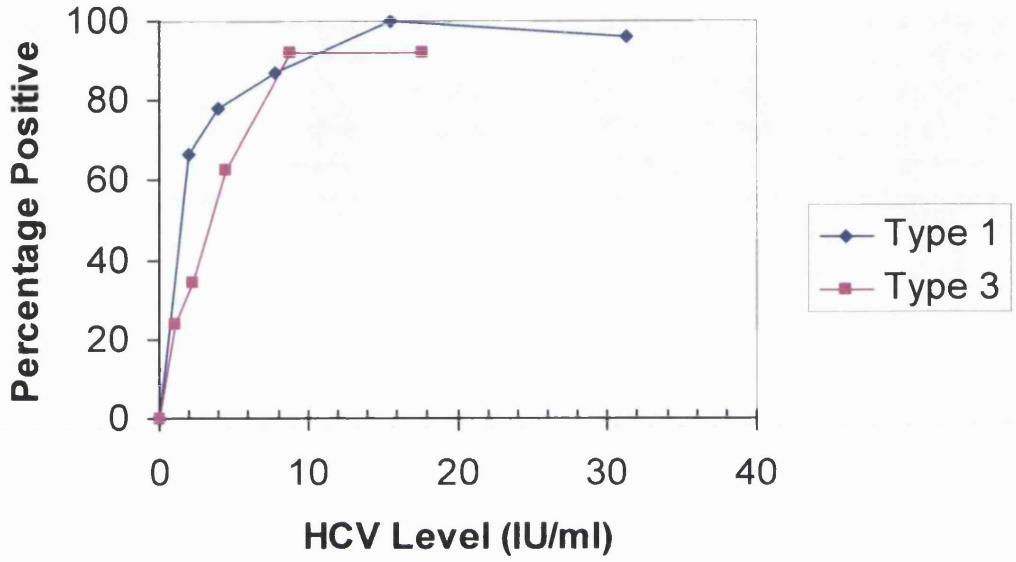


Figure 5.2: Comparison of detection of HCV genotypes 1 and 3 (IU/ml)

Using the same data as Figure 5.1 but plotted in IU/ml for the standards of genotype 1 and 3.

Of the 100 HCV positive minipools tested, 99 were reactive in the TMA assay and one was initially invalid, but when retested was also reactive. The signal to cut-off ratios of the reactive samples ranged from 3.8 to 24.3 with a median value of 20.2, and 95% of the values above 14.7 (Figure 5.3).

The minipools giving signal to cut-off ratios of 14.7 and below had low HCV RNA levels of between 64 and 380 IU/ml when tested by quantitative RT-PCR.

All 100 HCV PCR negative minipools were HCV un-reactive in the TMA assay. The signal to cut-off ratios of the un-reactive samples ranged from 0.03 to 0.58 with a median value of 0.17, and 97% were at or below 0.36 (Figure 5.4). The minipool which initially gave a signal to cut-off ratio of 0.58 gave a signal to cut-off ratio of 0.19 when retested.

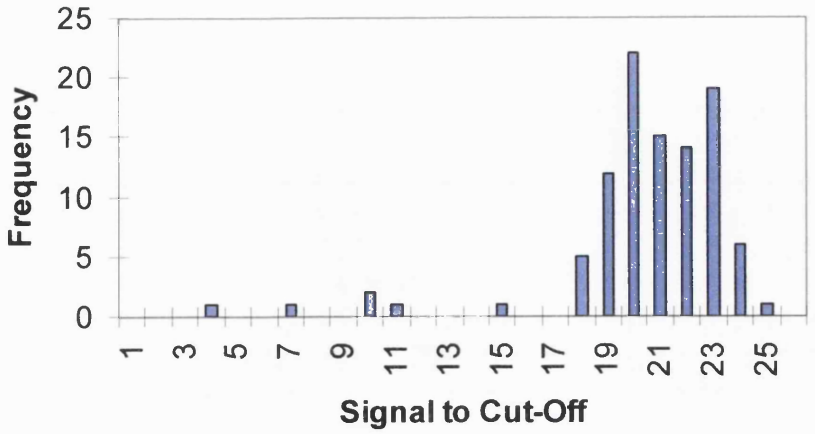


Figure 5.3: Signal to cut-off ratio of 100 HCV reactive minipools

The main population of positives is at a signal to cut off ratio of between 18 and 25, the positives between 4 and 15 have been shown to have low HCV RNA levels by quantitative PCR of between 64 and 380 IU/ml

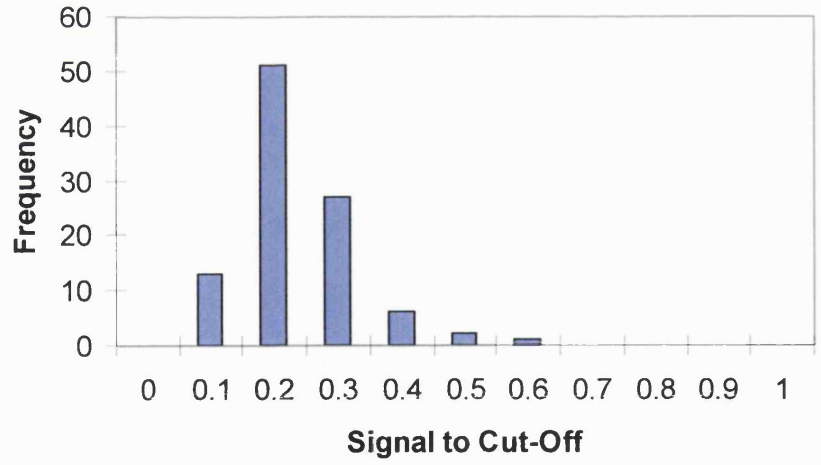


Figure 5.4: Signal to cut-off ratio of 100 HCV un-reactive minipools

5.1.4 Discussion

By combining the data for all three genotypes with probit data, an overall 95% sensitivity of the TMA assay of 85 copies/ml was calculated. By combining the data for types 1 and 3 an overall 95% sensitivity of 16 IU/ml was calculated. This level of sensitivity using the TMA assay compares to the 12.8 IU/ml 95% limit of detection which was achieved using the Qiagen QIAamp 96 Virus protocol with Roche COBAS HCV AmpliScreen v2.0 (section 4.8).

The 95% detection sensitivity of 16 IU/ml was sufficient to comply with the CPMP 100 IU/ml assay sensitivity and the PEI 5000 IU/ml per donation recommendations with a pool size of 48 or 96 donations.

There was no difference in detection between the four HCV genotypes. Any apparent discrepancy in sensitivity between the genotypes was probably due to differences in their calibration rather than a real effect. For example Table 5.2 shows a two fold better sensitivity in copies/ml for Type 1 than for Type 3, but if the IU/ml are plotted for the same data then the sensitivity is almost identical. This is because the PEI and NIBSC standards have been assigned IU/ml values based on calibration against the WHO international HCV RNA standard in a study by laboratories around the world (Saldanha *et al.*, 2000), whereas their copy/ml values have been assigned based on independent studies carried out by each institution.

Two recently published studies have found a better sensitivity using the WHO International standard of 6 IU/ml and 5 IU/ml respectively with equal detection of all the genotypes (Ross *et al.*, 2001; Krajden *et al.*, 2002). This compares to two studies of the HIV-1 / HCV multiplex assay which found 100% positives at 100 copies/ml of both targets with all of major genotypes (Linnen *et al.*, 2002; Giachetti *et al.*, 2002).

The TMA assay was able to detect HCV RNA in 96 donation minipools which had previously been found to contain HCV RNA during routine NBS PCR

screening. Maximum signal to cut-off ratios were given in 95% of these HCV positive minipools. The remaining 5% of minipools which gave lower signal to cut-off ratios had low HCV RNA levels.

The Bayer TMA assay was investigated to assess its suitability for use in the NBS as an HCV NAT screening assay. The TMA assay is able to process 100 samples and controls in four to five hours by one operator, which is faster than the six to seven hours for the BioRobot and COBAS assay in use in the NBS. However, the TMA assay is a manual process, which requires skilled operators to perform and requires considerable 'hands-on' time compared to the automated BioRobot and COBAS assay. The assay has to be performed to strict criteria to give valid results. As a manual assay electronic sample tracking cannot be used. It must also be noted that the Bayer assay used in this study is not available for routine blood screening and was used in place of the Chiron HCV/HIV-1 multiplex assay. These factors mean that in its manual form, the TMA assay cannot be considered as a replacement for the automated BioRobot and COBAS assays described in Chapter 4 and currently in use within the NBS. However if and when the fully automated version of this assay for the Tigris TMA system is developed it may warrant further investigation.

5.2 Detection of core antigen and quantification of RNA levels in hepatitis C virus pre-seroconversion donations

5.2.1 Introduction

The recent development of an ELISA for the detection of HCV antigen during the antibody negative phase of the acute infection (Peterson *et al.*, 2000) offers the possibility of screening for acute infections by detection of antigenaemia. The HCV core antigen assay is a sandwich ELISA with microwells coated with monoclonal antibodies (MAb) to the core antigen of HCV. The conjugate consists of MAb's with different epitope specificity to the capture MAb's, conjugated to horseradish peroxidase (HRP). The HRP is detected with OPD.

Originally, it was a widely held view that following initial detection of HCV RNA in the plasma of an infected donor, HCV RNA levels will rise rapidly with maximum load being attained within a very short period (Busch, 2000). With these characteristics in mind it has been suggested that the antigen assay could be used to detect window phase samples as efficiently but far more cost effectively than NAT.

In order to determine if core antigen testing was as effective as NAT screening in detection of HCV window phase donations a panel of HCV RNA positive window phase donations was tested for the presence of HCV core antigen. It was also decided to examine the time course of HCV RNA levels in these samples to gain more information on the viral dynamics in the HCV window phase. The window phase samples consisted of a non-commercially prepared panel of 41 donations from 17 plasma donors in the USA and one UK donor, all of which were identified by NAT during the seronegative phase of infection.

The studies described in this section were performed with the first generation free antigen assay, and have led to a publication in the journal Transfusion (Grant

et al., 2002b). A newer assay measuring total HCV core antigen, the Trak-C assay is described in section 5.3.

5.2.2 Methods

A total of 41 plasma donations from 17 US plasmapheresis donors (donors 1-17) and one English NBS donor (donor 18) all in the window phase of HCV pre-seroconversion were identified during the conduct of PCR screening. Most of the samples were detected by RT-PCR at a pool size of 512, however where this was not the case they were tested in pools of 96 and were found to be PCR positive at this dilution. The initial and subsequent plasma aliquots were taken from the plasmapheresis packs and stored between -40°C and -70°C until testing. They were not subjected to any further freeze thaw cycles.

All samples were tested in duplicate, in two assay runs, for HCV antigen by the prototype HCV antigen assay from Ortho Diagnostics according to the manufacturer's criteria as described in section 2.1.7. Samples were also tested for anti-HCV by Ortho v3.0 ELISA (Ortho Diagnostics, Raritan, NJ, USA) and by Murex v4.0 ELISA (Murex Biotech Ltd, Dartford, UK).

The samples were quantified by COBAS Amplicor HCV Monitor v2.0 (Roche Diagnostics, Branchburg, NJ, USA); (Yu *et al.*, 2000; Konnick *et al.*, 2002) and in duplicate by an in-house assay. The Roche Monitor assay was performed according to the manufacturer's instructions described in section 2.1.5. The in house assay was performed as described in section 2.2.

Line of best-fit analysis of HCV RNA and HCV antigen was performed using Microsoft Excel 2000.

5.2.3 Results

Good correlation was found between the Monitor and in-house quantitative assays with an average difference of 0.03 $\log_{10}$ IU/ml (range = 0.00 to 0.15 $\log_{10}$ IU/ml), the coefficient of variation (CV) of the replicates ranged from 0.4% to 41.9% with an average CV of 5.1% (Figure 5.5).

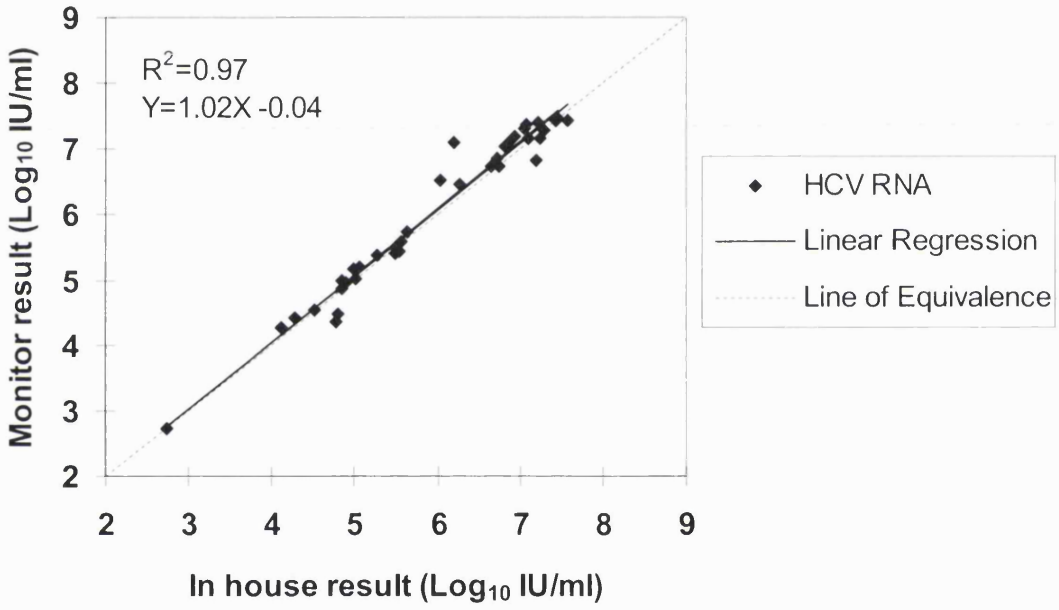


Figure 5.5: Comparison between Monitor and in house HCV qPCR assays.

The HCV RNA levels in the initial samples from the 18 donors shown in Table 5.3 ranged from 5.4 x 10^2 to 1.9 x 10^7 IU/ml with a median titre of 3.5 x 10^5 IU /ml. The median HCV titre calculated from all 41 samples was 3.7 x 10^5 IU /ml.

Using the Ortho HCV antigen assay, 11 (61%) of the 18 initial samples and 27 (67.5%) of 41 samples overall contained detectable HCV antigen by manufacturer's criteria. The limit of sensitivity of the antigen assay was calculated by comparing the signal to cut off ratio of the Ortho HCV antigen assay with the RNA titre (Figure 5.6). Samples above 10^7 IU/ml were excluded

because they gave antigen absorbance readings that were at or above the maximum range of the spectrophotometer used to read the plate, and would skew the linear regression line. Similarly samples giving absorbance readings at the level of the background of the assay were also excluded. A straight line drawn through these data using linear regression passes through the cut off of the antigen assay at approximately 1 x 10^5 HCV IU /ml.

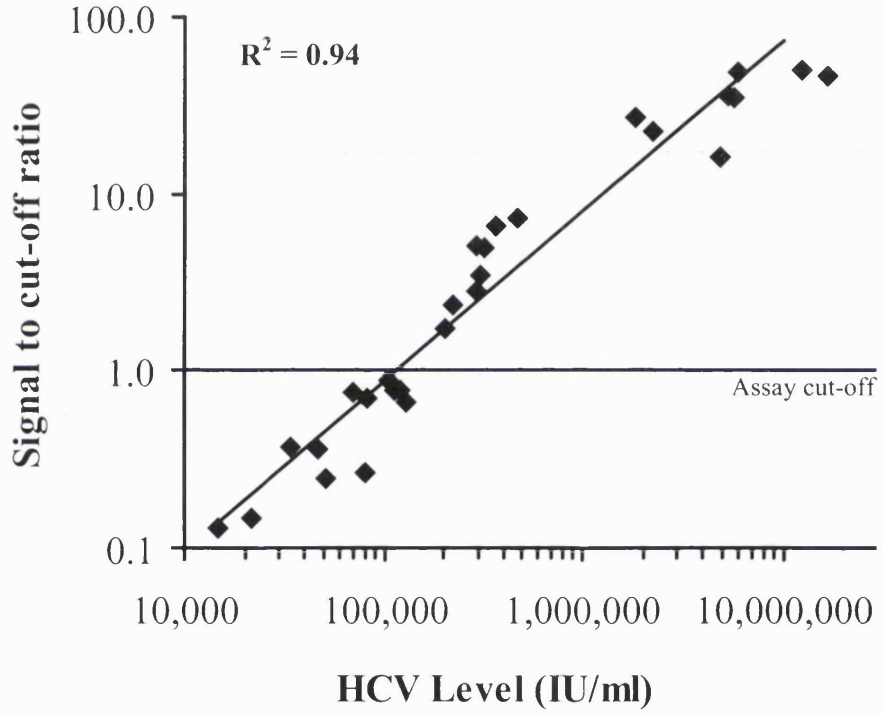


Figure 5.6: Line fit plot of signal to cut off against HCV RNA level.

The mean CV of the OD readings in the antigen positive samples was 5.2%. All antigen reactive samples were reactive in all replicates of the antigen assay.

It was noted that a number of HCV RNA positive samples tested by the antigen assay gave signal to cut-off ratios just below 1.0 (the assay cut-off). The HCV RNA positive samples that were negative in the antigen test were plotted in a histogram with the negative controls run in each assay (Figure 5.7). Two distinct populations can be seen in Figure 5.7 suggesting that the cut-off of the assay

could be lowered to include the group with higher ratios with the positives. The group with a higher signal to cut-off ratio contains six samples with significantly higher signal to cut-off ratios than the negatives, i.e. greater than the mean plus two standard deviations.

