Geno-Sen's

HCV

Real Time PCR Kit

Quantitative

For use with QIAGEN Rotor-Gene Q, Rotor-Gene 6000, ABI-7500, StepOne, StepOne Plus, QuantStudio™, Roche LightCycler®480, Bio-Rad CFX96™, I cycler, Analytik Jena qTOWER 3, Exicycler 96 (Bioneer), AriaMx (Agilent Technology) & Slan 48, ALTA 48, INSTA Q 96, etc.,

***In vitro* Diagnostic Use**

PACK INSERT

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(An ISO 13485:2012, 9001:2008 Certified Company)

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Geno-Sen's HCV Real Time PCR Kit

Table of Contents

1. Contents of the Kit:

Color Code	Contents	Cat. No. 9111007 100 rxns	Cat. No. 9111008 50 rxns	Cat. No. 9111009 25 rxns
R1 Blue	HCV Super mix.	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R2 Yellow	Mg Sol RT.	1 Vial	1 Vial	1 Vial
HCV-S1 Red	*HCV Standard 1* *1 X 10^5 IU/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
HCV-S2 Red	*HCV Standard 2* *1 X 10^4 IU/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
HCV-S3 Red	*HCV Standard 3* *1 X 10^3 IU/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
HCV-S4 Red	*HCV Standard 4* *1 X 10^2 IU/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
HCV-S5 Red	*HCV Standard 5* *1 X 10^1 IU/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
W White	Molecular Grade Water.	1 Vials of 1 ml	1 Vial of 1 ml	1 Vial of 1 ml
IC-1 (R3) Green	IC-1 RG (R3)	1 Vial of 1 ml	1 Vial of 1 ml	1 Vial of 1 ml

R = Reagents
S = Quantitation Standards
W = Molecular Grade Water.

All Vials have Color Coder tops to distinguish between different reagents.

2. Storage of the Kit.

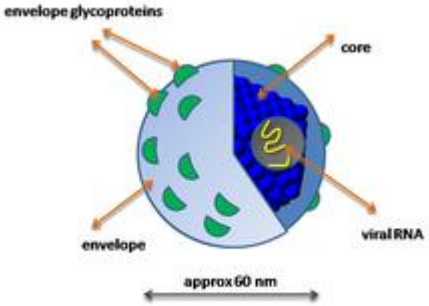
All the reagents of the kit should be stored at -20°C and is stable till expiry at this temperature. Repeated thawing and freezing (> 3x) should be avoided, as this may reduce the sensitivity of the assay. If the kit is to be used only occasionally, the reagents should be frozen in aliquots. Storage at +4°C is not recommended & should not exceed a period of 2 hours in any case.

3. HCV Information

Application

The hepatitis C virus belongs to the genus Hepacivirus a member of the family Flaviviridae. Until recently it was considered to be the only member of this genus. However a member of this genus has been discovered in dogs - canine hepacivirus. There is also at least one virus in this genus that infects horses.

Structure

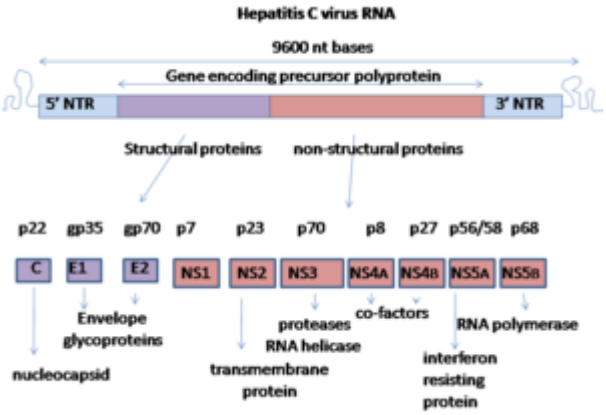


Structure of Hepatitis C Virus

Hepatitis C virus particle

The hepatitis C virus particle consists of a core of genetic material (RNA), surrounded by an icosahedral protective shell of protein, and further encased in a lipid (fatty) envelope of cellular origin. Two viral envelope glycoproteins, E1 and E2, are embedded in the lipid envelope.

Genome



Genome organisation of Hepatitis C virus

Hepatitis C virus has a positive sense single-stranded RNA genome. The genome consists of a single open reading frame that is 9600 nucleotide bases long. This single open reading frame is translated to produce a single protein product, which is then further processed to produce smaller active proteins.

At the 5' and 3' ends of the RNA are the UTR, that are not translated into proteins but are important to translation and replication of the viral RNA. The 5' UTR has a ribosome binding site. (IRES — Internal ribosome entry site) that starts the translation of a very long protein containing about 3,000 amino acids. The core domain of the hepatitis C virus (HCV) IRES contains a four-way helical junction that is integrated within a predicted pseudoknot. The conformation of this core domain constrains the open reading frame's orientation for positioning on the 40S ribosomal subunit. The large pre-protein is later cut by cellular and viral proteases into the 10 smaller proteins that allow viral replication within the host cell, or assemble into the mature viral particles.

Structural proteins made by the hepatitis C virus include Core protein, E1 and E2; nonstructural proteins include NS2, NS3, NS4, NS4A, NS4B, NS5, NS5A, and NS5B.

Molecular biology

The proteins of this virus are arranged along the genome in the following order: N terminal-core-envelope (E1)-E2-p7-nonstructural protein 2 (NS2)-NS3-NS4A-NS4B-NS5A-NS5B-C terminal. The mature nonstructural proteins (NS2 to NS5B) generation relies on the activity of viral proteinases. The NS2/NS3 junction is cleaved by a metal dependent autocatalytic proteinase encoded within NS2 and the N-terminus of NS3. The remaining cleavages downstream from this site are catalysed by a serine proteinase also contained within the N-terminal region of NS3.

The core protein has 191 amino acids and can be divided into three domains on the basis of hydrophobicity: domain 1 (residues 1-117) contains mainly basic residues with two short hydrophobic regions; domain 2 (resides 118-174) is less basic and more hydrophobic and its C-terminus is at the end of p21; domain 3 (residues 175-191) is highly hydrophobic and acts as a signal sequence for E1 envelope protein.

Both envelope proteins (E1 and E2) are highly glycosylated and important in cell entry. E1 serves as the fusogenic subunit and that E2 acts as the receptor binding protein. E1 has 4-5 N-linked glycans and E2 has 11 N-glycosylation sites.