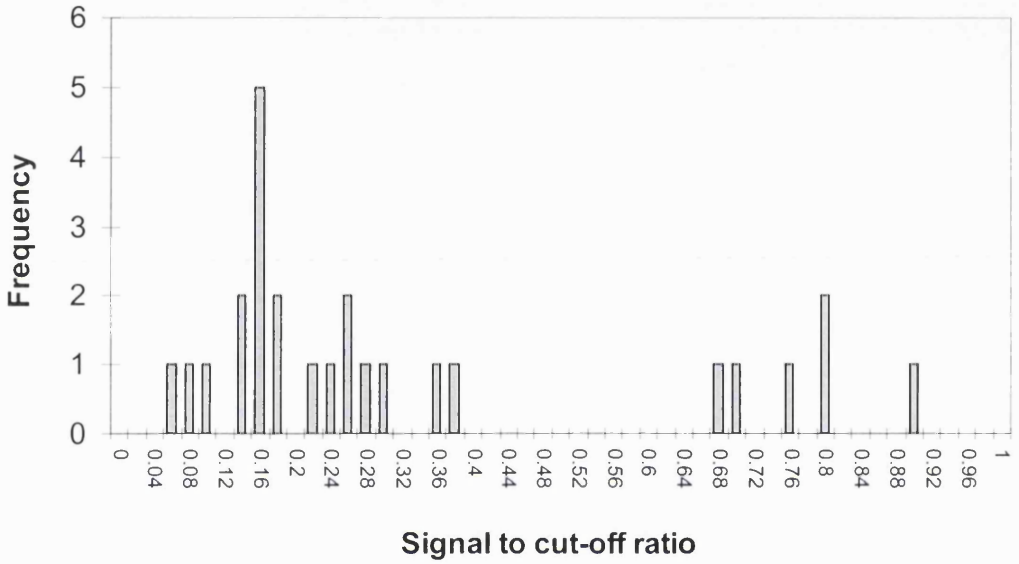


Figure 5.7: Histogram of signal to cut off ratios from samples below the cut off of the antigen assay

None of the initial donations contained detectable anti-HCV by the Ortho v3.0 ELISA. However donor 1 became anti-HCV positive by the Murex v4.0 ELISA four days after first detection by PCR and at least four days before the Ortho ELISA (Table 5.3). Donors 1 and 14 had elevated absorbance values using the Ortho anti-HCV ELISA that were below the assay cut off but were greater than the mean plus two times the standard deviation of the other values. Similarly the Murex assay showed elevated values for donors 4 and 6.

Table 5.3: HCV results for 18 window period donors.

Donor	Days after first PCR detection	HCV RNA Level (IU/ml)	Ortho antigen (S/CO)	Ortho Anti-HCV (S/CO)	Murex Anti-HCV (S/CO)
1	0	**11 000 000**	**64.39**	0.025	0.811
	4	**9 200 000**	**63.57**	0.105	**4.199**
	8	**7 970 000**	**62.36**	0.536	**3.850**
2	0	**324 000**	**4.90**	0.050	0.171
	2	**71 000**	0.76	0.087	0.150
	10	**306 000**	**3.48**	0.039	0.140
	17	**295 000**	**2.81**	0.029	0.135
3	0	**46 000**	0.36	0.050	0.198
	4	**293 000**	**5.05**	0.087	0.164
	7	**52 000**	0.25	0.039	0.158
	12	**105 000**	0.89	0.029	0.150
4	0	**19 200 000**	**64.72**	0.049	0.265
	2	**28 700 000**	**65.05**	0.066	0.362
5	0	**5 960 000**	**48.57**	0.021	0.162
	2	**13 400 000**	**62.11**	0.019	0.164
	7	**34 200 000**	**62.11**	0.012	0.151
	9	**26 800 000**	**62.11**	0.014	0.137
6	0	**15 700 000**	**61.92**	0.058	0.179
	5	**14 400 000**	**61.92**	0.019	0.369
	8	**19 500 000**	**60.13**	0.045	0.613
7	0	**114 000**	0.78	0.019	0.134
8	0	**81 000**	0.27	0.010	0.158
	2	**4 850 000**	**16.15**	0.023	0.138
9	0	**478 000**	**7.37**	0.064	0.178
	2	**83 000**	0.70	0.012	0.178
10	0	**16 600 000**	**45.78**	0.004	0.140
11	0	**540**	0.15	0.004	0.144
	6	**12 500 000**	**49.37**	0.023	0.145
12	0	**1 860 000**	**26.67**	0.019	0.208
	14	**5 360 000**	**35.69**	0.029	0.191
13	0	**131 000**	0.68	0.017	0.155
	7	**204 000**	**1.71**	0.045	0.155
	9	**2 240 000**	**22.51**	0.027	0.152
14	0	**15 000**	0.13	0.128	0.131
	2	**22 000**	0.15	0.109	0.134
	16	**1 300**	0.07	0.728	0.179
	21	**34 000**	0.37	0.672	0.191
15	0	**222 000**	**2.38**	0.033	0.167
16	0	**5 680 000**	**34.62**	0.060	0.177
17	0	**370 000**	**6.56**	0.012	0.158
18	0	**120 000**	0.79	0.022	0.101

The first donations detected by PCR are shown as day 0, subsequent donations show number of days after first donation. Positive HCV results are shown in **Bold.**

5.2.4 Discussion

The classic window phase model for HCV suggested that there would be a rapid increase in HCV RNA to very high levels before sero-conversion (Busch, 2000). From the data presented here, however, it appears that not all HCV infections result in such a rapid increase in viral titre to high levels as previously thought. Other studies have reported levels of 10^3 to 10^6 copies/ml (Lee *et al.*, 2001), 10^5 to 10^9 copies/ml (Nubling *et al.*, 1998) and 10^4 to 10^8 copies/ml (Busch *et al.*, 1997a) in window phase samples. In this study HCV levels ranging from 5.4 $x10^2$ to 3.4 $x10^7$ IU/ml were found.

Excluding the six donors from whom only one sample was available (7, 10, 15-18) the evolution of the HCV RNA in those donors with more than one sample investigated here falls into several distinct categories. Donors 1, 4, 5, 6, and 12 appear to be in the plateau of plasma viral load with high HCV levels of 10^6 IU/ml and above. Donors 2, 3, 9 and 14 also appeared to be in the plateau but at medium HCV levels of between 10^4 and 10^5 IU/ml. Donor 14 does not fit into the established window phase model having an initial HCV RNA level of 1.5 $x10^4$ IU/ml decreasing to 1.3 $x10^3$ IU/ml before rising again to 3.4 $x10^4$ IU/ml in spite of a follow up of 21 days from the initial detection of HCV RNA.

Only three donors (8, 11 and 13) appear to be undergoing the described rapid viral increase phase. Donor 11 shows the greatest rise from 5.4 $x10^2$ to 1.3 $x10^7$ IU/ml in six days. Even then one of these (donor 13) shows only a doubling of HCV RNA level over the first seven days of follow up.

All of the donors in this study, except donor 1, did not have detectable serum anti-HCV, rendering the failure to exhibit the 'usual' rapid increase in viral RNA level all the more remarkable. Interestingly the viral kinetics were paralleled both by RT-PCR for HCV RNA and the ELISA for HCV antigen. Thus some early modulation *in vivo* of viral replication seems likely and is perhaps more common than first thought. This means that estimates of the security of NAT testing

predicated using the established HCV window phase model may need to be treated with caution.

The antigen assay detected HCV antigen by manufacturer's criteria in 11 of the 18 (61%) donor samples initially identified by RT-PCR. Thus using the antigen assay alone would have lengthened the window phase of infection in these donors by an average of 2.5 days. In the seven cases (39%) where the antigen assay failed to detect HCV as early as PCR it took between 2 and over 21 days before HCV antigen was detected and the average extension of the window phase was eight days. Two donors were not detected by the presence of antigenaemia. One (18) was only sampled once, the other (14) was sampled four times over 21 days and antigen was not detected in any sample.

Using the antigen assay, 27 out of a total of 41 (65.9%) PCR-positive and sero-negative donations contained detectable antigen. Of the remaining 14 samples in which antigen was not detected, eight had mean OD's significantly higher than that of the negative control (i.e. greater than mean of the negatives plus two standard deviations) while still being less than the manufacturer's stated cut off. At the time of writing the assay was still undergoing clinical trial in the US. Data generated in this trial may result in the manufacturers reducing the assay cut-off from 0.04 plus the mean of the negative controls to 0.03 or 0.02 plus the mean of the negative controls. The results reported here were calculated according to the protocol provided but if the assay cut off were reduced as described above then recalculating the data would result in six of the previously negative samples becoming positive including three of the initial PCR detected samples. This would increase the number of donors in which HCV antigen was detected at the same time as HCV RNA to 14 out of 18 (77.8%) and the overall total from 27 to 33 out of 41 (80%) PCR-positive and sero-negative donations containing detectable antigen.

The situation in non-paid blood donors may be different to the plasmapheresis donors used in this study, because the samples used in this study were taken every few days and so the very low HCV levels at the beginning of infection would be represented by the first sample in the series to be detected by RT-PCR.

This allows an estimation of the difference in detection between RT-PCR and core antigen, but in the case of blood donors the chance of donation occurring within the first few days of the window phase is less.

The manufacturer has reported results using commercially available HCV window phase panels (Peterson *et al.*, 2000) with a total of 89 (85.6%) antigen positives out of 104 RNA positive anti-HCV negative samples, with only 4 out of 24 (16.7%) donors failing to detect antigen as early as RNA, with times ranging from 3 to 13 days. Another recently published study of HCV RNA positive pre-seroconversion samples showed antigen detection in 83% (5 of 6) in blood donors and 88% (81 of 92) of haemodialysis patients, with an average time from RNA detection to antigen detection of two days (Couroucé *et al.*, 2000).

The antigen assay is designed to screen individual donations and would certainly be more beneficial than conventional anti-HCV ELISA assays alone in a blood bank setting. However, with its current sensitivity, it would be unlikely to give an advantage over NAT because it is only able to detect antigen reliably in donations with 1 x 10^5 HCV IU/ml or more, a level which would easily be detectable by most NAT methods even at a pool size of 100 donations. The results of a recent international forum have shown that countries where NAT testing is already in place would not consider changing to antigen testing, but others without NAT infrastructure such as Spain and Greece are using or contemplating using antigen testing (Engelfriet and Reesink, 2002). In Spain the first HCV window phase blood donation was found using the core antigen test in routine screening (Sanz *et al.*, 2002). Other countries such as Italy (Piccoli *et al.*, 2001), Portugal (Lucas *et al.*, 2001), Croatia (Mahaljevic *et al.*, 2001), and Poland (Letowska *et al.*, 2001) are also using the antigen assay.

It was confirmed that all but two of the donations in this study were anti-HCV unreactive by the most sensitive assays. The anti-HCV v4.0 ELISA from Murex did detect anti-HCV in one of the donors (donor 1) at least four days before the Ortho v3.0 anti-HCV test, although the latter assay did show an elevated OD in the last sample. Samples from three donors (4 and 6 by Murex; 14 by Ortho) had elevated OD values below the manufacturer's cut-off. These observations as a

whole indicate that whilst the detection of anti-HCV fails in the window phase in most of these donors there is still room to improve antibody assays and the presence of low level reactivity for anti-HCV in 4 of these 18 donors indicates this might be possible. Combination HCV antigen and antibody ELISA tests are under development (Arcangel *et al.*, 2001).

The data presented here have an impact on NAT screening policy because the use of pooled sample testing is justified on the basis of high HCV titre in the window phase donations that could still be detected even after dilution in many other negative plasma samples in the pool. The PEI of Germany has set a level of 5000 IU/ml as the lower detection limit required for screening blood donations by NAT in Germany. Two of the samples quantified here fall below this limit, one was the initial sample from donor 11 (5.4×10^2 IU/ml) which had risen to 1.6 $\times 10^7$ IU/ml two days later in the rapid viral increase phase. Donor 14 in whom the second low sample occurs is a far more worrying pattern as the low specimen occurred 16 days after the initial detection and this donor maintained HCV RNA levels below 1×10^5 IU/ml for the 21 days that samples were available. The 1300 IU/ml in the sample on the 16th day after pick up would possibly have challenged NAT screening methods employing specimen mini pool testing although in this case this sample was identified by RT-PCR in a pool size of 96. Though NAT methods have improved greatly, careful monitoring of the sensitivity of the process is needed and reliance should not be placed upon an inevitable viral increase to high levels in all window phase donors.

5.3 The Ortho Trak-C HCV core antigen assay

5.3.1 Introduction

The Trak-C assay (Ortho Clinical Diagnostics. Raritan, NJ, USA) is a 96-well microplate based two step manual quantitative immunoassay for the measurement of total HCV nucleocapsid core antigen in human serum or plasma, in the presence or absence of anti-HCV antibodies.

The Trak-C assay is known as Ortho's second generation HCV core antigen assay. It differs from the first generation core antigen assay described in section 5.2 in that the samples and controls are incubated with a pre-treatment reagent to dissociate any immune complexes, thus allowing the HCV core antigen to be detected.

A recombinant antigen standard is supplied with the kit. This is run in the assay at several dilutions between 1.5 and 100 picograms per ml (pg/ml). The ODs from the standards are plotted against antigen level in pg/ml. The standards give a straight line and linear regression is used to calculate the slope of the curve allowing calculation of the antigen level in the unknown samples. The assay takes around four to five hours to perform.

The manufacturer has claimed that as well as being able to quantify the HCV viral quantities in clinical samples the sensitivity of the assay is significantly increased compared to the first generation antigen assay (Sentjens *et al.*, 2001).

The sensitivity of the assay was assessed with a well-characterised panel of HCV window phase donations described in section 5.2. Testing this panel of samples allowed comparisons to be drawn between the performances of the two antigen assays.

5.3.2 Methods

The window phase samples described in section 5.2 were tested by the Trak-C assay. The assay was performed according to the manufacturer's instructions as described in section 2.1.8. These were quantified using the Bayer Quantiplex bDNA v3.0 assay (Beld *et al.*, 2002).

A small stability study was performed to define the stability of HCV core antigen in plasma. A plasma sample was spiked with a known quantity of HCV, a time zero sample was taken immediately and frozen at -70°C, the rest was split into three aliquots and stored at 4°C, room temperature and 37°C. Samples were taken from each storage temperature at 4, 7 and 10 days and stored frozen at -70°C until testing. At the end of the study all samples were tested by the Trak-C assay.

After initial results suggested that the standard curve could be extended to give a higher upper limit of quantification for the assay the standard curve was extended to include 200, 400 and 800 pg/ml standards. The assay was performed as before with the exception of the plate being read at the standard 492 nm as well as an additional read at 450 nm.

5.3.3 Results

The assay was run with dilutions of the recombinant HCV core antigen standard supplied at 1.5, 5, 15, 50 and 100 pg/ml, according to the manufacturer's instructions (section 2.1.8). Linear regression was performed by the method of least squares in a Microsoft Excel 2000 spreadsheet to generate a standard curve with a straight line from 1.5 to 100 pg/ml (Figure 5.8).

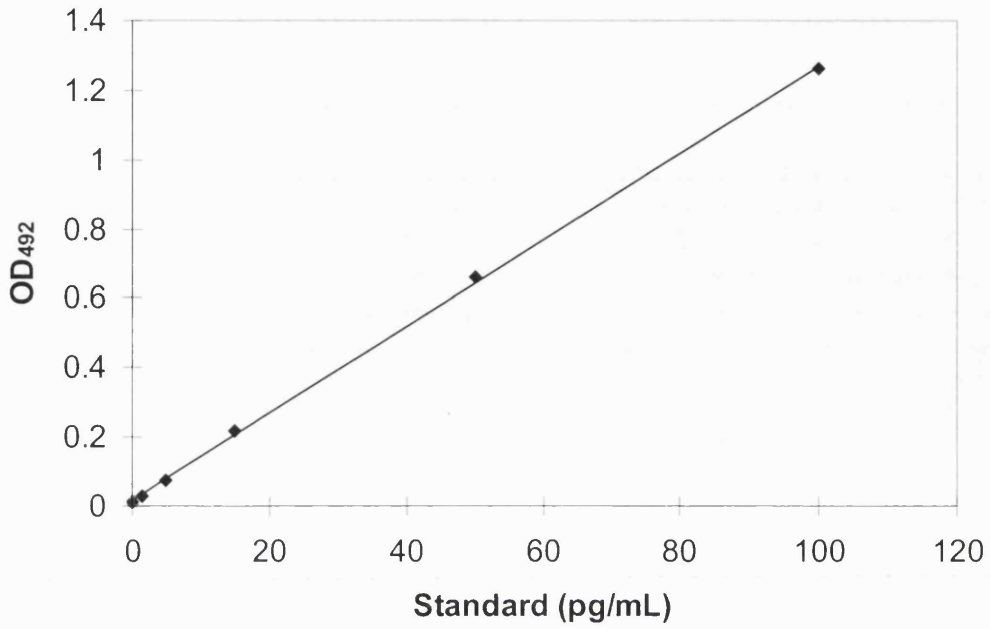


Figure 5.8: Ortho Trak-C HCV antigen standard curve

The top of the standard curve at 100 pg/ml was equivalent to approximately 1 x 10^6 IU/ml, however the OD reading at this level was only 1.260 suggesting that the standard curve could be extended to a higher level. Further evidence for this assumption was gained by running the in-house HCV RNA standard 'X' (section 2.2.4), which gave linear OD readings up to 2.616 at 2.5 x 10^6 IU/ml. It was also noted that samples containing a high level of HCV gave a much stronger colour by eye than the top of the standard curve.

It was therefore decided to extend the standard curve and at the suggestion from the manufacturer (Paul Wallis personal comment) to read the plate at a lower wavelength. The resulting standard curves from the two wavelengths are shown in Figure 5.9. The standard wavelength reached a plateau at the higher standards whereas the lower wavelength produced a much straighter line. However the lower wavelength could not resolve the lower standards from the background.