The p7 protein is dispensable for viral genome replication but plays a critical role in virus morphogenesis. This protein is a 63 amino acid membrane spanning protein which locates itself in the endoplasmic reticulum. Cleavage of p7 is mediated by the endoplasmic reticulum's signal peptidases. Two transmembrane domains of p7 are connected by a cytoplasmic loop and are oriented towards the endoplasmic reticulum's lumen.

NS2 protein is a 21-23 kiloDalton (kDa) transmembrane protein with protease activity.

NS3 is 67 kDa protein whose N-terminal has serine protease activity and whose C-terminal has NTPase/helicase activity. It is located within the endoplasmic reticulum and forms a heterodimeric complex with NS4A - a 54 amino acid membrane protein that acts as a cofactor of the proteinase.

NS4B is a small (27 kDa) hydrophobic integral membrane protein with 4 transmembrane domains. It is located within the endoplasmic reticulum and play an important role for recruitment of other viral proteins. It induces morphological changes to the endoplasmic reticulum forming a structure termed the membranous web.

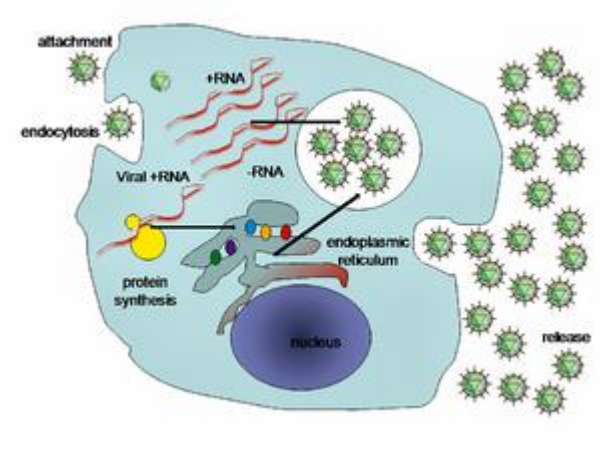
NS5A is a hydrophilic phosphoprotein which plays an important role in viral replication, modulation of cell signaling pathways and the interferon response. It is known to bind to endoplasmic reticulum anchored human VAP proteins.

The NS5B protein (65 kDa) is the viral RNA dependent RNA polymerase.

An 11th has also been described. This protein is encoded by a +1 frameshift in the capsid gene. It appears to be antigenic but its function is unknown.

Replication

Replication of HCV involves several steps. The virus replicates mainly in the hepatocytes of the liver, where it is estimated that daily each infected cell produces approximately fifty virions (virus particles) with a calculated total of one trillion virions generated. The virus may also replicate in peripheral blood mononuclear cells, potentially accounting for the high levels of immunological disorders found in chronically infected HCV patients. HCV has a wide variety of genotypes and mutates rapidly due to a high error rate on the part of the virus' RNA-dependent RNA polymerase. The mutation rate produces so many variants of the virus it is considered a quasispecies rather than a conventional virus species. Entry into host cells occur through complex interactions between virions and cell-surface molecules CD81, LDL receptor, SR-BI, DC-SIGN, Claudin-1, and Occludin.



HCV replication cycle

Once inside the hepatocyte, HCV takes over portions of the intracellular machinery to replicate. The HCV genome is translated to produce a single protein of around 3011 amino acids. The polyprotein is then proteolytically processed by viral and cellular proteases to produce three structural (virion-associated) and seven nonstructural (NS) proteins. Alternatively, a frameshift may occur in the Core region to produce an Alternate Reading Frame Protein (ARFP). HCV encodes two proteases, the NS2 cysteine autoprotease and the NS3-4A serine protease. The NS proteins then recruit the viral genome into an RNA replication complex, which is associated with rearranged cytoplasmic membranes. RNA replication takes places via the viral RNA-dependent RNA polymerase NS5B, which produces a negative strand RNA intermediate. The negative strand RNA then serves as a template for the production of new positive strand viral genomes. Nascent genomes can then be translated, further replicated or packaged within new virus particles. New virus particles are thought to bud into the secretory pathway and are released at the cell surface.

The virus replicates on intracellular lipid membranes. The endoplasmic reticulum in particular are deformed into uniquely shaped membrane structures termed 'membranous webs'. These structures can be induced by sole expression of the viral protein NS4B. The core protein associates with lipid droplets and utilises microtubules and dyneins to alter their location to a perinuclear distribution.

Release from the hepatocyte may involve the very low density lipoprotein secretory pathway.

Genotypes

Based on genetic differences between HCV isolates, the hepatitis C virus species is classified into seven genotypes (1-7) with several subtypes within each genotype (represented by lower-cased letters). Subtypes are further broken down into quasispecies based on their genetic diversity. Genotypes differ by 30-35% of the nucleotide sites over the complete genome. The difference in genomic composition of subtypes of a genotype is usually ~20-25%. Subtypes 1a and 1b are found worldwide and cause 60% of all cases.

Clinical importance of Genotypes.

Genotype is clinically important in determining potential response to interferon-based therapy and the required duration of such therapy. Genotypes 1 and 4 are less responsive to interferon-based treatment than are the other genotypes (2, 3, 5 and 6). Duration of standard interferon-based therapy for genotypes 1 and 4 is 48 weeks, whereas treatment for genotypes 2 and 3 is completed in 24 weeks. Sustained virological responses occur in 70% of genotype 1 cases, ~90% of genotypes 2 and 3, ~65% of genotype 4 and ~80% of genotype 6.

Infection with one genotype does not confer immunity against others, and concurrent infection with two strains is possible. In most of these cases, one of the strains removes the other from the host in a short time. This finding opens the door to replace strains non-responsive to medication with others easier to treat.

Evolution of HCV Genotypes.

Identifying of the origin of this virus has been difficult but genotypes 1 and 4 appear to share a common origin. A Bayesian analysis suggests that the major genotypes diverged about 300–400 years ago from the ancestor virus. The minor genotypes diverged about 200 years ago from their major genotypes. All of the extant genotypes appear to have evolved from genotype 1 subtype 1b.