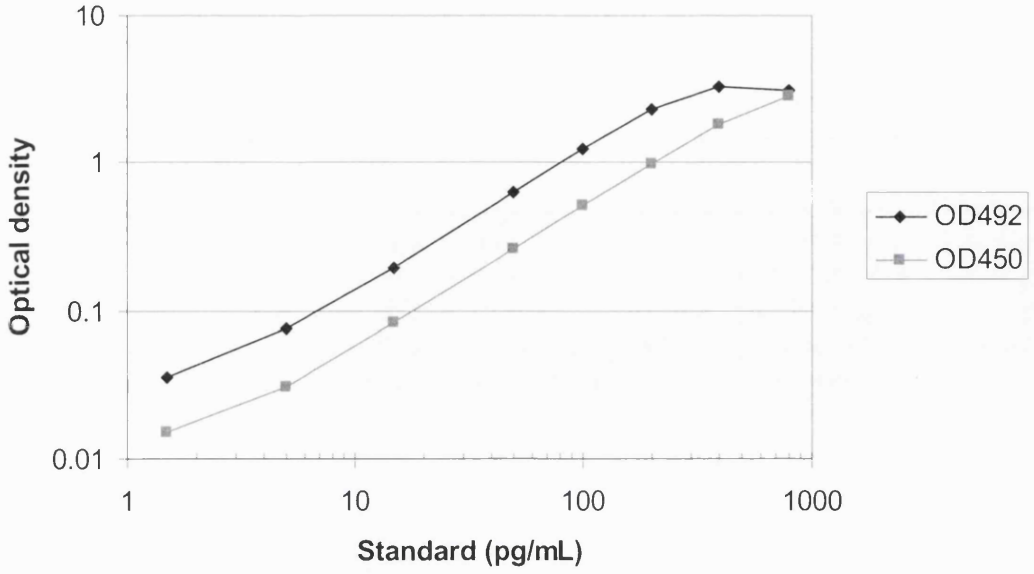


Figure 5.9: Effect of wavelength on standard curve

The standards were assayed and the plate was read at two wavelengths. At the standard 492nm there is a plateau at high antigen levels, this was not seen when the wavelength was reduced to 450nm.

The antigen levels are shown for pre-seroconversion window phase HCV samples of known HCV RNA concentration measured by the Quantiplex 3.0 HCV bDNA assay (Bayer Corporation, Tarrytown, NY, USA); (Figure 5.10). Using linear regression 1 pg/ml of HCV core antigen was shown to be equivalent to approximately 10 000 HCV RNA IU/ml. An in house HCV standard which had been calibrated against the WHO HCV International Standard 96/790 as described in section 2.2.4 was also run in this assay allowing calculation of the HCV levels in IU/ml. The IU/ml values closely matched the values previously measured by the Quantiplex v3.0 assay indicating that the levels of HCV core antigen closely match those of HCV RNA (Figure 5.11).

The lowest level of HCV that was detected by the antigen assay in this run was 7000 IU/ml. The WHO HCV RNA international standard (96/790) was also detected at a dilution of 1 in 10 or 10 000 IU/ml.

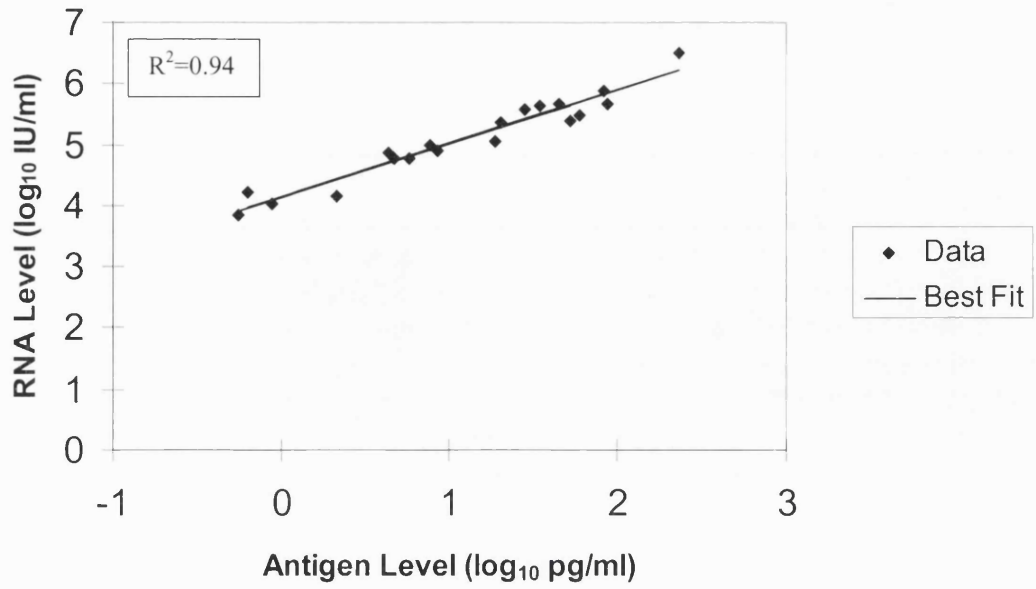


Figure 5.10: Relationship between HCV core antigen and HCV RNA

A line of best fit was calculated between the antigen level in pg/ml measured by the Trak-C antigen assay and the RNA level measured by the Quantiplex 3.0 bDNA assay

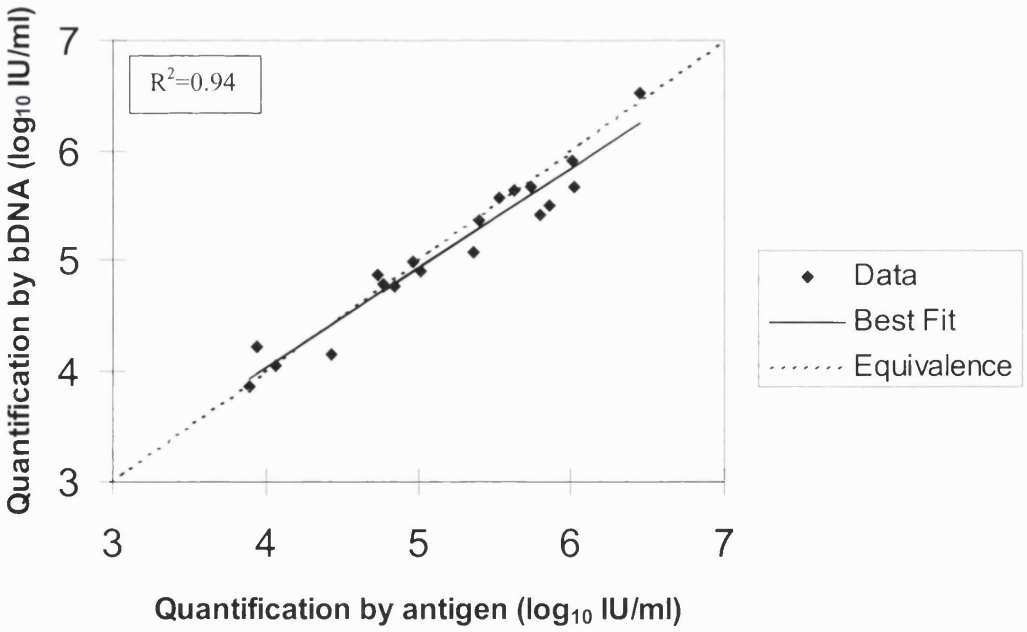


Figure 5.11: Comparison between HCV quantification in IU/ml using the Trak-C assay and the Quantiplex bDNA assay

A line of best fit was calculated between the estimate of HCV level from the Trak-C assay using an in-house standard quantified in IU/ml and the RNA level measured by the Quantiplex 3.0 bDNA assay

The stability of HCV core antigen was measured for up to 10 days storage at 4°C, room temperature and 37°C (Figure 5.12). Only storage at 37°C for 10 days resulted in a significant degradation in core antigen level of approximately 1 $\log_{10}$. At 37°C there was a rapid decline seen at the first time point on day 4 and then a levelling off in the rate of decline. Room temperature and 4°C storage gave declines in HCV core antigen level of 0.5 and 0.2 $\log_{10}$ respectively after 10 days.

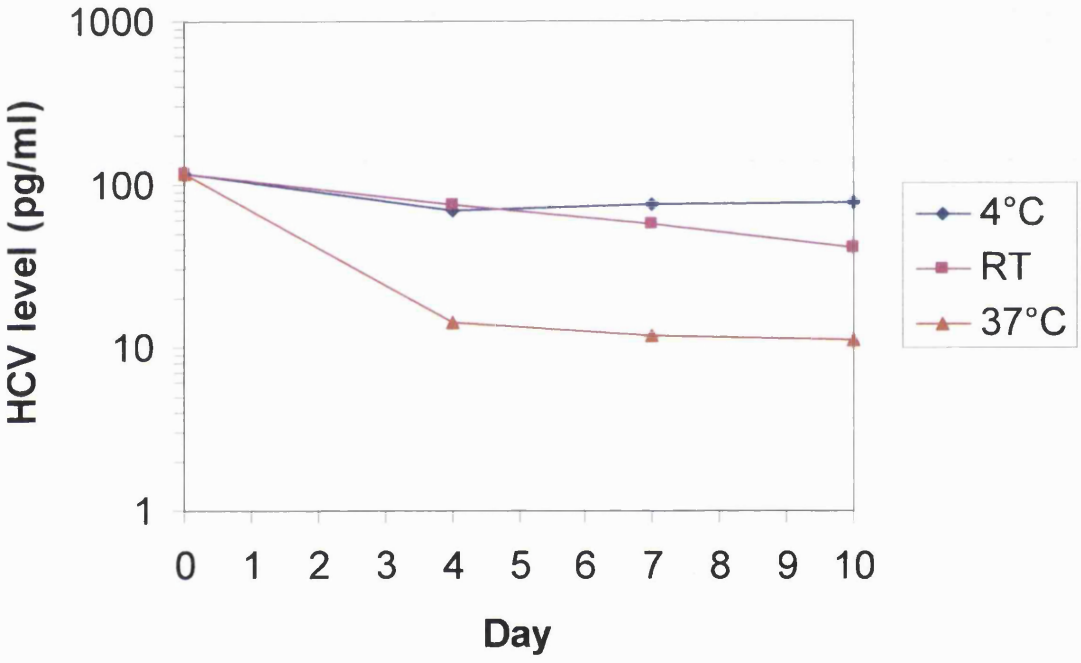


Figure 5.12: Stability of HCV core antigen

A plasma sample containing HCV was stored at 4°C room temperature and 37°C, the antigen levels were measured at various time points up to 10 days.

The Trak-C antigen results of the window phase panel described in section 5.2 were compared to the results with the first generation assay described in section 5.2. The Trak-C assay was able to detect antigen in 10 samples that had been negative by manufacturer's criteria with the first generation assay. The samples that had shown raised backgrounds with the first generation assay were all positive in the Trak-C assay with the exception of one sample which was

unavailable for testing by the Trak-C assay. The comparison of the signal to cut-off ratios between the two assays shows that the Trak-C assay is approximately 10 fold more sensitive than the previous version.

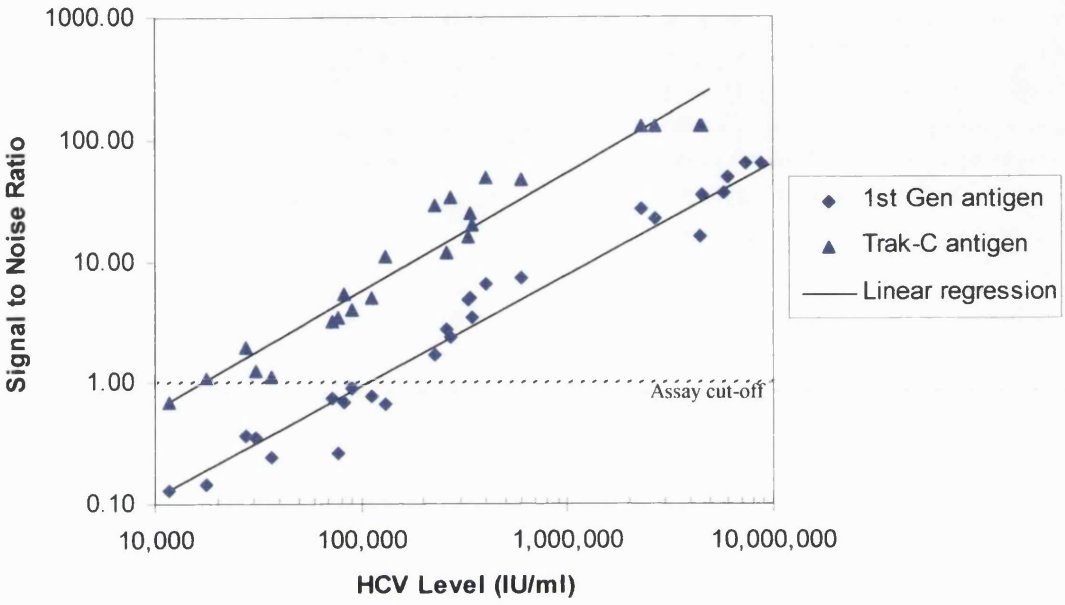


Figure 5.13: Comparison of sensitivity between first generation and Trak-C antigen assays in detection of HCV RNA positive window phase samples

The same panel of samples were tested by the 1st generation free antigen assay and the Trak-C total antigen assays. The difference in cut-off between the two assays was approximately 1 $\log_{10}$ IU/ml.

Using the Ortho Trak-C HCV antigen assay, 15 (88.2%) of the 17 samples that had been the initial time point detected by RT-PCR screening contained detectable levels of HCV core antigen. Overall, 37 (92.5%) of 40 HCV RNA positive window phase samples contained detectable HCV antigen by manufacturer's criteria. This represents a considerable improvement over the first generation assay where the equivalent figures where 11 (61%) of the 18 initial samples and 27 (67.5%) of 41 samples overall containing detectable HCV antigen.

5.3.4 Discussion

The Trak-C assay gives a quantitative measurement of total HCV core antigen. The standard curve gave a straight line up to 100 pg/ml. However by adding additional standards and reading the plate at two wavelengths the upper limit of quantification of the assay may be extended to 800 pg/ml.

By running HCV standards and samples with known HCV RNA levels calibrated in HCV IU/ml, a conversion factor was calculated so that 1 pg/ml of HCV core antigen was approximately equal to 10 000 IU/ml of HCV RNA.

Thus the upper limit of quantification of the assay was 1 x 10^6 IU/ml in the un-modified assay and up to 8 x 10^6 IU/ml with extra standards and the double wavelength read. The lower limit of detection of the assay was calculated using a linear regression line through a series of samples of known HCV RNA level. The lower limit of detection was found to be approximately 1 pg/ml or 10 000 IU/ml.

The dynamic range of the Trak-C assay is not quite as large as other commercially available HCV quantitative assays such as the HCV Quantiplex v3.0 (Bayer Corporation, Tarrytown, NY, USA) that will measure between 480 and 7.7 x 10^6 IU/ml, and the HCV Monitor v2.0 (Roche molecular systems, Inc. Pleasanton, CA, USA) which will measure between 600 and 8.5 x10^5 IU/ml.

As the Trak-C assay is intended for monitoring HCV virus levels in patients undergoing treatment, the accuracy of the Trak-C assay was assessed by testing samples that had previously been tested with the Quantiplex v3.0 bDNA assay. The HCV core antigen levels closely matched the HCV RNA levels.

The results of the stability study indicated that HCV core antigen has a similar stability to HCV RNA as defined in section 3.2. As expected the greatest loss in core antigen level occurred at the highest temperature storage (37°C) with minimal loss after 10 days of storage at 4°C.

The Trak-C assay is able to measure total core antigen due to a dissociation step, which removes any anti-HCV bound to the core antigen. It is at least 10 fold more sensitive than the first generation free core antigen assay discussed in section 5.2. The improvement in sensitivity would appear to be due to a lower background and lower cut-off in the Trak-C assay, enabled by the addition of urea to the wash buffer.

This improvement in sensitivity meant the Trak-C assay was able to detect 92.5% of the window phase samples detected by RT-PCR. There were only two donors out of 17 in which antigen was not detected as early as RT-PCR. In the first donor the initial donation was found positive by RT-PCR but at a very low HCV RNA level of 540 IU/ml. When re-tested six days later the donor had an HCV level of 1.2×10^7 IU/ml and contained over 100 pg/ml by the Trak-C test. In the second donor the initial RT-PCR positive sample contained an HCV RNA level of 1.5×10^4 IU/ml, but did not contain detectable antigen. When the donor re-donated two days later the HCV level had risen slightly to 2.2×10^4 IU/ml and antigen was detected (S:CO 1.09).

Although this assay represents a considerable improvement in sensitivity over the first generation assay, the sensitivity of 10 000 IU/ml achieved cannot match NAT and does not comply with the recommendation by the PEI of 5000 IU/ml per donation. The Trak-C assay did not detect some of the HCV window phase donations in this studyand a recent window phase donation detected by RT-PCR minipool screening in the NBS did not contain detectable antigen when subsequently tested with the Trak-C antigen assay (Section 6.4).

However the assay does offer a considerable improvement over anti-HCV screening tests and would be valuable in countries without the infrastructure for NAT blood screening.

Chapter 6

Results of NBS HCV NAT screening

6.1 Introduction of NAT assays into the NBS

The combination of the modified QIAamp viral RNA protocol with the COBAS Amplicor v2.0 assay described in section 4.3.7 was first introduced into the NBS in April 1999 for release of long shelf life (>35 days) blood components (Phase I). The assay was performed at BPL in Elstree, with pools of 96 donations. At BPL the NAT testing for England and Wales was performed in three separate rooms, a reagent preparation laboratory, a nucleic acid extraction laboratory housing two BioRobot 9604's and a PCR laboratory housing three COBAS Amplicor instruments.

Various procedures ensured that the possibility of amplicon or nucleic acid contamination was minimised. The PCR laboratory was under negative pressure to contain PCR amplicons and the reagent preparation laboratory was under positive pressure to prevent any contamination entering. Dedicated clothing was used in each area. A strict staff movement pattern was used so that staff having entered the extraction or PCR laboratories could not enter the reagent preparation laboratory, and staff having entered the PCR laboratory could not enter either the reagent preparation laboratory or the extraction laboratory unless they first took a shower.

The NAT laboratory at BPL continued to screen all blood donated in England and Wales for release of long shelf life components until Phase II of the NBS NAT screening plan was introduced. The NAT laboratory at Brentwood in Essex took over HCV NAT testing of all donations from the London and South East area on July 4th 2000 for release of long shelf life blood components using the same assay as BPL. On December 18th 2000 the NAT lab at Leeds in Yorkshire took over HCV NAT testing of all blood donations in the Northern area using the COBAS Amplicor HCV v2.0 assay with a manual Amplicor extraction. BPL

stopped testing blood donations for the NBS at the end of 2000 with Brentwood taking on the testing for Manchester and the Southwest.

From February 22nd 2001 Brentwood began releasing short shelf life blood components except platelets for the London and South East area on the basis of the HCV NAT test. Shortly afterwards on March 4th Brentwood introduced 24 hour shift working at the laboratory in order to cut down the time taken from receipt of samples to release of NAT result.

The combination of the QIAamp 96 Virus protocol with COBAS HCV AmpliScreen v2.0 described in section 4.8 was introduced into the NBS at the Brentwood NAT laboratory on June 18th 2001. At the same time the minipool size was reduced from 96 to 48 donations per pool. These changes allowed the release of all blood components with a shelf life of greater than 24 hours, which enabled the release of platelets on the basis of the NAT result.

Shortly after the introduction of the QIAamp 96 Virus protocol with COBAS HCV AmpliScreen v2.0 combination the third NBS NAT laboratory at Birmingham in the West Midlands, began NAT testing on June 25th 2001 using the same assay with minipools of 48 donations for release of short shelf life components (>24 hours). The Leeds laboratory began short shelf life component (>24 hours) release on minipools of 48 donations from July 11th 2001, although they continued NAT testing using the COBAS Amplicor HCV v2.0 assay with a manual Amplicor extraction until July 22nd 2002 when they began testing with the QIAamp 96 Virus protocol with COBAS HCV AmpliScreen v2.0 combination.

6.1.1 Sample collection and pooling

As a result of the studies described in Chapter 3 the NBS implemented the following sample handling procedures. A dedicated blood sample was taken into a dry K_3EDTA vacuum tube at the time of donation. The samples were sent to the closest pooling laboratory at ambient temperature and then stored at 4°C

upon arrival. The pooling laboratories were located at Brentwood, Birmingham and Leeds.

At the pooling laboratories the samples were separated and 50 µl of plasma were taken from each using a Tecan Genesis 200 Robotic Sample Processor (RSP; Tecan Group Ltd. Zurich, Switzerland) to create a 4.8 ml minipool containing 96 samples. A 1 ml aliquot of each sample was also archived in a 96 deep well plate at this time. The archive plate was stored frozen at –40°C and the minipools were sent in cool boxes to BPL, or later to the NBS NAT laboratories.

Anti-HCV positive donations were not withheld from the minipool for two reasons. Firstly, it would have taken up much of the available shelf life to test the samples by serology, and then make the minipools and NAT test. With a shelf life of only five days for platelets the speed of testing was critical, therefore serological testing and NAT testing were performed in parallel. Secondly, including sero-positives in the minipools ensured a steady supply of positive minipools so that the resolution of positive samples became a routine event, testing the NAT screening process. As HCV prevalence is low in UK blood donors this did not cause a significant number of positive minipools.

In the case of a positive minipool result the minipool was retested in duplicate and if two out of three or more results were positive the minipool was considered a repeat reactive. If only the initial result was positive this was considered to be a false positive result. This system was changed when the NBS laboratories took over testing so that only the initial result was required for the minipool to be considered positive.

In the case of a repeat reactive minipool the archive plate was thawed at the pooling laboratory and the Tecan Genesis RSP was used to create 8 cross pools of 12 samples and 12 cross pools of 8 samples from the rows and columns of the archive plate (Figure 6.1). These 20 cross pools were then sent to the NAT laboratory and NAT tested with the intersect of the positive row and column pools used to identify the individual well containing the positive sample.

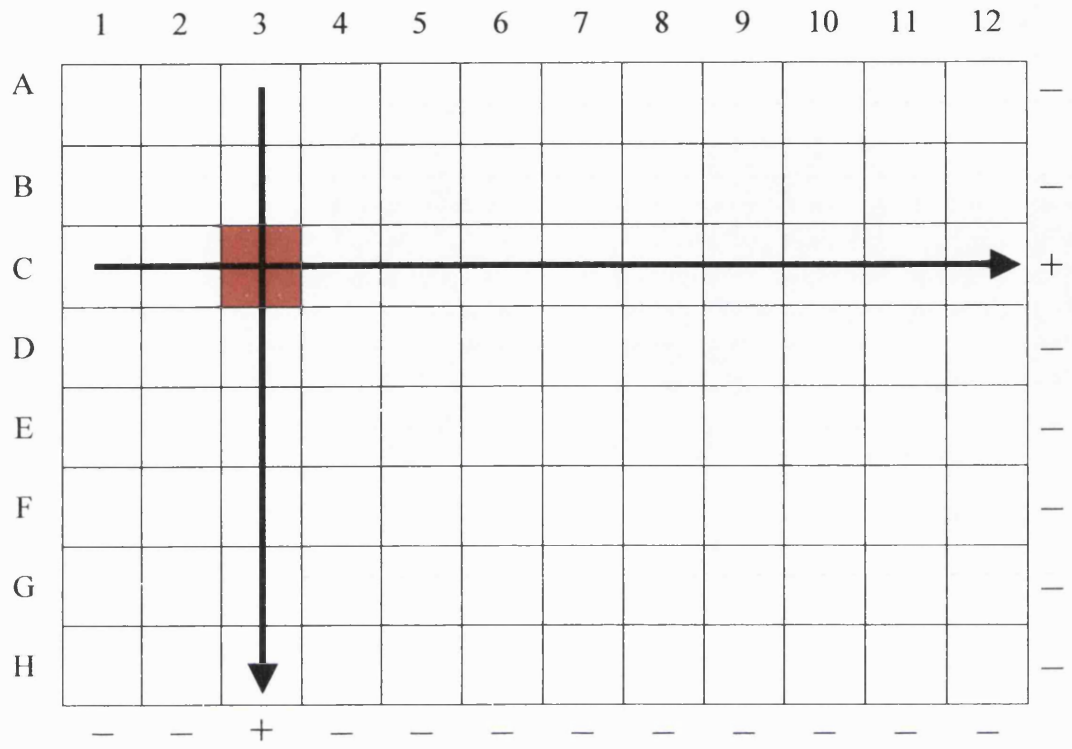


Figure 6.1: Cross-pooling of a 96 well archive plate with a single positive.

Two-dimensional analysis resolving identity of the NAT positive donation. Cross-pool 3 (column) and C (row) identify the sample in position C3.

The positive donation identified at the intercept of the positive cross pools was checked against the serology result and if the intercept position was found sero positive the donation is removed and no further action is taken. If the positive donation was not found to be sero-positive it is removed from the archive plate and referred to the NAT reference laboratory at UCL for confirmatory NAT testing. These cases are also referred to the NBS donor recipient outcome group (DROG) chaired by Dr. P Hewitt. The positive donation was matched to the serology data to determine if the sample was in the window phase of infection.

In rare cases more than one positive sample occurred in a single pool giving multiple positive cross pools. A minipool containing two positive donations has only occurred four times since testing was started in the NBS, and in three of these cases four cross pools have been found positive as shown in Figure 6.2.

Two of the intersects, indicated in red have also been sero-positive, however, the other two intersects, indicated in grey were also NAT tested just in case they contained a window phase sample.

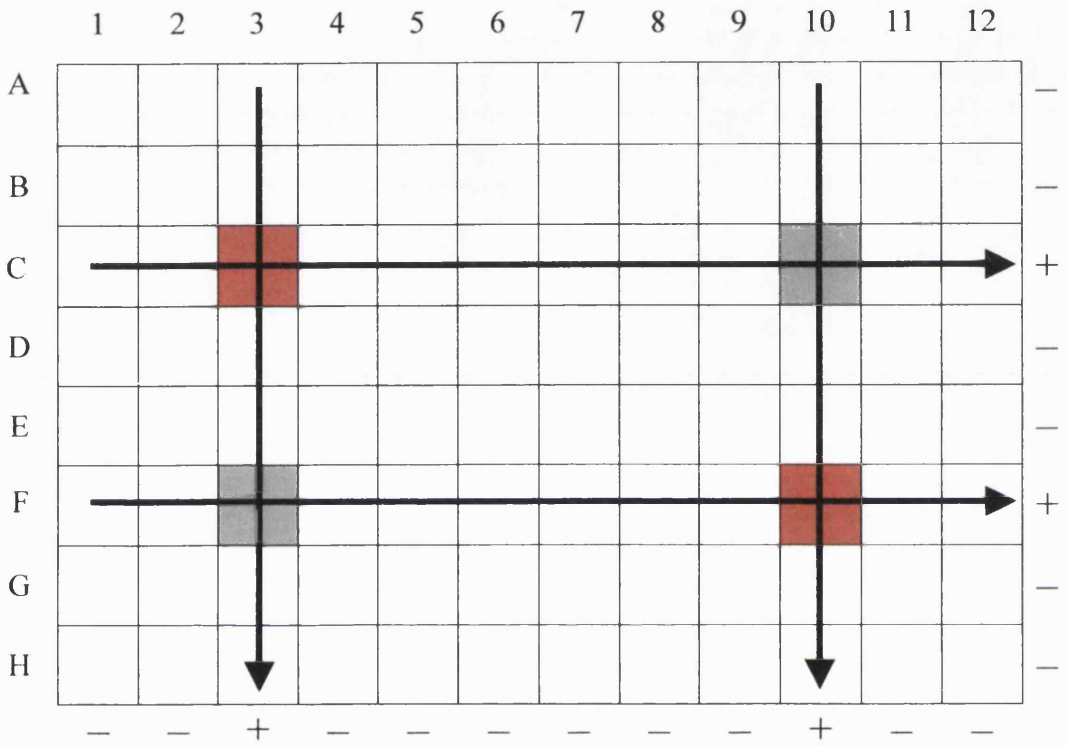


Figure 6.2: Cross pooling of a 96 well archive plate with two positives.

Four positive cross-pools (C, F, 3 and 10) indicate four possible positions of the two positive donations. If two positions (marked in red) are found sero-positive the other two positions (marked in grey) must still be NAT tested to discount the 'unlikely' possibility of a window phase donation in either position.

In the other case where two positive donations occurred in a minipool only three cross pools were found positive. In theory two positive donations in a minipool could result in only three cross pools being positive if the two donations are on the same row or column of the archive plate (Figure 6.3). However in this case when the individual samples were tested the two positive were not in the same row or column indicating an error in the cross pooling. Although there have been nine other cases with three positive cross pools these have all been due to contamination of the third cross pool rather than two positive samples.

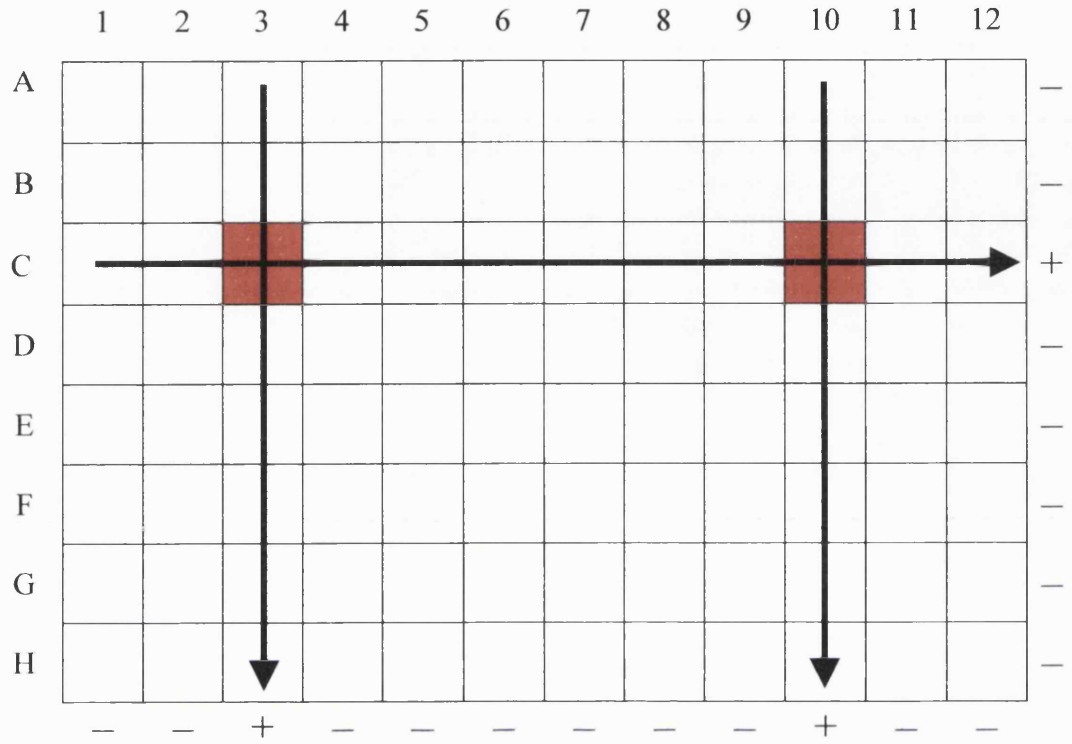


Figure 6.3: Cross pooling of a 96 well archive plate with two positives.

Three positive cross-pools (C, 3 and 10) indicate the positions of the two positive donations (marked in red).

With the introduction of short shelf life component release the pool size was reduced to 48 donations. In this case two minipools were stored in one 96 well archive plate and the cross pooling was performed in an 8 by 6 matrix.

6.2 Sensitivity Monitoring

Each BioRobot extraction of 96 samples and controls was amplified and detected in four COBAS runs of 24 samples and controls. Each COBAS run consisted of two A-rings of 12 samples and controls which were amplified in two separate thermalcyclers within the COBAS instrument. Each A-ring contained a negative, high positive and low positive control plasma. The high positive control was regarded as a 'go or no go' control i.e. if this control failed then the samples in that A-ring must be retested. The level of the 'go or no go' positive control was 71 IU/ml at BPL (a 1:10 dilution of the NIBSC working reagent) and 100 IU/ml when testing was introduced into the three NBS laboratories.

The low or monitoring control was 3.5 IU/ml (a 1:200 dilution of the NIBSC working reagent); this was also included on every A-ring. Another monitoring positive control of 50 IU/ml was run once per extraction run. The monitoring controls were not used as validation criteria for the individual assay run, but their frequency of detection was monitored over time to check that the assay sensitivity remained constant.

At the end of the run each A-ring was assessed individually for acceptance criteria. The 71 or 100 IU/ml positive "go / no-go" control was required to be positive for the A-ring to pass (i.e. OD $\geq$ 0.15 for COBAS Amplicor v2.0). The validity of the entire extraction run was assessed on the basis of the 71 IU or 100 IU/ml 'go or no-go' positive control results. In an extraction run containing one to four A-rings it was considered that all A-rings must have valid 'go or no-go' controls, if one A-ring failed then the entire run failed. In an extraction run of five to six A-rings, one A-ring was allowed to fail but two or more failures failed the extraction run. In an extraction run of seven to eight A-rings, two A-rings were allowed to fail but three or more failures failed the extraction run.

These were arbitrary criteria, put in place because during periods of poor performance of the assay in routine use at BPL it had been noted that A-rings with failed 100 IU/controls and low IC values were grouped by extraction runs,

and not usually found in single A-rings or COBAS runs. This meant that in extraction runs where some A-rings had failed, samples in other A-rings from the same extraction may have been tested with lower sensitivity despite the controls passing.

All HCV negative sample results required a valid internal control result. The validity criteria imposed on the internal control results were far more strict than those of the manufacturer because it had been found that the performance of the internal control was linked to the sensitivity of the assay (Figure 6.4). In a study of the performance of the 3.5 IU/ml control sample during routine use at Brentwood, out of 698 analyses, 455 were found negative and 243 were found positive. When the IC values obtained from these analyses were compared it was noted that the IC OD was significantly lower in the negative assays than in the positives (P= <0.0001 by normal distribution Z test) indicating that low IC OD can signify a loss of HCV sensitivity in the assay. Only 17 of the 698 results analysed had IC levels between 0.150 and 1.000 (2.4%) of these two were HCV positive (11.8%) compared to 239 (36.7%) HCV positives in the 652 with IC OD's above 1.000. (P=0.026 by Fishers' exact test). Of the 29 controls that had an IC OD lower than 0.150 (i.e. failed by manufacturer's criteria) two were HCV positive (6.9%).

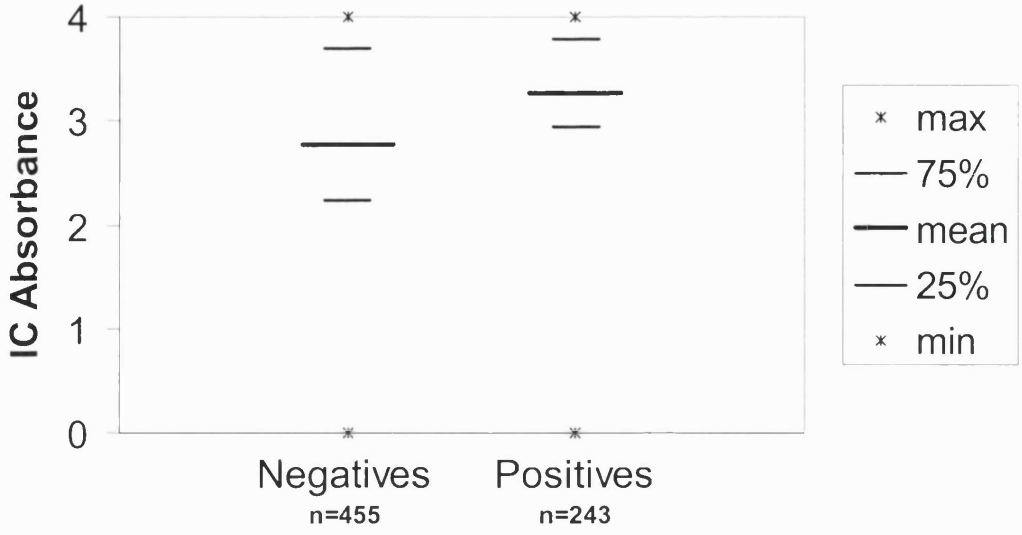


Figure 6.4: Relationship between IC OD and HCV sensitivity

Analysis of IC absorbance in 3.5IU/ml HCV controls

Therefore the validity of the internal control was not based on the manufacturer's criteria, but was calculated on the basis of the other samples as follows: The internal control results in all HCV negative test samples in each A-ring were averaged and if two or more individual internal control results were less than two thirds of the mean then the A-ring was failed. If only one sample had an IC OD of less than two thirds of the mean then this sample was failed and the mean IC OD for the rest of the negative test samples was re-calculated. The mean IC OD_{660} for the A-ring was required to be greater or equal to 3.5, or the A-ring was failed.

All failed samples or the samples in failed A-rings or extraction runs based on the criteria described above were required to be retested from the start, i.e. the whole extraction, amplification and detection were repeated.

The criteria were later changed when the assay was introduced into the Brentwood NAT laboratory so that the internal control was only valid if its OD_{660} was greater or equal to 1.000, as opposed to the manufacturer's cut off of 0.150. The negative control was also omitted as it was felt that it was not necessary due to the vast majority of the test samples being HCV negative.