An study of genotype 6 strains suggests an earlier date of evolution: ~ 1,100 to 1,350 years before the present (95% credible region, 600 to >2,500 years ago). The estimated rate of mutation was 1.8 × 10−4 (95% credible region 0.9 × 10−4 to 2.9 × 10−4). This genotype may be the ancestor of the other genotypes. A study of European, USA and Japanese isolates suggested that the date of origin of genotype 1b was ~1925. The estimated dates of origin of types 2a and 3a were 1917 and 1943 respectively. The time of divergence of types 1a and 1b was estimated to be 200–300 years.

A study of genotype 1a and 1b estimated the dates of origin to be 1914-1930 (95% credible interval: 1802-1957) for type 1a and 1911-1944 (95% credible interval: 1806-1965) for type 1b. Both types 1a and 1b underwent massive expansions in their effective population size between 1940 and 1960. The expansion of HCV subtype 1b preceded that of subtype 1a by at least 16 years (95% credible interval: 15–17 years). Both types appear to have spread from the developed world to the developing world.

The genotype 2 strains from Africa can be divided into four clades that correlate with their country of origin: (1) Cameroon and Central African Republic (2) Benin, Ghana and Burkina Faso (3) Gambia, Guinea, Guinea-Bissau and Senegal (4) Madagascar.

Genotype 3 is thought to have its origin in South East Asia.

These dates from these various countries suggests that this virus may have evolved in South East Asia and was spread to West Africa by traders from Western Europe. It was later introduced into Japan once that country's self imposed isolation was lifted. Once introduced to a country its spread has been influenced by many local factors including blood transfusions, vaccination programmes, intravenous drug misuse and treatment regimes. Given the reduction in the rate of spread once screening for Hepatitis C in blood products was implemented in the 1990s it would seem that at least in recent times blood transfusion has been an important method of spreading for this virus. Additional work is required to determine the dates of evolution of the various genotypes and the timing of their spread across the globe.

Hepatitis C infection facts

- HCV is one of several viruses that cause hepatitis (inflammation of the liver).
- Up to 85% of individuals who are initially (acutely) infected with HCV will fail to eliminate the virus and will become chronically infected.

- HCV is spread most commonly through inadvertent exposure to infected blood. Intravenous drug abuse is the most common mode of transmission. The risk of acquiring HCV through sexual contact is low.

- Generally, patients do not develop symptoms of chronic infection with HCV until they have extensive scarring of the liver (cirrhosis). Some individuals, however, may have fatigue and other non-specific symptoms in the absence of cirrhosis. A minority of patients with HCV have symptoms from organs outside of the liver.

- In the U.S., Infection with HCV is the most common cause of chronic hepatitis and the most common reason for liver transplantation.

- HCV is diagnosed by determining levels in the blood of antibodies to the virus and then confirmed with other tests for viral RNA. The amount of viral RNA in the blood (viral load) does not correlate with the severity of the disease but can be used to track the response to treatment.

- A liver biopsy may be used to assess the amount of liver damage (liver cell injury and scarring), which can be important in planning treatment.

- Considerable progress has been made in the treatment of HCV, although response rates remain imperfect, approximately 50%-60% for genotype 1. Combined therapy with pegylated interferon and ribavirin is the standard treatment regimen.

- Treatment results in reduced inflammation and scarring of the liver in most sustained responders and also occasionally (and to a much lesser extent) in those who relapse or do not respond.

Hepatitis C infection

Hepatitis C infection is an infection of the liver caused by the hepatitis C virus (HCV). It is difficult for the human immune system to eliminate HCV from the body, and infection with HCV usually becomes chronic. Over decades, chronic infection with HCV damages the liver and can cause liver failure. In the U.S., the number of new cases of HCV infection has declined from a peak of 200,000 annually to about 17,000 in 2007. When the virus first enters the body, however, there usually are no symptoms, so these numbers are estimates. Up to 85% of newly-infected people fail to eliminate the virus and become chronically infected. In the U.S., more than three million people are chronically infected with HCV. Infection is most commonly detected among people who are 40 to 60 years of age, reflecting the high rates of infection in the 1970s and 1980s. There are 8,000 to 10,000 deaths each year in the U.S. related to HCV infection. HCV infection is the leading cause of liver transplantation in the U.S and is a risk factor for liver cancer.

Nature of the hepatitis C virus

'Hepatitis' means inflammation of the liver. HCV is one of several viruses that can cause hepatitis. It is unrelated to the other common hepatitis viruses (for example, hepatitis A or hepatitis B). HCV is a member of the Flaviviridae family of viruses. Other members of this family of viruses include those that cause yellow fever and dengue.

Viruses belonging to this family all have ribonucleic acid (RNA) as their genetic material. All hepatitis C viruses are made up of an outer coat (envelope) and contain enzymes and proteins that allow the virus to reproduce within the cells of the body, in particular, the cells of the liver. Although this basic structure is common to all hepatitis C viruses, there are at least six distinctly different strains of the virus which have different genetic profiles (genotypes). In the U. S., genotype 1 is the most common form of HCV. Even within a single genotype there may be some variations (genotype 1a and 1b, for example). Genotyping is important to guide treatment because some viral genotypes respond better to therapy than others. The genetic diversity of HCV is one reason that it has been difficult to develop an effective vaccine since the vaccine must protect against all genotypes.

Liver damage may occur in hepatitis C infection

The presence of HCV in the liver triggers the human immune system, which leads to inflammation. Over time (usually decades), prolonged inflammation may cause scarring. Extensive scarring in the liver is called cirrhosis. When the liver becomes cirrhotic, it fails to perform its normal functions, (liver failure), and this leads to serious complications and even death. Cirrhotic livers also are more prone to become cancerous.

Spread of HCV & Prevention

- HCV is spread (transmitted) most efficiently through exposure to infected blood.
- The most common route of transmission is needles shared among users of illicit drugs.

- Accidental needle-sticks in health care workers also have transmitted the virus. The average risk of getting HCV infection from a stick with a contaminated needle is 1.8% (range 0% to 10%)

- Prior to 1992, some people acquired the HCV infection from transfusions of blood or blood products. Since 1992, all blood products have been screened for HCV, and cases of HCV due to blood transfusion now are extremely rare.

- HCV infection also can be passed from mother to unborn child. Approximately 4 of every 100 infants born to HCV-infected mothers become infected with the virus.

- A small number of cases are transmitted through sexual intercourse. The risk of transmission of HCV from an infected individual to a non-infected spouse or sexual partner without the use of condoms over a lifetime has been estimated to be between 1% and 4%.

- Finally, there have been some outbreaks of HCV when instruments or exposed to blood have been re-used without appropriate cleaning between patients.

Transmission of HCV can be prevented in several ways.

Prevention programs have been aimed at avoiding needle sharing among drug addicts. Needle exchange programs and educational interventions have reduced transmission of HCV infection. However, the population of drug addicts is a difficult population to reach, and rates of HCV remain high among addicts (30% of younger users).

Among healthcare workers, safe needle-usage techniques have been developed to reduce accidental needle-sticks. Newer syringes have self-capping needle systems that avoid the need to manually replace a cap after drawing blood and reduce the risk of needle-sticks.

There is no clear way to prevent transmission of the HCV from mother to child.
Persons with multiple sexual partners should use barrier precautions such as condoms to limit the risk of HCV as well as other sexually-transmitted diseases.
If one partner is infected, monogamous couples should consider the low risk of transmission of HCV infection when deciding whether to use condoms during intercourse. Some couples may decide to use them and some may not.

Screening tests for blood products have almost eliminated the risk of transmission of HCV infection through transfusion, estimated by the CDC to be less than one in two million transfused blood products.

People with HCV infection should not share razors or toothbrushes with others.
It is critical that physicians and clinics follow manufacturer's directions for sterilizing/cleaning instruments and that disposable instruments be discarded properly.

It is important to realize that HCV is not spread by casual contact. Thus, shaking hands, kissing, and hugging are not behaviors that increase the risk of transmission.

There is no need to use special isolation procedures when dealing with infected patients.

Symptoms of HCV

About 75% of people have no symptoms when they first acquire HCV infection. The remaining 25% may complain of

- fatigue,
- loss of appetite,
- muscle aches or fever.

Yellowing of the skin or eyes (jaundice) is rare at this early stage of infection.
Over time, people with chronic infection may begin to experience the effects of the persistent inflammation of the liver caused by the immune reaction to the virus. Blood tests may show elevated levels of liver enzymes, a sign of liver damage, which is often the first suggestion that the infection may be present. Patients may become easily fatigued or complain of nonspecific symptoms.

As cirrhosis develops, symptoms increase and may include:

- weakness,
- loss of appetite,
- weight loss,
- breast enlargement in men,
- a rash on the palms,
- difficulty with the clotting of blood, and
- spider-like blood vessels on the skin.

In patients with advanced cirrhosis, the liver begins to fail. This is a life-threatening problem. Confusion and even coma (encephalopathy) may result from the inability of the liver to process certain toxic substances.

Increased pressure in the blood vessels of the liver (portal hypertension) may cause fluid to build up in the abdominal cavity (ascites) and result in engorged veins in the swallowing tube (esophageal varices) that tear easily and can bleed suddenly and massively. Portal hypertension also can cause kidney failure or an enlarged spleen resulting in a decrease of blood cells and the development of (anemia), or the development of low platelets (thrombocytopenia), which can promote bleeding.

In advanced cirrhosis, liver failure causes decreased production of clotting factors. Patients with advanced cirrhosis often develop jaundice because the damaged liver is unable to eliminate a yellow compound, called bilirubin that is formed from the hemoglobin of old red blood cells.

Conditions outside the liver Associated with HCV

Most of the signs and symptoms of HCV infection relate to the liver. Less commonly, HCV infection causes conditions outside of the liver.

HCV infection can cause the body to produce unusual antibodies called 'cryoglobulins'. These cryoglobulins cause inflammation of the arteries (vasculitis) which may damage the skin, joints, and kidneys. Patients with cryoglobulinemia (cryoglobulins in the blood) may have joint pain, arthritis, a raised purple rash on the legs, generalized pain or swelling. In addition, these patients may develop Raynaud's phenomenon in which the fingers and toes turn color (white, then purple, then red) and become painful at cold temperatures.

Two skin conditions, lichen planus and porphyria cutanea tarda, have been associated with chronic infection with HCV.

For reasons that are unclear, diabetes is three times more common among patients with chronic HCV infection than in the general population.

Low platelet counts may occur as a result of the destruction of platelets by antibodies.
HCV also is associated with B-cell lymphoma, a cancer of the lymph system.

Progression of chronic HCV

Understanding of the natural progression (history) of HCV infection still is evolving. Of 100 people infected with HCV, it is estimated that 75 to 85 will become chronically infected, 60 to 70 will develop liver disease, 5 to 20 will develop cirrhosis and 1 to 5 will die from complications of liver disease such as cirrhosis or liver cancer.

Scientists are learning more about what causes some people to have milder problems and others to have serious complications. Drinking alcohol and acquiring other hepatitis viruses are risk factors for severe disease. Thus, persons who have chronic hepatitis C infection should avoid drinking and should be vaccinated against the other hepatitis viruses (A and B).

Liver cancer (hepatocellular carcinoma) is associated with cirrhosis due to chronic HCV infection. Some experts recommend screening patients with HCV infection and cirrhosis for liver cancer every six months with abdominal ultrasound examinations and a blood test for alpha-fetoprotein (a marker for liver cancer). The effectiveness of this screening is unclear.

People at high risk and should be tested for HCV

- Currently, screening for HCV is not recommended as part of a routine physical examination. Rather, testing should be done among:
- Individuals at high risk for infection including current and past users of injectable drugs and persons exposed to infected blood or organs from infected persons
- Children born to chronically infected mothers
- People who received blood, blood products, or transplanted organs prior to 1992
- Persons with abnormal levels of liver enzymes in the blood
- These are not the only circumstances under which testing for HCV infection may be done. In general, testing is recommended when exposure to the virus is suspected.

Diagnostic tests for HCV and their relevance.

Several diagnostic tests currently are available for the diagnosis of HCV infection.

They can be categorized according to the way the tests are used.

Screening tests

Screening tests are tests that are used to diagnose a condition or disease among individuals not known to have the disease. They are is particularly useful for individuals who have risk factors for the condition or disease.

The first step in screening for HCV infection is to test blood for the antibody to HCV using an enzyme immuno-assays (EIAs). If the EIA test is negative (does not find the antibody), the patient is assumed to be free of HCV. It takes several weeks (up to six months) for antibodies to develop after the initial infection with HCV, so this screening test may miss a few newly-infected individuals. The EIA screening tests are very good (specific); if the test is positive the probability of having HCV infection is greater than 99%.