6.2.1 Development of automated results analysis

Excel spreadsheets were developed to assist in the analysis of the raw data from the NBS NAT assays. These performed two functions, the first being to view the OD data graphically from an individual run to aid in the analysis of the assay performance. The second function was to monitor the performance of the positive and internal controls over time to detect any changes in the performance of the assay, i.e. changes in sensitivity over time.

An example of the raw data text file output from a COBAS Amplicor analyser is shown in Figure 6.5. This COBAS text file shows the OD result of each test (HCX = AmpliScreen HCV, HXC = Internal control) with sample and A-ring ID

and details of time and date. The layout of the text file is quite complicated and it is difficult to pick out the relevant data easily. For this reason Microsoft Excel macros were written to import the text file and sort the data contained into HCV and IC OD for the standards and samples (Appendix B; macro 2). These data were then represented in a graphical form to aid the analysis of the run. Example HCV and IC plots are shown for a successful assay run in Figure 6.6 and for a failed assay run in Figure 6.7.

The difference in performance between the runs shown in Figure 6.6 and Figure 6.7 can be easily judged at a glance with the data represented graphically rather finding the OD data in the raw data files for each A-ring.

The example of a valid run in Figure 6.6 shows the HCV and IC data for a BioRobot extraction run with five A-rings i.e. 60 test results. The 100 IU go or no-go controls are all at or near maximum OD. The 50 IU monitoring control has amplified with medium OD. Four out of five 3.5 IU monitoring controls have amplified; at this low HCV level less than 100% amplification and low ODs are expected. A minipool has also tested positive in this run. The IC ODs are all at a high level apart from one sample. The low IC ODs on this minipool sample, which was not the HCV positive minipool, meant that this minipool must be repeated.

The example of a failed run in Figure 6.7 also shows the HCV and IC data for a BioRobot extraction run with five A-rings. Four out of five 100 IU go or no-go controls have failed to amplify, which would mean the run had failed the acceptance criteria. Most of the internal controls failed to amplify and those that did had mostly medium to low OD levels.

Sample Id	Test	Result	Unit	Flags	DateTime	A-ring ID	A-tube Pos	LoadDateTime	Instrument ID	Accepted Op	AcceptedDateTime	Comment	Sample Value	Blank Value
P10099/738	HCX	POS	-	___	16/01/01 10:25:33	976487	1	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		3.865	0.049
P5099/738	HCX	POS	-	___	16/01/01 10:26:45	976487	2	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		2.543	0.049
P3.599/738	HCX	NEG	-	___	16/01/01 10:27:57	976487	3	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		0.005	0.049
P010130900	HCX	NEG	-	___	16/01/01 10:29:09	976487	4	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		0.005	0.049
P010131000	HCX	NEG	-	___	16/01/01 10:30:21	976487	5	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		0.004	0.049
P010131100	HCX	NEG	-	___	16/01/01 10:31:33	976487	6	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		0.005	0.049
P010131200	HCX	NEG	-	___	16/01/01 10:32:45	976487	7	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		0.004	0.049
P010131300	HCX	NEG	-	___	16/01/01 10:33:57	976487	8	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		0.004	0.049
P010131400	HCX	NEG	-	___	16/01/01 10:35:09	976487	9	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		0.005	0.049
P010131500	HCX	NEG	-	___	16/01/01 10:36:21	976487	10	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		0.005	0.049
P010131600	HCX	NEG	-	___	16/01/01 10:37:33	976487	11	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		0.005	0.049
S010150100	HCX	NEG	-	___	16/01/01 10:38:43	976487	12	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		0.004	0.049
P10099/738	HXC	POS	-	___	16/01/01 10:54:16	976487	1	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		3.865	0.049
P5099/738	HXC	POS	-	___	16/01/01 10:55:28	976487	2	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		*.***	0.049
P3.599/738	HXC	POS	-	___	16/01/01 10:56:40	976487	3	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		3.865	0.049
P010130900	HXC	POS	-	___	16/01/01 10:57:52	976487	4	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		3.865	0.049
P010131000	HXC	POS	-	___	16/01/01 10:59:04	976487	5	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		*.***	0.049
P010131100	HXC	POS	-	___	16/01/01 11:00:16	976487	6	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		2.99	0.049
P010131200	HXC	POS	-	___	16/01/01 11:01:28	976487	7	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		3.865	0.049
P010131300	HXC	POS	-	___	16/01/01 11:02:39	976487	8	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		2.887	0.049
P010131400	HXC	POS	-	___	16/01/01 11:03:52	976487	9	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		3.865	0.049
P010131500	HXC	POS	-	___	16/01/01 11:05:04	976487	10	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		2.522	0.049
P010131600	HXC	POS	-	___	16/01/01 11:06:15	976487	11	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		3.865	0.049
S010150100	HXC	POS	-	___	16/01/01 11:07:28	976487	12	16/01/01 07:27:06	36-2831	2 sd	16/01/01 11:48:12		3.865	0.049

Figure 6.5: COBAS analyser raw data text file

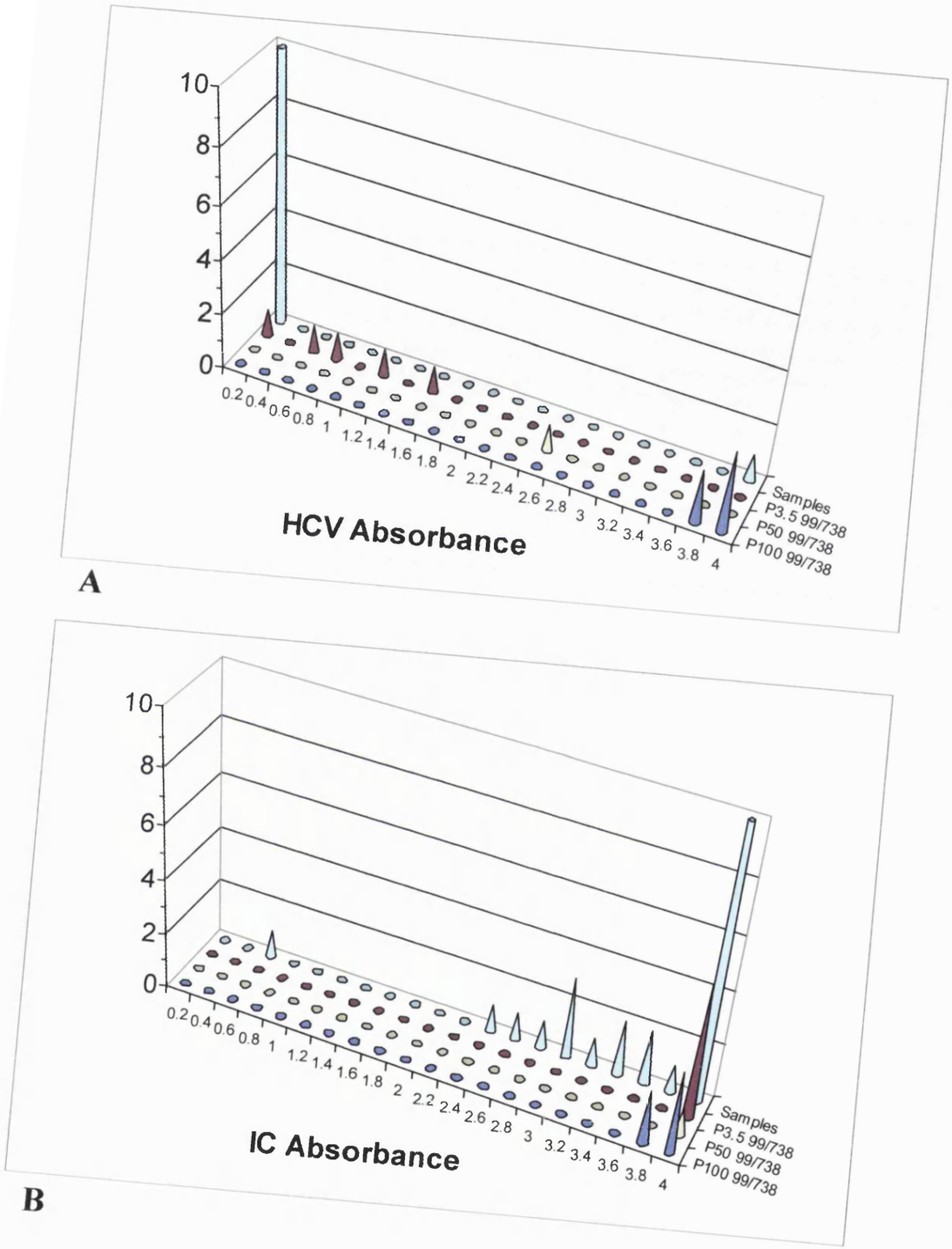
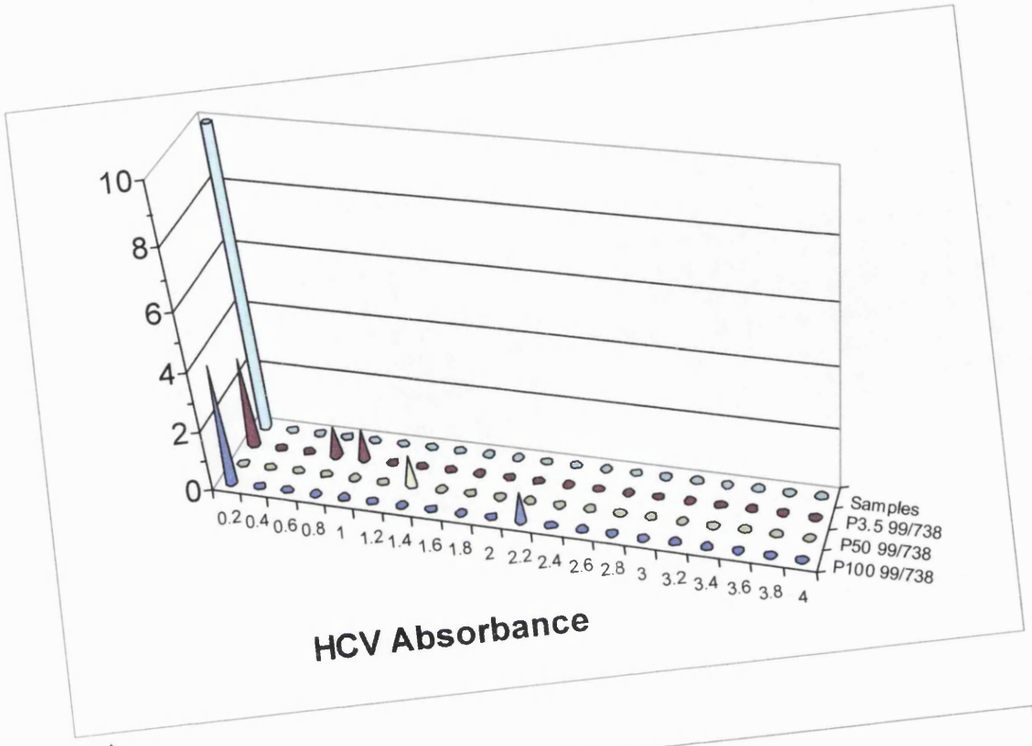


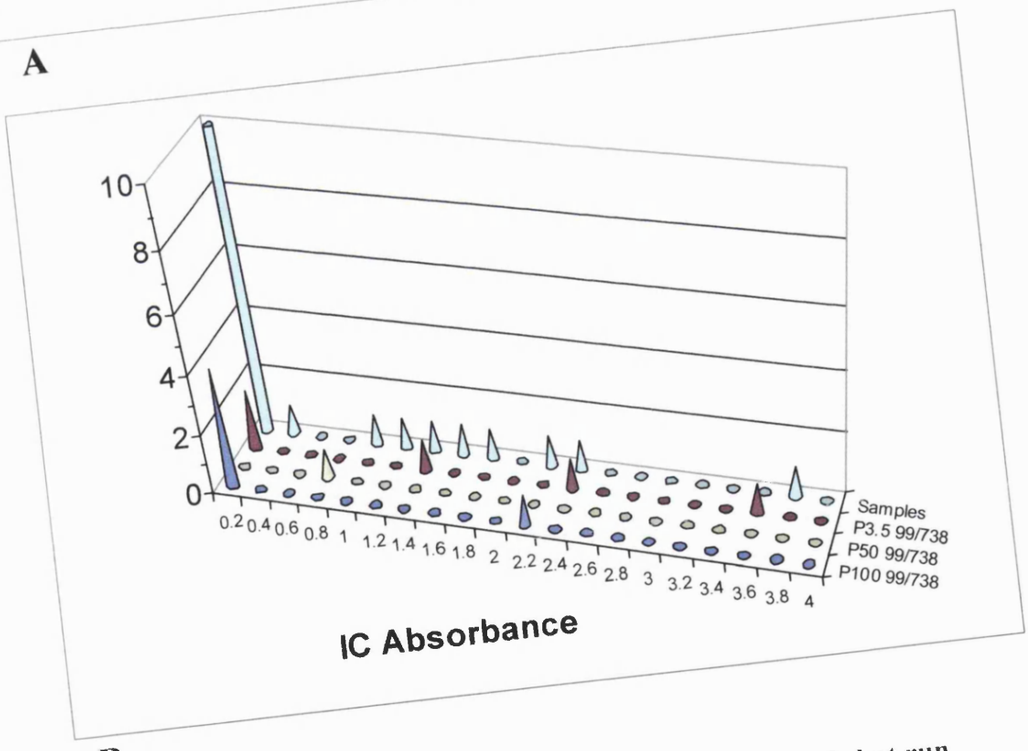
Figure 6.6: A plot of the COBAS OD results for a valid BioRobot run

A: HCV data: All P100 IU controls have amplified with high ODs. Four out of five P3.5 IU monitoring controls have amplified. One positive minipool has been found.

B: IC data: The IC has amplified with high ODs in most cases; one sample has low IC OD requiring a retest.



A



B

Figure 6.7: A plot of the COBAS OD results for a failed BioRobot run

A: HCV data: Four out of five P100 controls have failed to amplify failing the extraction.

B: IC data: the IC has failed to amplify in many cases.

In this run the entire extraction amplification and detection must be repeated.

Trend analysis

As well as monitoring the results of each individual assay run, the results were also monitored over time to identify any trends in the performance of the controls. It was important to monitor the performance of the assay to identify deterioration in sensitivity due to batch changes of critical reagents or declining efficiency of the equipment used for extraction or amplification and detection.

Examples are shown for the period before and after the change of assay from the QIAamp 96 viral RNA and Amplicor HCV v2.0 method to the QIAamp 96 Virus and AmpliScreen HCV v2.0 method at the Brentwood laboratory. The change in mean internal control OD is shown in Figure 6.8 and similarly the change in the mean OD of the 100 IU/ml go or no go positive control is shown in Figure 6.9. These plots show a noticeable improvement in the ODs for the 100 IU/ml controls and the internal control at the time of the change to the Virus and AmpliScreen assay, which resulted in considerably less samples requiring repeat testing (Section 6.3).

The trend analysis plots were generated using macros in Microsoft Excel (Appendix B; Macro 3) to analyse the data in multiple COBAS text files (as shown in Figure 6.5) and to convert this data into graphical plots.

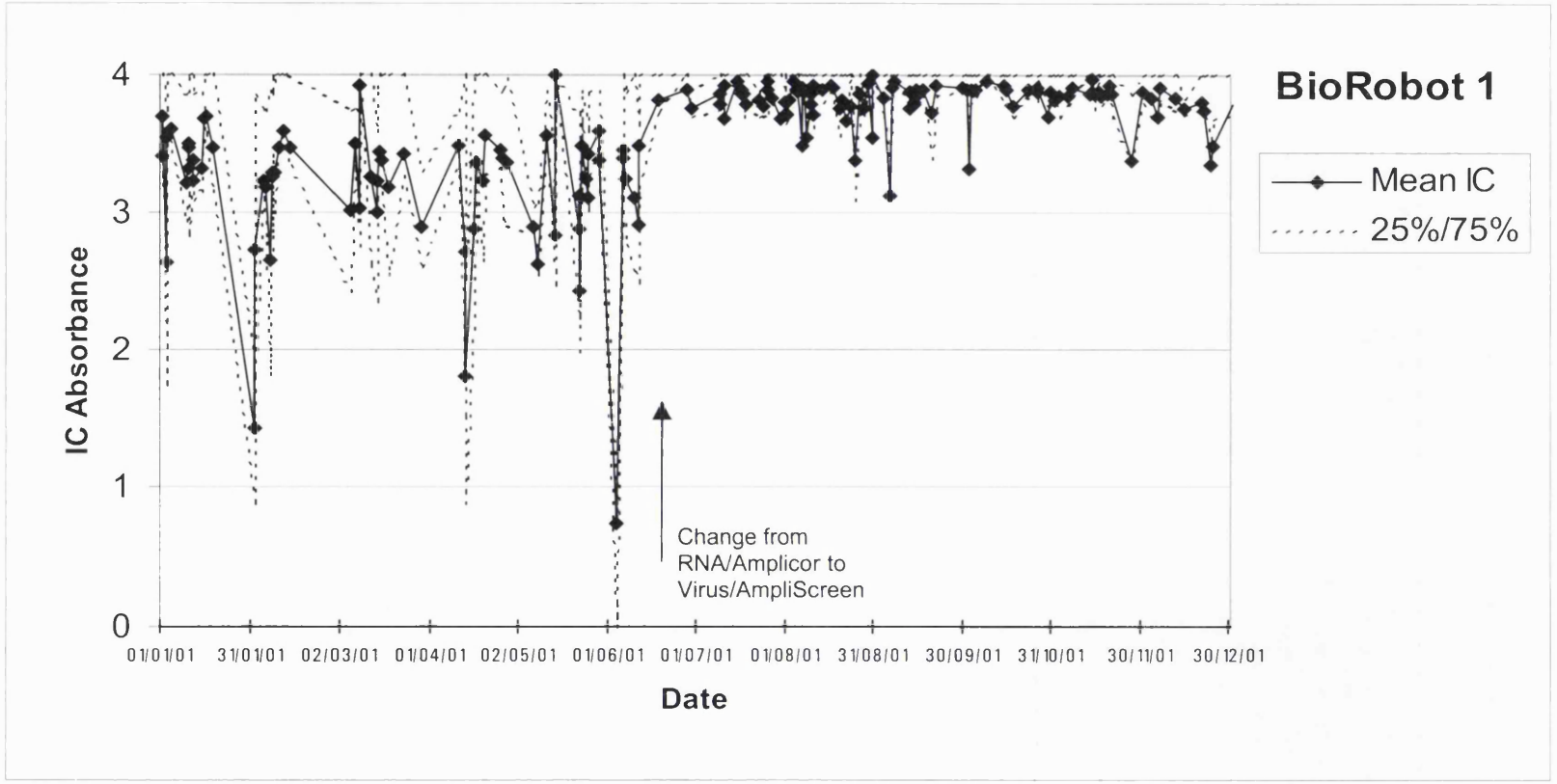


Figure 6.8: Change in mean internal control OD before and after the change of assay from RNA/Amplicor to Virus/AmpliScreen at the Brentwood NAT laboratory