Recombinant immunoblot assay (RIBA) is used to confirm the positive results of EIAs since occasionally a positive EIA is a false positive, that is, the test is positive when HCV is not present. Although the direct detection of HCV RNA (HCV PCR) also is widely used to confirm the HCV infection, RIBA is still useful to differentiate false positive results in the few individuals whose immune systems have eliminated the virus but still have antibodies left over from the resolved infection.

As previously described, HCV contains RNA. Several tests (assays) are available to measure the amount of HCV RNA in a person's blood. These tests are referred to as molecular tests because they examine the virus at the molecular level. A single negative test for RNA does not mean that there is no infection because the virus may appear in the blood intermittently or may exist in small amounts. Newer tests have helped by detecting smaller and smaller amounts of virus in the blood.

Testing for RNA is useful in determining whether or not a patient has circulating virus in the blood (viremia). Hence, it can be used to confirm that a positive EIA/ELISA truly reflects active HCV infection.

RNA testing also should be done in individuals who may have been recently exposed to HCV. HCV RNA testing is more sensitive (that is, will detect more cases) than the conventional EIA testing in this setting. The reason for this greater sensitivity is that it may take a person several weeks after exposure to HCV to develop the antibodies, whereas HCV RNA becomes detectable one to three weeks after exposure. Finally, HCV RNA testing may be helpful to assess a patient's response to treatment at certain time points during antiviral therapy (see treatment of HCV below).

Blood tests also have been developed to identify the HCV genotype. This information is used to help guide treatment.

The table below provides guidelines for interpreting the results of testing for HCV antibodies by EIA and RIBA and for hepatitis C virus RNA. These are standard interpretations, but it is important to remember that the diagnosis of HCV infection should be made by an experienced clinician who is familiar with the patient's medical history.

Anti-HCV (ELISA/EIA)	Anti-HCV (RIBA)	HCV RNA	Interpretation
Negative	Negative	Negative	No infection
Positive	Positive	Positive	Ongoing infection
Positive	Positive	Negative	Past or current infection. Additional or repeat testing should be done to exclude fluctuating or low levels of viremia.
Positive	Negative	Negative	False positive ELISA; no infection
Positive	Indeterminate	Negative	Situation unclear, consider additional testing
Negative	Negative	Positive	New (acute) HCV infection or chronic HCV infection in an immunocompromised person unable to make adequate antibodies.

Role of a liver biopsy in the management of chronic HCV

Blood tests can tell the clinician whether HCV is present but cannot tell the level of liver damage that has occurred. Liver biopsy allows the clinician to determine how much inflammation and scarring is present by examining a small sample of liver tissue. Liver biopsy gives information useful in the decision to initiate therapy. Significant liver damage is a risk factor for other conditions such as hepatocellular carcinoma and esophageal varices. Liver biopsy may be recommended when the clinician is uncertain about whether to begin treatment or wishes to monitor the response within the liver to therapy.

It is possible to measure liver stiffness with transient elastography, a non-invasive test. Stiffer livers mean that advanced liver fibrosis may be present. However such tests do not completely replace the need for liver biopsy in routine clinical practice.
Several batteries of blood tests also have been found to be useful in diagnosing cirrhosis; however, like transient elastography, these tests have not completely replaced the need for liver biopsy.

Treatment for HCV & AntiViral Therapy.

Current treatment options are imperfect. Patients with HCV infection should discuss treatment options with a physician who is experienced in treating the disease.

Treatment is recommended in patients at increased risk for cirrhosis unless there are reasons that would make treatment unsafe. According to the National Institutes of Health (NIH) persons at increased risk for cirrhosis would include those with HCV infection and:

Persistent elevation of ALT (alanine aminotransferase, a liver enzyme in the blood)

High levels of HCV RNA in the blood

Evidence of early fibrosis (scarring) or moderate inflammation and injury of liver cells on liver biopsy

The NIH also recommends treatment for patients who are co-infected with HCV and HIV, because these patients have a more rapid course of liver injury.

These are general guidelines. Patients and providers may decide that treatment is needed for other reasons. For example, HCV infection may cause cryoglobulinemia. Persistent cryoglobulinemia may be a reason for treating hepatitis C. Newer therapies may be offered to selected patients in research settings.

Individuals who should not be treated with antiviral therapy include those who are unable to comply with the treatment schedule, have reasons that may make treatment unsafe (for example, allergy to the medications), have received a solid organ transplant, are pregnant or unwilling to practice adequate contraception during treatment, or who have reversible serious untreated conditions such as unstable heart disease, uncontrolled high blood pressure, or untreated major depression.

Patients with unstable (decompensated) cirrhosis are at high risk for complications for treatment and usually do not receive medical treatment except in research settings. Fundamentally, the decision regarding antiviral therapy in chronic HCV infection should be tailored to the individual patient with careful consideration of the risks and benefits.

All patients with HCV should be vaccinated against hepatitis B and hepatitis A. They also should be counseled on measures to prevent the spread of HCV and eliminating alcohol use. Finally, risk behaviors for HCV overlap with those of HIV, and all patients with HCV should be tested for HIV.

Pattern of response to antiviral treatment

Treatment responses are mainly defined by results of the HCV RNA testing. Four patterns of response to antiviral treatment have been described:

- sustained virologic response,
- relapse,
- partial response, and
- no response.

Sustained virologic response: The optimal response is a sustained virologic response (SVR), defined as the absence of detectable HCV RNA in serum using a sensitive test at the end of the treatment and six months later. Most of these individuals will remain in remission (no signs of the disease) indefinitely, with no detectable hepatitis C virus RNA in the blood or liver.

Moreover, follow-up biopsies show a marked reduction in inflammation and there even can be regression of scarring. Longer follow-up of these patients is necessary, however, to evaluate definitively whether sustained responders will avoid the complications of cirrhosis and live longer.

Relapse: Relapsers are patients who initially eliminate the RNA from their blood but then develop detectable RNA again shortly after discontinuing therapy. The RNA becomes detectable again within six months and usually within the first three months of stopping treatment.

Partial responders: Patients whose HCV RNA levels decline (two log decrease) but never become undetectable at 24 weeks are referred to as partial responders.