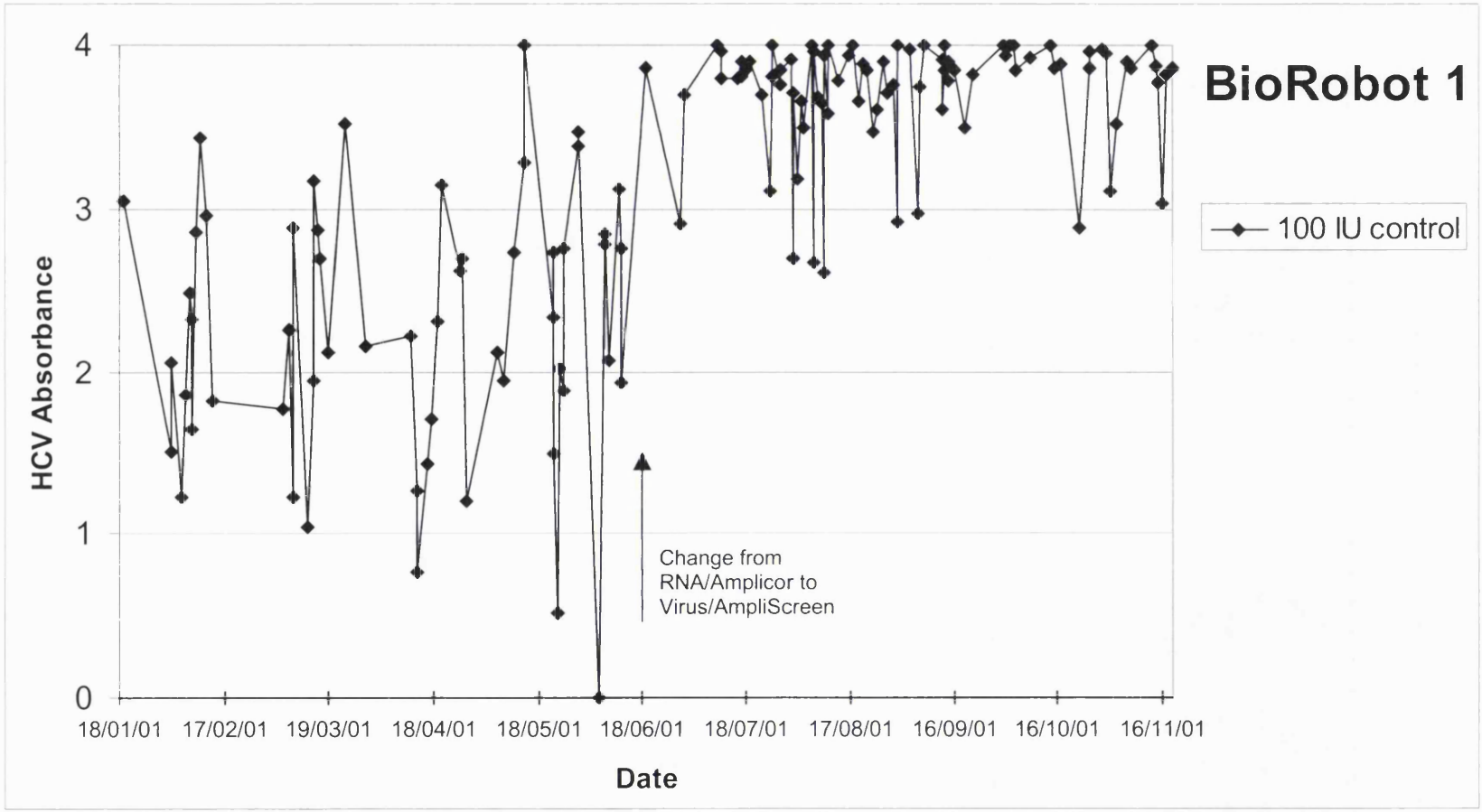


Figure 6.9: Change in mean 100 IU/ml control OD before and after the change of assay from RNA/Amplicor to Virus/AmpliScreen at the Brentwood NAT laboratory

6.3 Assay performance in routine use

When the assays were introduced into routine use the results showed that they were not as robust and sensitive as they had been during the validation. The reasons for this were never fully elucidated. At least one period of poor performance at BPL was due to ethanol contamination of the BioRobot eluate (Dr. C. Sims, BPL; personal communication).

The residual ethanol present in the eluate was caused by a subcontractor manufacturer changing the composition of the glue on the porous tape used to cover the QIAamp plate during the drying spin. The change in glue caused clogging of the pores of the tape so that the ethanol could not evaporate efficiently during the drying spin.

When the eluates from failed tests were tested at BPL, ethanol levels of up to 10% were found. This level of ethanol was certainly enough to inhibit the PCR reaction (R. Sun, Roche Molecular Systems Inc.; personal communication). Further studies were performed to determine the optimal drying spin times during extraction. A QIAamp 96 viral RNA extraction was performed as described in section 2.1.2 up to the drying spin which was modified as follows: The plate was spun for 5 minutes and samples 1 to 24 were eluted as normal. The spin was repeated for 5 minutes and the next 24 were eluted. This was repeated twice so that the final 24 samples had a total of 20 minutes drying centrifugation. The amount of ethanol in the eluates from the plate was analysed at BPL with the results shown in Figure 6.10. Ethanol levels above 1% were detected in eluates from the central wells of the plate in all but the final 24 samples which had been centrifuged for 20 minutes.

In view of this data Qiagen changed the protocol to omit the use of the porous tape during the drying spin, which remained at 10 minutes.

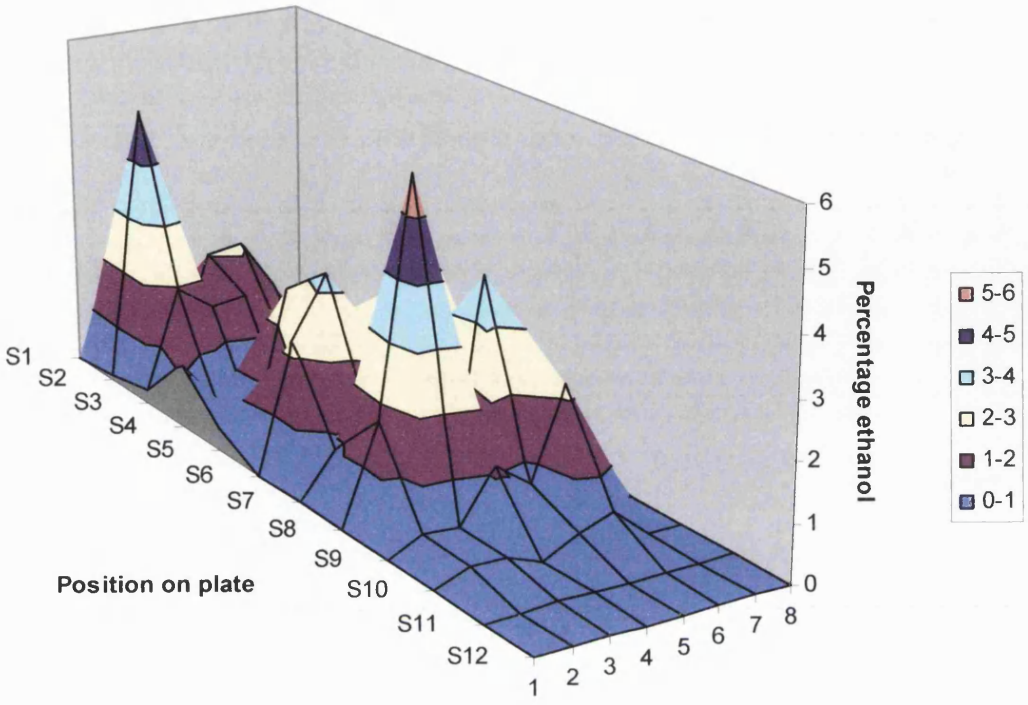


Figure 6.10: Effect of drying time on ethanol contamination

The amount of ethanol contaminating the BioRobot eluate is shown for each position of a 96-well plate. S1-3 had 5 minutes drying. S4-6 had 10 minutes drying. S7-9 had 15 minutes drying and S10-12 had 20 minutes drying. (Data from Dr. C. Sims BPL.)

The new QIAamp 96 Virus protocol and the COBAS AmpliScreen assay combination produced much better results when introduced at the Brentwood NAT laboratory. The detection rate of the 100 IU/ml control at Brentwood averaged 96.2% for the six months before introduction of the new assay combination, in the six months after this introduction the average detection rose to 97.2%, and detection of the 3.5 IU/ml control had risen from 34.1% to 41.9%. Similarly the internal control detection rate had risen from 97.8% to 99.2%. These increases in performance represent a reduction in the number of repeat assays from 9.6% to 5.2%. These changes in performance can clearly be seen in the mean internal control and 100 IU/ml OD changes in Figure 6.8 and Figure 6.9.

6.4 Results of NAT testing in the NBS

To date (Dec 2002) some nine million blood donations have been screened in England and Wales by HCV NAT using the methods described above. Out of 401 confirmed NAT positive pools seven confirmed HCV RNA positive, anti-HCV negative donations have been detected (Table 6.1). The window phase donations were subjected to several tests as follows: The quantification was performed as described in section 2.2. The genotyping was performed as described in section 2.3. The antigen testing was performed as described in sections 2.1.7 and 2.1.8. The anti-HCV data and donor details were provided by Dr. Pat Hewitt of the NBS donor and recipient outcome group (DROG).

Components were transfused from the first window case, as at the time NAT was only used to release frozen components with a shelf life of >35 days, however the recipient was thallasaemic and already HCV infected from previous multiple transfusions. No components from the other window cases were transfused. Five of the seven window phase donors have since sero converted. The other two donors had not sero converted when re-sampled, however one donor was retested after only nine days and the other after 27 days.

In addition 575 sero positive HCV samples have been identified. Of these 534 have been tested by NAT, 406 (76%) were NAT positive and the remaining 128 were NAT negative (Data from NBS/PHLS-CDSC monthly donation testing report). All sero-positive samples in NAT negative minipools are tested as individual donations by NAT at the NAT reference laboratory at UCL. Of the 128 sero-positive donations in NAT negative minipools five were detected by individual donation NAT only i.e. they were not detected in 96 donation pools.

A selection of 86 randomly selected sero-positive samples were tested by the in-house HCV quantitative PCR as described in section 2.2. The mean value of HCV RNA in these positive minipools was 17 600 IU/ml (range 44 to 140 000 IU/ml) but as these were 96 donation minipools the mean value of the positive donations equates to 1 690 000 IU/ml.

Table 6.1: HCV window phase samples identified during NAT screening.

No.	Centre	Sex	Age	Geno-Type	Donation Date	QPCR result	Antigen result	Trak C result	Anti-HCV result	Comments
1	Cardiff	M	21.5	3	04/08/99	16 000 000 copies/ml	Positive	Positive	Negative	
					N/A	N/A	N/A	N/A	Positive	Seroconverted
2	B'ham	M	29.7	3	04/02/00	350 000 copies/ml	Negative	Negative	Negative	Had been in prison with possible IVDU history
					24/02/00	527 000 copies/ml	Negative	N/A	Negative	
					04/06/00	N/A	N/A	N/A	Positive	Seroconverted
3	B'ham	F	27.2	1	01/09/00	1 200 000 IU/ml	N/A	Positive	Negative	Partner has Christmas disease
					N/A	N/A	N/A	N/A	Positive	Seroconverted
4	Cardiff	M	40.4	3	23/10/01	2000 IU/ml	N/A	Negative	Negative	Donor had new partner whose previous partner had IVDU history
					30/10/01	37 500 IU/ml	N/A	Positive	Negative	Not yet seroconverted
5	Leeds	F	21.1	3	07/02/02	60 700 IU/ml	N/A	Positive	Negative	Donor had new partner who had been in prison
					N/A	N/A	N/A	N/A	Indeterm	Seroconverted
6	Bristol	M	28.7	1	10/05/02	5300 IU/ml	N/A	Positive	Negative	Bitten by drug crazed man with jaundice
					06/06/02	N/A	N/A	N/A	Negative	Not yet seroconverted
7	B'wood	F	35.6	1	05/07/02	33 400 IU/ml	N/A	Positive	Negative	Partner of a soldier from Kosovo
					20/07/02	N/A	N/A	N/A	Indeterm	Seroconverted

Donor and Anti-HCV Data from Dr. Pat Hewitt (DROG)

6.5 Discussion

Since the assays described in Chapter 4 have been introduced into the NBS NAT laboratories, their performance has been carefully monitored both at the outset and once established. The protocols used have combined two different assay systems and therefore the manufacturers' criteria for acceptability may no longer be valid. For this reason the use of controls and their interpretation has been strictly defined.

When considering controls for an assay run, the entire assay is usually considered to mean the extraction, amplification and detection. In the case of the NBS NAT assay the BioRobot extraction may have up to 96 samples and controls but each COBAS instrument can only amplify and detect 24 samples and controls per run. The extract from a single BioRobot run may require up to four COBAS runs for amplification and detection. As each NAT laboratory has three COBAS instruments, the RNA from a single BioRobot run is unlikely to be amplified and detected by the same COBAS instrument. Obviously this meant that controls were needed for each COBAS run. However, the 24 COBAS tests are amplified in two separate thermal cycler units, each with a capacity of 12 using an A-ring consisting of 12 PCR tubes with integral caps. Therefore when considering the smallest test unit that needs controls the 12 test A-ring was used. In this way if a situation occurred where one thermal cycler was not performing as efficiently as the other the sensitivity controls would reflect this.

Each A-ring had to be separately validated and therefore consisted routinely of a 'go or no go' positive control (71 or 100 IU/ml), a monitoring control (3.5 IU/ml) and a negative control, plus nine minipool samples. The negative control was later discarded as the majority of the minipool samples were negative, leaving two controls for 10 minipool samples. In addition a 50 IU/ml monitoring control was run once per BioRobot extraction. The position of the positive controls in the extraction was varied so that any difference in efficiency from well to well could be detected.

The performance of the positive and internal controls was carefully monitored on a run by run basis as well as collated over time for trend analysis. The samples contained in an A-ring were passed or failed on the basis of the 'go or no go' control and the internal controls of that A-ring. The samples contained in an extraction run were passed or failed based on the validation of the individual A-rings. Any failed samples (e.g. IC failures) were repeated from the extraction stage.

The ODs of the internal controls were carefully monitored and acceptance criteria which were far more strict than the manufacturer's criteria were applied. This was because it had been noted that the performance of the internal control correlated with the performance of the HCV positive controls. Thus, when the internal control OD's were lower and showed a greater spread in value, the HCV detection was more likely to fail. This was later borne out by statistical analysis of the results as described in section 6.2.

Over time, problems with equipment were overcome and gaining familiarity with the assay meant that the performance gradually improved and the number of samples or runs requiring repeat testing dropped. The introduction of the QIAamp Virus and COBAS AmpliScreen assay combination also greatly improved the performance (Figure 6.8 and Figure 6.9).

Seven window phase donations have been detected by NAT screening so far. The HCV RNA levels found in these samples has varied from 2.0×10^3 to 3.2×10^6 IU/ml. The average HCV level found in the initial positive donations was 6.5×10^5, this is a similar level to the 3.5×10^5 IU/ml found for the US donations in section 5.2. The NBS window donations have been of the expected genotypes for the UK i.e. mostly types 1 and 3.

The rate of window phase positives has been less than expected before the testing began, the current rate is 1 window phase per 1.3 million donations (7 cases in 9 million), this compares to the rate of 1 per 225 000 donations that had been estimated for the UK before NAT testing was introduced (Soldan and Barbara, 1999).

However, the rates of HCV window phase donations in most other European countries are similar to the NBS current rate. The SNBTS has found one window phase donation in approximately one million donations screened (Brian Dow; personal communication). In the Red Cross blood centres in Germany the window phase donation rate is 1 in 1.2 million (11 cases in 12.7 million); (Seifried and Roth, 2001). In the Netherlands no window phase donations had been found after screening 1.5 million donations (Cuijpers *et al.*, 2001).

Other countries have reported higher rates. In the Finland a rate of 1 in 170 000 (three cases in 510 000 donations) has been found: S. Wessberg & T. Krusius in: (Engelfriet and Reesink, 2002). In Japan, using an automated extraction system developed by Roche coupled with the ABI 7700 (Applied Biosystems; Foster City, CA, USA) the rate has been reported as 1 in 480 000 (15 in 7.2 million) (Japanese Red Cross NAT screening research group, 2001). In North America a rate of 1 in 263 000 (62 cases in 16.3 million) has been found (Stramer *et al.*, 2000).

These differences in the number of window phase samples detected by NAT screening may in part be due to differences in the serological assays in use and also reflect the incidence of the infection in the donor populations. The incidence is determined by the general prevalence of the infection in the population, and the selection of the donors. Blood donors need careful selection to avoid certain risk groups thereby reducing the risk of window phase donations occurring. For example if the NBS had refused donations on the basis of having been in prison or having a partner who had been in prison then at least two of the NBS windows may have been avoided. However when defining risk groups from which donations will be refused, consideration must be given to the number of donations that will be lost. When the supply of donated blood is already very low and only just meets the demands of the service, imposition of further restrictions on donors should not be introduced unless there is good reason.

Chapter 7

General Discussion

The protocols developed in this thesis have been successfully introduced into routine use within the National Blood Service in England and Wales from 1999. The work on sample stability and handling described in Chapter 3 has defined the procedures used in the NBS from the type of blood collection tube used to take the NAT sample, and the handling of that sample through transport pooling and up until the extraction of the nucleic acid.

The development of the assay systems is described in Chapter 4. Initially the BioRobot viral RNA and COBAS Amplicor v2.0 combination was introduced into the NBS. The increased sensitivity and robustness gained with the new BioRobot Virus and COBAS AmpliScreen v2.0 combination also described in Chapter 4 was used as the justification for the change to this new assay combination within the NBS. The work with HIV-1 and HBV showed that the BioRobot protocols could also be used for the detection of these viruses if the need ever arose.

Alternative systems were investigated and discussed in Chapter 5. The main alternative to the AmpliScreen system is the TMA assay developed by Gen-Probe and marketed by Chiron, this assay is a multiplex for detection of both HCV and HIV-1 RNA. The Bayer assay described in Chapter 5 is the HCV discriminatory assay used in the multiplex system. The performance of the Bayer HCV TMA assay was investigated in preference to the Chiron HCV/HIV-1 multiplex TMA assay so that HIV-1 NAT testing would not necessarily need to be introduced. To have some donations in the country screened for HIV-1 but not others would have been an unacceptable situation. Furthermore, the NBS was considering having both the TMA and BioRobot/COBAS systems in use at the same time as security against a problem occurring with a single assay system. The TMA assay was not introduced into routine use within the NBS due to the lack of positive sample ID and problems with robustness as well as for financial

and licensing reasons. The HCV core antigen detection assays were also investigated and have been described in Chapter 5 as a potentially less costly alternative to NAT screening, however these assays proved to have inadequate sensitivity, compared to NAT testing.

The phased introduction of HCV NAT screening into routine use began with phase I, a single laboratory at BPL testing all donations from England and Wales for HCV RNA to release the long shelf life blood components i.e. fresh frozen plasma. The NAT testing was then moved to three NBS laboratories to enable the introduction of phase II, which included the release of short shelf life components including platelets. The use of these three regional laboratories around the country enabled a reduction of the transport time from donation centre to the combined pooling and testing centre, thereby reducing the total time from donation to release of result. Shift working with 24 hour cover was also required to reduce the overall time from donation to result, with the aim of release of results by 1pm on day 1 (where samples are taken on day 0). Reduction of testing time was important for the shortest shelf life components where the NAT testing time could use up a significant amount of the useful shelf life.

When NAT testing for the release of short shelf life components was introduced the pool size was reduced from 96 to 48 samples, so that fewer donations were held while a positive minipool was resolved. This also had the advantage of doubling the effective sensitivity on a per donation basis. The reduction in pool size ensured that the continuity of supply of components was maintained and the likelihood of detecting reactive units was increased. The disadvantage was that the cost of testing was increased.

Having three laboratories also has the advantage of operational flexibility, e.g. disaster recovery, so if one NAT laboratory was rendered non-functional the others could take on the extra work. This scenario became more realistic when the roof of the Birmingham NAT laboratory collapsed during the validation phase before NAT testing became live, setting the work back by several months.

7.1 Other test systems

The HCV NAT blood screening method developed and described in this thesis compares well to the methods developed in other countries around the world. Several other European countries have developed screening assays based on combinations of methods or in-house assembled assays. The advantage of these modified or in-house NAT assays is that users can define their own assay characteristics; however, the disadvantage is that the blood services introducing these methods effectively become assay manufacturers themselves, with the consequence of being responsible for the full validation and support of these tests.

One of the first such assays to be described was at a blood bank in Germany (Roth *et al.*, 1999). In this method nucleic acid was extracted from pools of 96 donations using the Qiagen spin column method. The extracted nucleic acid was tested for HCV RNA using the Roche COBAS Amplicor HCV v1.0 assay described above. HBV and HIV-1 was amplified using in house developed PCR. The same group has recently described a TaqMan assay for detection of HIV-1 (Drosten *et al.*, 2001; Roth *et al.*, 2002a; Roth *et al.*, 2002b).

The Roche Amplicor HCV v2.0 is commonly used in modified NAT assays. The amplification and detection steps of this assay can be automated using the COBAS analyser (DiDomenico *et al.*, 1996), and the manual extraction procedure can be replaced by automated nucleic acid extraction procedures from other manufacturers. One automated nucleic acid extraction system used in several locations is the NucliSens Extractor (van Buul *et al.*, 1998) which allows the automation of the Boom nucleic extraction method of the NASBA assay (Boom *et al.*, 1990). The NucliSens Extractor will extract the DNA or RNA from up to 2 ml sample volume in cartridges, and can process ten cartridges at a time, taking approximately 45 minutes per run. The manual NucliSens procedure was used at CLB as a substitute for the manual extraction method of the Roche HCV Amplicor v1.0 for screening of plasma pools for fractionation. Using these

methods gave a 95% end point of 47-80 copies/ml which represented a 28 to 88 fold improvement over the Amplicor v1.0 manual method (Lelie *et al.*, 1998).

The NucliSens Extractor method is now used in combination with the COBAS HCV Amplicor v2.0 assay to screen donated blood in pools of 48 donations for HCV in four laboratories in the Netherlands, allowing the release of all labile blood components on the basis of the NAT result (Beld *et al.*, 2000; Cuijpers *et al.*, 2001). The reported 95% sensitivity of this method is 8 IU/ml. The assay used in the Netherlands has recently been updated to replace the HCV Amplicor v2.0 with HCV AmpliScreen v2.0 assay, and the HIV-1 AmpliScreen v1.5 assay is now also used in combination with the NucliSens Extractor with a sensitivity of around 30 copies/ml (Jongerius *et al.*, 2002). The NucliSens Extractor and COBAS HCV Amplicor v2.0 assay combination has also been used to screen blood for HCV RNA in a blood bank in Germany where a 95% sensitivity was reported as 30 IU/ml (Cardoso *et al.*, 1998a; Cardoso *et al.*, 1999a; Cardoso *et al.*, 2000).

In the Scottish National Blood Transfusion Service the NucliSens Extractor was initially used in combination with an in house PCR for screening blood donations for HCV (Jarvis *et al.*, 2000) with a 95 % sensitivity of 7.3 IU/ml. A nested RT-PCR for HIV-1 has been described by the same group with an HIV-1 sensitivity of 24 IU/ml (Cleland *et al.*, 2001). Both methods now use real time fluorometric detection of the second round products using a LightCycler (Roche Diagnostics, Basel Switzerland); (Jarvis and Simmonds, 2002).

The NucliSens Extractor has been used in combination with TaqMan RT-PCR on the Prism 7700 (Applied Biosytems, Foster City, CA, USA) for detection of HCV RNA in donated blood in a German blood bank (Hennig *et al.*, 2001). This method uses multiple TaqMan probes specific for different genotypes of HCV and has a reported high specificity for HCV genotypes 1 to 5 and a sensitivity of 20 IU/ml. TaqMan PCR is also used by the Japanese Red Cross, who use an automated extraction system (GT-X) built by Roche in Japan. A Prism 7700 is then used to detect HBV DNA, HCV RNA and HIV-1 RNA, and an internal control making the system totally automated. The combination of the GT-X

sample preparation station and the PRISM 7700 amplification and detection system is termed AMPLINAT MPX assay system and is able to generate results for 96 samples and controls in around four hours. The reported sensitivity of this system is 30 copies/ml for HBV, 77 IU/ml for HCV and 42 copies/ml for HIV-1 (Meng *et al.*, 2001).

In the USA, screening is restricted to assays licensed by the US Food and Drug Administration (FDA). The first to be licensed was an in-house sample pooling and PCR system developed by the National Genetics Institute (NGI; Los Angeles CA, US). The NGI assay is approved exclusively for screening 512 member pools of source or recovered plasma intended for fractionation. As all plasma for fractionation in England is sourced in the US it is also NAT tested by the NGI assay.

The second licensed NAT assay procedure is the GenProbe/Chiron TMA system, which is licensed for screening pools of up to 16 samples from donors of whole blood and blood components intended for use in transfusion (FDA, 2002). The FDA stated that the use by blood banks of this test for HIV-1 will allow the discontinuation of the controversial HIV-1 p24 antigen test.

The Gen-Probe/Chiron TMA and Roche AmpliScreen assay systems (the later is under FDA Investigational New Drug [IND] exemption with a license application pending at the time of writing) described above are widely used in the U.S. for HCV and HIV-1 RNA screening. The Chiron system is also used exclusively in Australia, while the Roche AmpliScreen assay is used exclusively in Canada (Stramer *et al.*, 2000). Both assays test pooled donation samples. The TMA assay uses pools of 16 donations (24 donations in Australia) made by a Tecan Genesis robotic sample processor (Tecan Group Ltd, Mannedorf, Switzerland). The Roche AmpliScreen assay is used to screen pools of 24 samples made by an automated liquid handling workstation (Hamilton Company, Reno, NV, USA).

The various NAT assays in use around the world have similar sensitivities with differences being generally due to the amount of plasma processed, these assays

are capable of achieving the sensitivity requirements of the regulatory authorities (Lelie *et al.*, 2002). Fully automated commercial NAT systems are under development, and it will only be a matter of time before blood donation NAT screening occurs in 'black box' type technology. As NAT technology evolves it is likely that there will be a transition from pooled to individual donation NAT, as well as incorporation of detection capacity for additional viruses and other pathogens including parasites and bacteria (Busch and Dodd, 2000). Perhaps most important, the existence of NAT technology in routine blood bank screening should allow for rapid introduction of screening for major new pathogens that may emerge in the future.

7.2 Final Conclusions

In the last few years NAT blood screening systems for the detection of blood borne viruses have been successfully implemented in blood banks and blood services around the world (Engelfriet and Reesink, 2002). Many developed countries now release blood components for transfusion on the basis of a valid negative NAT test for HCV RNA, and in some countries other viruses as well (HIV-1, HBV, Parvo-B19, HAV). Current NAT test systems generally screen pooled donations, with pool sizes ranging from 16 to 96 donations. At present the in-house or modified NAT systems are widely used as they allow greater sensitivity, flexibility and throughput, often at lower costs than the commercial systems. By combining automated extraction systems, such as the NucliSens Extractor or the BioRobot, with automated amplification and detection systems, such as the COBAS Amplicor, AmpliScreen or TaqMan assays, fully automated NAT systems have been developed and validated by the blood services of several countries.

The safety of blood has undoubtedly been enhanced by the introduction of NAT testing, although perhaps not as much as had been thought before it was introduced. The yield of HCV window phase samples in the UK has been less than estimated, probably due to inaccuracies in the methods used in these estimations. The rate of window phase samples found during the course of NAT testing has varied throughout the world, and is dependent upon the prevalence of the disease in the donor populations and front line screening assays.

Even with NAT testing there is still a risk of HCV infected blood being transfused, and cases have been reported (Schuttler *et al.*, 2000). There is still a period early in the infection when the viral levels are below that which can be detected by NAT tests but nevertheless the blood may be infectious. There is still debate as to whether to strive for even more sensitivity in the tests used to catch infections earlier. Calculations have been made indicating the benefit of single donation NAT testing versus minipool testing and how many days earlier an infection may be detected based upon viral replication rates. However, it was

shown that even after virus concentration and large scale plasma extraction giving a theoretical 95% sensitivity of between 4 and 12 genomes per ml, HCV still could not be detected in a donation that had caused HCV transmission to a recipient (Schuttler *et al.*, 2000). So with the current NAT technology the risk of HCV transmission still cannot be completely removed.

Although NAT testing is more costly than serological testing, the cost per donation tested by NAT may be reduced by pooling. However this may be changed as Chiron imposes a royalty charge for use of its patent on HCV. This charge will not be levied per test as the Roche charge for the PCR patent but per donation tested (Simmonds *et al.*, 2002). For example if the charge was to be $10 per donation the NBS would be expected to pay $480 per minipool at the current pool size. These charges may lead some countries to stop NAT testing and would certainly influence the decision of others to implement NAT testing, thereby leading to a reduction in blood safety.

NAT testing might not have been implemented in the first place if the HCV core antigen assay had been available at the time. It has been argued by some that NAT testing should be abandoned in favour of antigen testing which would be expected to be charged at a similar level to other serological assays.

It may be argued that as long as all reasonable steps have been taken to ensure safety any residual risk must be accepted, particularly as the transfusion will only be given in the case of serious and sometimes life threatening illness. However this issue was further complicated in a legal ruling by Mr. Justice Burton in a case brought by those that had been infected with HCV by blood transfusions or products (A and Others v. National Blood Authority and Another, 3 All E.R. 289, 2001). In this case 114 claimants successfully sued the National Blood Authority under the Consumer Protection Act legislation of 1987 establishing that blood was a product and that HCV infected blood was a defective product and those injured by it were liable to compensation. The claimants had transfusions after the 1st of March 1988 when the Act came into effect, and although the defendants claimed that they could not detect the defect at the time, the legislation imposes liability irrespective of fault. It was ruled that since the presence of an as yet

unidentified unknown virus as the cause of NANB hepatitis was known, the NBA should have implemented screening earlier then it did, once it had become available. The compensation and legal costs are likely to run into millions of pounds. The Consumer Protection Act is based on the European Council Directive 85/374 (EEC) and therefore this successful case may lead other plaintiffs in the European Union to seek similar claims.

Although an immense effort has been made to reduce the number of viral infections resulting from transfusions, the SHOT reports have shown that far more infections result from bacterial contamination of blood rather than viral contamination (Williamson *et al.*, 1999). Other transfusion complications have resulted from the incorrect blood being given to the patient through errors made at the bedside. The overall safety of blood transfusion could be improved if these areas were concentrated on as well as viral infections. It could be argued that money spent on NAT testing could be better spent on other aspects of blood transfusion such as systems or procedures to minimise the risk of errors in matching units to patients. The decision by Mr. Justice Burton makes this argument unlikely to be politically acceptable.

Another area of blood safety that is being developed is viral inactivation. This has been used for many years in the plasma fractionation industry (Pamphilon, 2001). Most fractionation processes employ viral inactivation procedures such as solvent detergent that will reduce the viral load by several logs (Horowitz *et al.*, 1993). Viral inactivation strategies are under development that will be applied to individual components for transfusion. Methylene blue and photo-inactivation have been shown to be effective for viral inactivation of units of fresh frozen plasma (Lambrecht *et al.*, 1991) and this method is now used in Europe and the US. Another method under development is psoralen S-59 in combination with ultraviolet A (UV-A) light which is able to inactive viruses and bacteria in platelet concentrates or plasma (Lin *et al.*, 1997; Wollowitz, 2001). Both the agents described above work by binding to the nucleic acid of any infectious agents present, and there are concerns that they may be carcinogenic in humans. These viral inactivation procedures are unlikely to be accepted as a replacement for viral screening, however they can still be used in parallel.

NAT has established itself as a valuable technology in the pursuance of safer blood. The work described in this thesis has made a significant contribution to the NAT screening policy in this country and may have influenced others. It may be possible to reduce the risk even further using this technology but as the risk gets smaller the effort required to reduce it further gets greater and more costly. An analysis of cost versus benefit must be employed, as greater gains in safety may be available elsewhere. It will be interesting to see how the transfusion services meet these challenges and others in the future.

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Appendix A

Buffers

Buffers Used in the ELONA:

Phosphate Buffered Saline (PBS):

1.5×10^{-1} M NaCl

1.5×10^{-3} M KH_2PO_4

6.3×10^{-3} M $Na_2HPO_4.12H_2O$

2.6×10^{-3} M KCl

pH7.2

2x PBS is made up by adding 2 PBS tablets (Oxoid) to 100 ml sterile water (Baxter) and allow to dissolve before mixing thoroughly.

SSC (20X)

3.0 M NaCl

3.0 x 10-1 M sodium citrate

pH 7.0

ELONA diluent:

5% (v/v) casein blocking buffer (Genosys Biotechnologies)

0.05% (v/v) Tween-20

Diluted in PBS

For a full plate, Add 10 mls of 2X PBS to a sterile universal, add 1ml of Casein blocking buffer (Genosys Biotechnologies) add 100 µl Tween 20. Make up to 20 mls with sterile water (Baxter).

Hybridisation buffer:

5% (v/v) casein blocking buffer (Genosys Biotechnologies)

0.05% (v/v) Tween-20

Diluted in 10X SSC (from 20X stock)

Place 5ml of 20X SSC (Sigma) in a sterile Universal and add 500µl of casein blocking buffer. Make up to 10 ml with Baxter water. Add 50µl of Tween 20 and mix thoroughly to dissolve. The Tween 20 will dissolve more easily if added last when making this buffer.

ELONA wash buffer (TTA):

10 mM Tris-HCl (pH 7.5)

0.1% (w/v) sodium azide

0.05% (w/v) Tween-20

Gel electrophoresis:

50X TAE:

1.0 M Tris

6% acetic acid

5.0 x 10^{-2} M EDTA

(from pH 8.0 stock)

Agarose Gel:

1X TAE (from 50X stock)

2.5% (w/v) agarose

0.05% (w/v) ethidium bromide

For 100ml: 2mls 50X TAE 2.5 g agarose, made up to 100mls with deionised water. Boil in a microwave oven until agarose has completely dissolved. Allow to cool to around 50°C then add 5µl of a 10 mg/ml ethidium bromide solution.