No response: Patients who have sustained levels of detectable HCV RNA during therapy are known as non-responders. Patients in whom HCV RNA becomes undetectable during the early period of treatment but reappears before the end of therapy, should probably likewise be considered non-responders. This reappearance of HCV RNA during therapy is referred to as a 'break through' of HCV.

Goals of therapy for HCV

The ultimate goals of antiviral therapy are to eliminate HCV, prevent transmission, improve or normalize the liver tests and histology (microscopic appearance), prevent progression to cirrhosis and liver cancer, prolong survival, and improve the quality of life.

As already stated, only a sustained virologic response provides the possibility of achieving all of these goals since most patients who have a sustained response will remain in remission indefinitely. The rest of the patients (non-responders, partial responders and relapsers) may show improvement in blood tests with or without relief of symptoms.

Therapy options for previously untreated patients with chronic hepatitis C infection.

For previously untreated patients who are candidates for therapy, the optimal approach is combined treatment with pegylated interferon and ribavirin (Rebetol, Copegus). Patients who have reasons not to receive ribavirin may be treated solely with pegylated interferon. Older preparations (nonpegylated forms)of interferon are less effective and less commonly used.

Pegylated interferon: Interferons are a family of naturally occurring proteins that are produced by the body to fight viral infections. To produce pegylated interferon, the interferon is processed by attaching ethylene glycol to it. This process is called pegylation and it slows the elimination of interferon from the body so that its effects are more prolonged. There are currently two types of pegylated interferon: pegylated interferon alpha 2b (Peg-Intron A) and pegylated interferon alpha 2a (Pegasys). Both pegylated interferon alpha 2b and 2a; are given as a subcutaneous injection once a week.

Optimally, pegylated interferon therapy should be combined with ribavirin. In persons who cannot take ribavirin, monotherapy with pegylated interferon may be used. Monotherapy has been shown to achieve sustained virologic response rates of 23% to 25% in patients.

Ribavirin: The antiviral agent, ribavirin (Rebetol, Copegus), is a nucleoside analogue that is taken by mouth. Nucleoside analogues are man-made molecules that closely resemble the biochemical units that make up genetic material (RNA and DNA). Ribavirin works by fooling the virus into using it instead of the normal building blocks, thereby slowing viral reproduction. Ribavirin has not worked well when used alone for hepatitis C.

Combined pegylated interferon and ribavirin: Combined therapy with both pegylated interferon and ribavirin produces a sustained virologic response in 28% to 50% of patients with genotype 1. Genotype 1 is the most common genotype in the U.S., but also the most resistant to treatment. For unknown reasons, response rates are lower in African American persons and higher in Caucasians. In patients with genotype 2, sustained response rates are higher (76% to 82%).

The duration of therapy depends on the genotype of the HCV.

Hence the recommended duration of treatment for HCV genotype 2 and 3 is 24 weeks and for genotype 1 is 48 weeks.

Sustained virologic response usually is accompanied by a return to normal serum ALT levels and improvement in inflammation within the liver.

Combination therapy is associated with more side effects than therapy with single drugs (see below). In research studies, up to 20% of patients receiving combination therapy required a reduction in the doses or discontinuation of therapy because of the side effects. Nevertheless, combination therapy represents significant progress in the treatment of chronic HCV and is the current standard of care.

Some patients treated successfully with combination therapy still have detectable virus after 12 weeks of treatment but go on to have a sustained response. Therefore, patients on combination therapy should have hepatitis C virus RNA measured at 24 weeks of therapy. In those who are still positive for the virus at that time, consideration is given to stopping treatment, since the chance of sustained response is small.

Relapses and non-responders treatment options.

The optimal treatment for nonresponders and relapsers is not well established.
A minority of nonresponders (6% to 12%) will respond to a second course of pegylated interferon and ribavirin.

Patients initially treated with older nonpegylated interferon can be considered for the therapy with either pegylated interferon or pegylated interferon plus ribavirin therapy.

Newer preparations of interferon and protease inhibitors are being studied and show promise in persons who did not respond to combination therapy.

Despite the failure to achieve sustained virologic response, treatment may slow the progression of HCV to cirrhosis, although this has not been shown for certain.

Treatment for acute HCV infection.

When people first acquire HCV, the infection is said to be 'acute'. There is no standard approach to treatment for acute HCV. Most patients with acute HCV do not have symptoms, so they are not recognized as being infected. However, some have low-grade fever, fatigue or other symptoms that lead to an early diagnosis. Others who become infected have a known exposure to an infected source, such as a needlestick injury, and are monitored closely. Treatment decisions should be made on a case-by-case basis. Response to treatment is higher in acute hepatitis infection than chronic infection. However, many experts prefer to hold off treatment for 8-12 weeks to see whether the patient eliminates the virus without treatment.

Side effects of treatment for HCV

Flu-like symptoms, hair thinning and depression are common side effects of interferon or pegylated interferon. Depression may be serious and is common enough that patients should be monitored for this side effect.

Interferons may cause transient bone marrow suppression resulting in reduced white blood cell and/or red blood cell counts (leucopenia and anemia, respectively). Reductions in white blood cell counts may cause increased susceptibility to infection. Growth factors (erythropoietin) can be used to improve the anemia associated with interferon. Death rarely occurs as a result of therapy, but may occur from progressive liver failure in patients with advanced cirrhosis.

Certain side effects are attributed to the addition of ribavirin to interferon, including nausea, cough, shortness of breath, rash, itching, insomnia, and loss of appetite.

Ribavirin also causes anemia due to the destruction of red blood cells (hemolysis). This anemia is usually mild but can become clinically significant. Ribavirin particularly may cause destruction of red blood cells (hemolysis) in people with kidney failure. Anemia improves with a reduction in the dose of ribavirin.

Ribavirin also accumulates in the testicles and ovaries and causes birth defects in animals. Although no birth defects have been reported in humans as yet, both men and women should use contraceptive measures to avoid pregnancy during and for at least six months after ribavirin treatment.

Liver transplantation options for HCV patients.

HCV is the leading reason for liver transplantation in the U.S., accounting for 40% to 45% of transplants. HCV routinely recurs after transplantation and infects the new liver.

Approximately 25% of these patients with recurrent hepatitis will develop cirrhosis within five years of transplantation. Despite these findings of recurrence, the five-year survival rate for patients with HCV is comparable to that of patients who are transplanted for other types of liver disease.

Treatment for recurrent hepatitis after transplantation is not a simple issue. Most transplant centers delay therapy until recurrent disease is confirmed. Treatment of recurrent hepatitis is complicated with interferon, an important drug for treatment, is an immune modulator (modifier) that may promote rejection of the transplanted liver. Furthermore, interferon and ribavirin may not be well tolerated by patients who just underwent transplantation and are taking many different kinds of medications.

Current research and future for HCV Patients

As our awareness of HCV infection increases, more and more patients are being diagnosed with this condition. Current research includes diagnosis, natural history, treatment, and vaccine development.

Diagnosis: More accurate tests are being developed to detect even smaller amounts of the virus.

Natural history: There is much we do not know about the natural history of chronic HCV.

- Why do some people clear the virus spontaneously.
- What makes some people develop cirrhosis when others appear to have little liver damage.
- What predicts response to treatment or re-treatment.

Treatment: New formulations of interferon are being developed in the hopes of improving response rates. In addition, new agents are being tested in combination with pegylated interferon and ribavirin. Some of these agents, like telaprevir or boceprevir inhibit protease enzymes that HCV needs in order to reproduce.

Vaccine development: Scientists have not been able to develop an effective vaccine against HCV. This is partly due to the ability of the HCV to change (mutate) and evade the body's immune responses.

The ***Geno-Sen's HCV*** *Real Time PCR* RG Kit Quantification assay is developed for laboratory scale or high-throughput quantitative transcript analysis by real time quantitative fluorescence PCR.

Geno-Sen's HCV *Real Time PCR* RG Kit standardized ready-to-use Control and Reaction mix allow fast processing of the samples.

Samples which can be used for Extraction: Serum, plasma, whole blood, Biopsies etc.

4. Precautions for PCR

The following aspects should always be taken care of:

- Store positive material (Specimens, Standards or amplicons) separately from all other reagents and add it to the reaction mix in a separate facility.
- Thaw all components thoroughly at room temperature before starting the assay.

- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice or in the Cooling Block.
- All the reagents including the NTC (except for standards & specimens) should be mixed & dispensed in pre-mix area.
- All the standards & specimens should be mixed & dispensed in extraction area.
- Use pipette tips with filters only.
- Always use disposable powder-free gloves

5. Additionally Required Materials and Devices

- RNA isolation kit (see **8.a. RNA extraction**)
- 0.2 ml PCR tubes for use with 36-well rotor (Corbett Research, Cat.-Nr.: SE-1003F) alternatively 0.1 ml PCR tubes for use with 72-well rotor (Corbett Research, Cat.-Nr.: ST-1001)
- Micro Pipettes Variable Volume 2-20µl, 10-100µl, 100-1000µl,
- Sterile pipette tips with aerosol barrier 2-20µl, 10-100µl, 100-1000µl,
- Disposable powder-free gloves
- Vortex mixer
- Centrifuge Desktop with rotor for 1.7 ml reaction tubes

6. Principle of Real-Time PCR

The robust assay exploits the so-called Taqman principle. During PCR, forward and reverse primers hybridize to a specific sequence product. A TaqMan probe, which is contained in the same reaction mixture and which consists of an oligonucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher dye, hybridizes to a target sequence within the PCR product. A Taq polymerase which possesses 5' - 3' exonuclease activity cleaves the probe. The reporter dye and quencher dye are separated upon cleavage, resulting in an increase in fluorescence for the reporter. Thus, the increase in fluorescence is directly proportional to the target amplification during PCR.

7. Description Of the Product.

The ***Geno-Sen's HCV*** *PCR* Reagents constitute a ready to use system for detection and quantification of HCV using Polymerase chain reaction (PCR) in the Rotor Gene 2000/3000/6000 (Corbett Research). *The Specific Master* mix contains reagents and enzymes for the specific amplification of HCV and for the direct detection of the specific amplicon in fluorescence channel Cycling A.FAM of the *Rotor Gene 2000/3000/6000 & the Reference gene on* Cycling A. Joe. External positive

Standards (HCV S 1-5) are supplied which allow the determination of the gene load. For further information, please refer to section 8.c Quantitation.

8. Procedure

8.a RNA Extraction

RNA Extraction kits are available from various manufacturers. Sample volumes for the RNA Extraction procedure depend on the protocol used. Please carry out the RNA Extraction according to the manufacturer's instructions. The recommended Extraction kits are the following:

Sample Material	**Nucleic Acid Isolation Kit**	**Cat. Num. REF**	
Serum,plasma, Whole Blood,semen, tears	***Geno Sen's*** ® Viral RNA Extraction Mini Kit (Columns based)	98001 or 98002	Genome Diagnostics Pvt. Ltd. India.
	OR		
	QIAamp Viral RNA Mini extraction Kit (50)	52904	QIAGEN

The ***Geno Sen's*** ® ***HCV Real Time PCR RG Kit*** has been optimized with the above mentioned extraction kit. The ***Geno Sen's*** ® Viral RNA Extraction Mini Kit provides a relatively higher yield than most of the commercial extraction kits available on the world market & hence is the preferred Kit for extraction of Viral RNA. However the customer can use their own extraction systems depending on how good the yield is. Always use an extraction kit with a higher RNA yield otherwise the low positives will not be detected.

Blood collection tubes coated with anticoagulants may inhibit the PCR, However these inhibitors will be eliminated by the use of the isolation kits given above. It is recommended to avoid the usage of heparin blood.

When using Extraction protocols with ethanol-containing washing buffers, please carry out an additional centrifugation step before the elution to remove any remaining ethanol. This prevents possible inhibition of PCR.

The *HCV Rotor Gene PCR Reagents* should not be used with phenol based isolation methods.

8.b *Inhibition Control:*

Inhibition Control Gene allows the user to determine & control possible PCR inhibition. The Inhibition Control gene reagents are in built in the premix provided and need not be run separately. There is need to add internal control Gene in the reaction mix which has been provided as IC-1 RG (R3). To add the IC Gene 0.5 µl/rxn of R3 is added to the premix as shown in the Figure 4 & 5. The results can be visualized in the Joe channel. However if there is a need for the Internal control to be used as an extraction efficiency control, then 5 µl of R3 i.e. Inhibition control should be added to each sample before adding the Buffer MBR in the extraction process.

However in such a case, the IC-1 (R3) should not be mixed with the pre-mix as described above & instead 0.5 µl/rxn Molecular Grade water should be added to the pre-mix.