Appendix B

Microsoft Excel Macros

Macro 1:

```
Filename = ActiveWorkbook.Name


   fileToOpen = Application _
   .GetOpenFilename("Text Files (*.txt), *.txt")
   If fileToOpen = False Then End
   Workbooks.OpenText Filename:= _
      fileToOpen, Origin:=xlWindows, _
      StartRow:=1, DataType:=xlDelimited, TextQualifier:=xlDoubleQuote, _
      ConsecutiveDelimiter:=False, Tab:=True, Semicolon:=False, Comma:=False _
      , Space:=False, Other:=False, FieldInfo:=Array(1, 1)
   textfile = ActiveWorkbook.Name
   Range("A1:A48").Select
   Selection.Copy
   Windows(Filename).Activate
   Sheets("Data").Select
   Range("A2").Select
   ActiveSheet.Paste
   Windows(textfile).Activate
   Range("A49:A96").Select
   Selection.Copy
   Windows(Filename).Activate
   Sheets("Data").Select
   Range("A50").Select
   ActiveSheet.Paste
   Range("A1").Select
   Cells(1, 1).Value = textfile
   Sheets("Results").Select
   Windows(textfile).Activate
   ActiveWorkbook.Close


   Sheets("Analysis").Select
   Range("A1").Select
   ConfideL = Cells(45, 6).Value
   Range("F47:N66").Select
   Selection.ClearContents
   Workbooks.Open(Filename:= _
      "C:\Program Files\Microsoft Office\Office\Library\Analysis\ATPVBAEN.XLA"). _

      RunAutoMacros Which:=xlAutoOpen
   Windows(Filename).Activate
   Application.Run "ATPVBAEN.XLA!Regress", ActiveSheet.Range("$b$26:$b$29"), ActiveSheet.Range("$a$26:$a$29"), False, False, ConfideL, _
      ActiveSheet.Range("$F$47"), False, False, False, False, , False
   Sheets("Results").Select


Sheets("Analysis").Select
ActiveSheet.ChartObjects("Chart 9").Activate
   ActiveChart.ChartArea.Select
   ActiveChart.ChartTitle.Select
   Selection.Characters.Text = textfile
   ActiveWindow.Visible = False
   Windows(Filename).Activate
   Range("k55").Select
   Sheets("Graph").Select
   ActiveChart.ChartTitle.Select
   Selection.Characters.Text = textfile
   ActiveChart.ChartArea.Select
   Sheets("Results").Select
   Range("B3").Select


fName = Application.GetSaveAsFilename(, "Excel Files (*.xls), *.xls")
   If fName = False Then End
   ActiveWorkbook.SaveAs Filename:=fName

End Sub
```

Macro2:

```
NatMonitor = ActiveWorkbook.Name
fileToOpen = Application _
.GetOpenFilename("Excel Files (*.xls), *.xls")
If fileToOpen = False Then End
Workbooks.OpenText Filename:= _
fileToOpen, Origin:=xlWindows, _
StartRow:=1, DataType:=xlDelimited, TextQualifier:=xlDoubleQuote, _
ConsecutiveDelimiter:=False, Tab:=True, Semicolon:=False, Comma:=False _
,        Space:=False,        Other:=False,

FieldInfo:=Array(1, 1)

Filename = ActiveWorkbook.Name
Sheets(1).Select
Set NewSheet = Sheets.Add(Type:=xlWorksheet)
  NewSheet.Name = "master"
n = 1
For i = 2 To Sheets.Count
Sheets(i).Select
Range("a1:o25").Select
Selection.Copy
Sheets("master").Select
Cells(n, 1).Select
ActiveSheet.Paste
n = n + 25
Next i
Sheets("master").Select
Range("A:B,N:N").Select
Selection.Copy
Windows(NatMonitor).Activate
Sheets("sheet1").Select
Range("a1").Select
ActiveSheet.Paste
Application.DisplayAlerts = False
Workbooks(Filename).Close
Application.DisplayAlerts = True
Cells.Select
' filter controls
Selection.AutoFilter
Selection.AutoFilter Field:=2, Criteria1:="HCX"
Selection.AutoFilter Field:=1, Criteria1:="P10099*"
Range("D:D").Select
ActiveCell.FormulaR1C1 = "P100 99/738"
Range("M1").Select
ActiveCell.FormulaR1C1 = "P50 99/738"
Range("O1").Select
ActiveCell.FormulaR1C1 = "P3.5 99/738"
Range("Q1").Select
ActiveCell.FormulaR1C1 = "Samples"
ActiveSheet.ChartObjects("Chart 2").Activate
ActiveChart.ChartArea.Select
With ActiveChart
.ChartTitle.Characters.Text = Filename
End With
Range("a1").Select
'
' Internal Control DATA
'
'
Sheets("sheet1").Select
Selection.AutoFilter Field:=2, Criteria1:="HXC"
Selection.AutoFilter Field:=1, Criteria1:="P10099*"
Range("D:D").Select
Selection.Copy
Sheets("IC OD").Select
Range("a1").Select
ActiveSheet.Paste
Cells(1, 1).Value = ("P10099/738")
Sheets("sheet1").Select
Selection.AutoFilter Field:=1, Criteria1:="P5099*"
Range("D:D").Select
Application.CutCopyMode = False
Selection.Copy
Sheets("IC OD").Select
Range("b1").Select
ActiveSheet.Paste
Cells(1, 2).Value = ("P5099/738")
Sheets("sheet1").Select
Selection.AutoFilter Field:=1, Criteria1:="P3.5*"
Range("D:D").Select
Application.CutCopyMode = False
Selection.Copy
Sheets("IC OD").Select
Range("c1").Select
```

```
Selection.Copy
Sheets("HCV OD").Select
Range("a1").Select
ActiveSheet.Paste
Cells(1, 1).Value = ("P10099/738")
Sheets("sheet1").Select
Selection.AutoFilter Field:=1,
Criteria1:="P5099*"
Range("D:D").Select
Application.CutCopyMode = False
Selection.Copy
Sheets("HCV OD").Select
Range("b1").Select
ActiveSheet.Paste
Cells(1, 2).Value = ("P5099/738")
Sheets("sheet1").Select
Selection.AutoFilter Field:=1,
Criteria1:="P3.5*"
Range("D:D").Select
Application.CutCopyMode = False
Selection.Copy
Sheets("HCV OD").Select
Range("c1").Select
ActiveSheet.Paste
Cells(1, 3).Value = ("P3.599/738")
' filter samples
Sheets("sheet1").Select
Selection.AutoFilter Field:=1,
Criteria1:="<>P10099*",
Operator:=xlAnd _
, Criteria2:="<>P5099*"
Range("A:A,D:D").Select
Range("D1").Activate
Application.CutCopyMode = False
Selection.Copy
Sheets("sheet4").Select
Range("a1").Select
ActiveSheet.Paste
Range("A:B").Select
Selection.AutoFilter
Selection.AutoFilter Field:=1,
Criteria1:="<>P3.5*", Operator:=xlAnd
_
, Criteria2:="<>N2099/738"
Range("A:B").Select
Application.CutCopyMode = False
Selection.Copy
Sheets("sheet5").Select
Range("a1").Select
ActiveSheet.Paste
```

```
ActiveSheet.Paste
Cells(1, 3).Value = ("P3.599/738")
' filter samples
Sheets("sheet1").Select
Selection.AutoFilter Field:=1,
Criteria1:="<>P10099*",
Operator:=xlAnd _
, Criteria2:="<>P5099*"
Range("A:A,D:D").Select
Range("D1").Activate
Application.CutCopyMode = False
Selection.Copy
Sheets("sheet4").Select
Range("a1").Select
ActiveSheet.Paste
Range("A:B").Select
Selection.AutoFilter
Selection.AutoFilter Field:=1,
Criteria1:="<>P3.5*", Operator:=xlAnd _
, Criteria2:="<>N2099/738"
Range("A:B").Select
Application.CutCopyMode = False
Selection.Copy
Sheets("sheet5").Select
Range("a1").Select
ActiveSheet.Paste
Range("A:B").Select
Selection.AutoFilter
Selection.AutoFilter Field:=1,
Criteria1:="<>NEG*", Operator:=xlAnd
_
, Criteria2:="<>PNEG*"
Range("B:B").Select
Selection.Copy
Sheets("IC OD").Select
Range("d1").Select
ActiveSheet.Paste
Cells(1, 4).Value = ("Samples")
' histogram
Sheets("IC OD").Select
Range("j1:u22").Clear
Application.Run
"ATPVBAEN.XLA!Histogram",
ActiveSheet.Range("$A$2:$A$9"), _
ActiveSheet.Range("$J$1"),
ActiveSheet.Range("$H$2:$H$21"),
False, False, _
False, False
Application.Run
"ATPVBAEN.XLA!Histogram",
```

```
Range("A:B").Select
Selection.AutoFilter
Selection.AutoFilter Field:=1,
Criteria1:="<>NEG*", Operator:=xlAnd
_
, Criteria2:="<>PNEG*"
Range("B:B").Select
Selection.Copy
Sheets("HCV OD").Select
Range("d1").Select
ActiveSheet.Paste
Cells(1, 4).Value = ("Samples")
' histogram of data
Workbooks.Open(Filename:= _
"C:\Program Files\Microsoft
Office\Office\Library\Analysis\ATPVB
AEN.XLA"). _
RunAutoMacros Which:=xlAutoOpen
Windows(NatMonitor).Activate
Sheets("HCV OD").Select
Range("j1:u22").Clear
Application.Run
"ATPVBAEN.XLA!Histogram",
ActiveSheet.Range("$A$2:$A$9"), _
ActiveSheet.Range("$J$1"),
ActiveSheet.Range("$H$2:$H$21"),
False, False, _
False, False
Application.Run
"ATPVBAEN.XLA!Histogram",
ActiveSheet.Range("$b$2:$b$9"), _
ActiveSheet.Range("$l$1"),
ActiveSheet.Range("$H$2:$H$21"),
False, False, _
False, False
Application.Run
"ATPVBAEN.XLA!Histogram",
ActiveSheet.Range("$c$2:$c$9"), _
ActiveSheet.Range("$n$1"),
ActiveSheet.Range("$H$2:$H$21"),
False, False, _
False, False
Application.Run
"ATPVBAEN.XLA!Histogram",
ActiveSheet.Range("$d$2:$d$96"), _
ActiveSheet.Range("$p$1"),
ActiveSheet.Range("$H$2:$H$21"),
False, False, _
False, False
```

```
ActiveSheet.Range("$b$2:$b$9"), _
ActiveSheet.Range("$l$1"),
ActiveSheet.Range("$H$2:$H$21"),
False, False, _
False, False
Application.Run
"ATPVBAEN.XLA!Histogram",
ActiveSheet.Range("$c$2:$c$9"), _
ActiveSheet.Range("$n$1"),
ActiveSheet.Range("$H$2:$H$21"),
False, False, _
False, False
Application.Run
"ATPVBAEN.XLA!Histogram",
ActiveSheet.Range("$d$2:$d$96"), _
ActiveSheet.Range("$p$1"),
ActiveSheet.Range("$H$2:$H$21"),
False, False, _
False, False
'  Chart output
Sheets("IC OD").Select
Range("K1").Select
ActiveCell.FormulaR1C1 = "P100
99/738"
Range("M1").Select
ActiveCell.FormulaR1C1 = "P50 99/738"
Range("O1").Select
ActiveCell.FormulaR1C1 = "P3.5
99/738"
Range("Q1").Select
ActiveCell.FormulaR1C1 = "Samples"
ActiveSheet.ChartObjects("Chart
5").Activate
ActiveChart.ChartArea.Select
With ActiveChart
.ChartTitle.Characters.Text = Filename
End With
Range("a1").Select
Sheets("HCV OD").Select
Range("a1").Select
End Sub
```

Macro3:

```
NatMonitor = ActiveWorkbook.Name
   Sheets("Means").Select
   Range("a1").Select
   RunNumber = Cells(1, 1).Value
   If RunNumber = 999 Then GoTo Full
   Cells(RunNumber, 1).Select
   fileToOpen = Application _
   .GetOpenFilename("Excel Files
(*.xls), *.xls")
   If fileToOpen = False Then End
   Workbooks.OpenText Filename:= _
      fileToOpen, Origin:=xlWindows, _
      StartRow:=1,
DataType:=xlDelimited,
TextQualifier:=xlDoubleQuote, _
      ConsecutiveDelimiter:=False,
Tab:=True, Semicolon:=False,
Comma:=False _
      , Space:=False, Other:=False,
FieldInfo:=Array(1, 1)
   Filename = ActiveWorkbook.Name
'copy all data to one sheet
   Sheets(1).Select
   Set NewSheet =
Sheets.Add(Type:=xlWorksheet)
  NewSheet.Name = "master"
   n = 1
   For i = 2 To Sheets.Count
   Sheets(i).Select
   Range("a1:o25").Select
   Selection.Copy
   Sheets("master").Select
   Cells(n, 1).Select
   ActiveSheet.Paste
   n = n + 25
   Next i
   Sheets("master").Select
   ' Copy data to spreadsheet
   Range("A:B,N:N").Select
   Selection.Copy
   Windows(NatMonitor).Activate
   Sheets("sheet1").Select
   Range("a1").Select
   ActiveSheet.Paste

   ' Copy rundate
   Windows(Filename).Activate
   rawdate = Cells(2, 6)
   If rawdate = "" Then rawdate =
      False, Transpose:=False
'  copy min data
   Sheets("Sheet3").Select
   Range("A16:b16").Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("Means").Select
   Cells(RunNumber, 15).Select
   Selection.PasteSpecial
Paste:=xlValues, Operation:=xlNone,
SkipBlanks:= _
      False, Transpose:=False
'  copy max data
   Sheets("Sheet3").Select
   Range("A20:b20").Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("Means").Select
   Cells(RunNumber, 17).Select
   Selection.PasteSpecial
Paste:=xlValues, Operation:=xlNone,
SkipBlanks:= _
      False, Transpose:=False
'  copy 25% data
   Sheets("Sheet3").Select
   Range("A17:b17").Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("Means").Select
   Cells(RunNumber, 19).Select
   Selection.PasteSpecial
Paste:=xlValues, Operation:=xlNone,
SkipBlanks:= _
      False, Transpose:=False
'  copy 75% data
   Sheets("Sheet3").Select
   Range("A19:b19").Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("Means").Select
   Cells(RunNumber, 21).Select
   Selection.PasteSpecial
Paste:=xlValues, Operation:=xlNone,
SkipBlanks:= _
      False, Transpose:=False
'  copy IC data
   Sheets("Sheet3").Select
   Range("e100:h100").Select
   Application.CutCopyMode = False
```

```
Cells(27, 6)
   Application.CutCopyMode = False
   Selection.Copy
   Windows(NatMonitor).Activate
   Sheets("Means").Select
   Cells(RunNumber, 1).Value =
Filename
   Cells(RunNumber, 8).Value = rawdate
   RunDate = Cells(RunNumber, 12)
   Cells(RunNumber, 2).Value =
RunDate
   Application.DisplayAlerts = False
   Workbooks(Filename).Close
   Application.DisplayAlerts = True
   ' filter data
   Sheets("sheet1").Select
   Cells.Select
   Selection.AutoFilter
   Selection.AutoFilter Field:=2,
Criteria1:="HCX"
   Selection.AutoFilter Field:=1,
Criteria1:="P100*"
   Range("D:D").Select
   Selection.Copy
   Sheets("sheet2").Select
   Range("a1").Select
   ActiveSheet.Paste
   Cells(1, 1).Value = ("P100")
   Sheets("sheet1").Select
   Selection.AutoFilter Field:=1,
Criteria1:="P3.5*", Operator:=xlOr, _
      Criteria2:="n20*"
   Range("D:D").Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("sheet2").Select
   Range("b1").Select
   ActiveSheet.Paste
   Cells(1, 2).Value = ("P3.5")
   Sheets("sheet1").Select
   Selection.AutoFilter Field:=1,
Criteria1:="P50*"
   Range("D:D").Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("sheet2").Select
   Range("c1").Select
   ActiveSheet.Paste
   Cells(1, 3).Value = ("P50")
   Selection.Copy
   Sheets("Means").Select
   Cells(RunNumber, 23).Select
   Selection.PasteSpecial
Paste:=xlValues, Operation:=xlNone,
SkipBlanks:= _
      False, Transpose:=False

'  copy Percentages data
   Sheets("Sheet3").Select
   Range("A14:b14").Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("Percentages").Select
   Range("a1").Select
   Cells(RunNumber, 3).Select
   Selection.PasteSpecial
Paste:=xlValues, Operation:=xlNone,
SkipBlanks:= _
      False, Transpose:=False
'  copy filename and date to percentages
   Sheets("Means").Select
   Cells(RunNumber, 1).Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("Percentages").Select
   Cells(RunNumber, 1).Select
   ActiveSheet.Paste
   Sheets("Means").Select
   Cells(RunNumber, 2).Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("Percentages").Select
   Cells(RunNumber, 2).Select
   ActiveSheet.Paste
'  Increase run number and save
   Sheets("means").Select
   Range("a1").Select
   RunNumber = (RunNumber + 1)
   Cells(1, 1).Value = (RunNumber)
   Application.DisplayAlerts = False
   Workbooks(NatMonitor).Save
   Application.DisplayAlerts = True
Exit Sub
ErrorHandler:
   Dim Msg, Style, Title, Help, Ctxt,
Response, MyString
Msg = "Sheet 1 could not be found"    '
Define message.
Style = vbOKOnly + vbCritical    ' Define
```

```
'  filter IC data
   Sheets("sheet1").Select
   Selection.AutoFilter Field:=2,
Criteria1:="HXC"
   Selection.AutoFilter Field:=1
   Range("D:D").Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("sheet2").Select
   Range("d1").Select
   ActiveSheet.Paste
   Cells(1, 4).Value = ("IC")
'  copy Means data
   Sheets("sheet2").Select
   Range("A1:c9").Select
   Selection.Copy
   Sheets("Sheet3").Select
   Range("A1").Select
   ActiveSheet.Paste
   Sheets("sheet2").Select
   Range("d1:d96").Select
   Selection.Copy
   Sheets("Sheet3").Select
   Range("d1").Select
   ActiveSheet.Paste
   Range("A11:c11").Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("Means").Select
   Cells(RunNumber, 3).Select
   Selection.PasteSpecial
Paste:=xlValues, Operation:=xlNone,
SkipBlanks:= _
      False, Transpose:=False
   Sheets("Sheet3").Select
   Range("d100").Select
   Application.CutCopyMode = False
   Selection.Copy
   Sheets("Means").Select
   Cells(RunNumber, 6).Select
   Selection.PasteSpecial
Paste:=xlValues, Operation:=xlNone,
SkipBlanks:= _
```

```
buttons.
Title = "Error"    ' Define title.
Help = "DEMO.HLP"    ' Define Help
file.
Ctxt = 1000    ' Define topic
      ' context.
      ' Display message.
Response = MsgBox(Msg, Style, Title,
Help, Ctxt)



Exit Sub
Full:
Msg = "Sheet is full, please save and
clear"    ' Define message.
Style = vbOKOnly + vbCritical    ' Define
buttons.
Title = "Error"    ' Define title.
Help = "DEMO.HLP"    ' Define Help
file.
Ctxt = 1000    ' Define topic
      ' context.
      ' Display message.
Response = MsgBox(Msg, Style, Title,
Help, Ctxt)
If Response = vbYes Then    ' User chose
Yes.
   MyString = "Yes"    ' Perform some
action.
Else    ' User chose No.
   MyString = "No"    ' Perform some
action.
End If
Exit Sub
End Sub
```