Also Note that in such a case there will be no amplification of Internal control in the Joe Channel in the NTC, & Standards. There will only be amplification in the Joe Channel in the Samples to which the IC-1 (R3) has been added before extraction.

Whereas if the IC-1 (R3) is added to the premix then there will be amplification in Joe Channel in all the samples, standards & NTC.

8.c Quantitation

The quantitation standards provided in the kit *(HCV S 1-5)* are treated in the same way as extracted samples and the same volume is used i.e. (15µl) instead of the sample. To generate a standard curve in the *RotorGene™ 2000/3000/6000*, all 5 Standards should be used as defined in the menu window *Edit Samples* of the *RotorGene™* software. The same should also be defined as standards with the specified concentrations (see *RotorGene™* Manual). The standard curve generated as above can also be used for quantitation in subsequent runs, provided that at least one standard is used in the current run. For this purpose, the previously generated standard curve needs to be imported (see *Rotor Gene™ 2000/3000/6000 Manual*). However, this quantitation method may lead to deviations in the results due to variability between different PCR runs & due to varying Reaction efficiencies.

Attention: The standards are defined as IU/µl. The following formula has to be applied to convert the values determined using the standard curve into IU/ml of sample material:

$$\textbf{Result (IU/ml)} = \frac{\textbf{Result (IU/µl) x Elution Volume (µl)}}{\textbf{Sample Volume (ml)}}$$

Or else direct conversion can be done keeping in mind the starting volume of the sample & the final eluted Volume e.g.

In case ***Geno Sen's*** ® Viral RNA Extraction Mini Kit is being used where the starting volume is 150µl & the final Eluted Volume is 60µl then to obtain the direct values i.e. IU/ml for the patient samples the following values for the standards can be fed directly into the operating software which is again based on the above formula and will yield direct conversion of per ml of patient sample data.

S1: 10^5 **IU**/µl = 40000000 **IU** /ml
S2: 10^4 **IU** /µl = 4000000 **IU** /ml
S3: 10^3 **IU** /µl = 400000 **IU** /ml
S4: 10^2 **IU** /µl = 40000 **IU** /ml
S5: 10^1 **IU** /µl = 4000 **IU** /ml

In case of Qiagen Viral RNA extraction kit is being used where the starting volume is 140µl & the final Eluted Volume is 50µl then to obtain the direct values i.e. IU/ml for the patient samples the following values for the standards can be fed directly into the operating software which is again based on the above formula and will yield direct conversion of per ml of patient sample data.

S1: 10^5 IU/µl = 35750000 IU/ml
S2: 10^4 IU/µl = 3575000 IU/ml
S3: 10^3 IU/µl = 357500 IU/ml
S4: 10^2 IU/µl = 35750 IU/ml
S5: 10^1 IU/µl = 3575 IU/ml

8.d Preparation for PCR & amplification.

First make sure that the Cooling Block (accessory of the *Rotor Gene*™, Corbett Research) is pre-cooled to +4°C in a Refrigerator or Deep Freezer. Place the desired number of PCR tubes into the Cooling Block. Make sure that the tubes for standards & at least one negative control (*Water, PCR grade*) are included per PCR run. To generate a standard curve, use all supplied Standards *(HCV S 1-5)* for each PCR

run. Before each use, all reagents need to be thawed completely and mixed (by pipetting or by brief vortexing).

Please follow the pipeting scheme mentioned below for each sample depending upon the number of samples a mix can be prepared as follows. Each sample

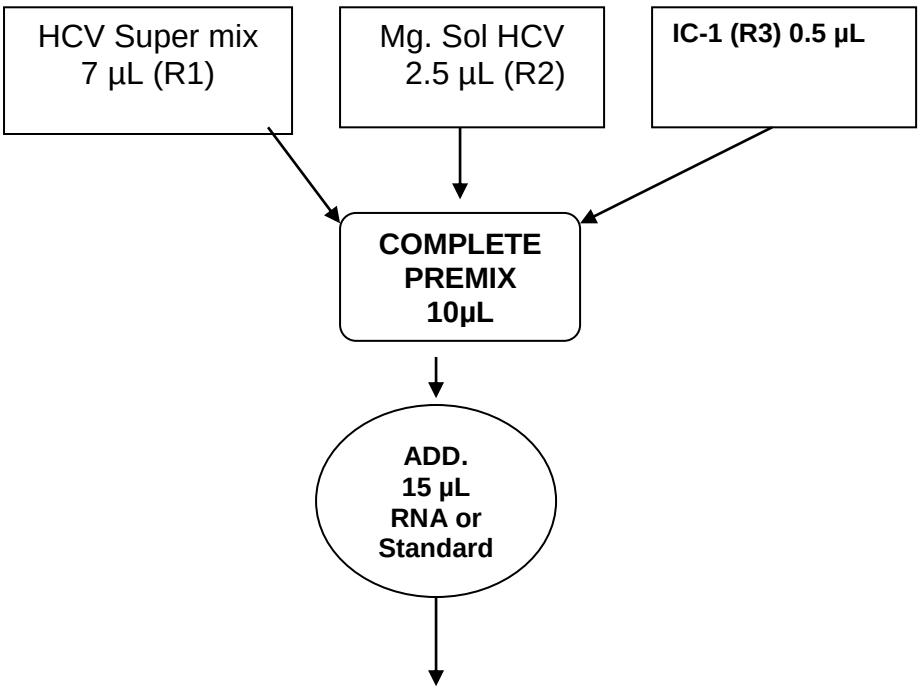


Fig. 4.

Depending upon the number of samples the following pipetting scheme can be followed. e.g. for 10 rxns.

HCV MASTER MIX	1 rxns.	10 rxns.
HCV Super Mix (R1)	7 µL	70 µL
HCV Mg Sol. (R2)	2.5 µL	25 µL
IC-1 (R3)	0.5 µL	5 µL
Total	10µL	100µL

Fig. 5.

Pipette 10 µl of the Master Mix into each labelled PCR tube. Then add 15 µl of the earlier extracted RNA to each sample tube and mix well by pipeting up and down. Correspondingly, 15 µl of the Standards (HCV *S1-5*) must be used as a positive control and 15 µl of water (*Water, PCR grade*) as a negative control. Close the PCR tubes and transfer the same into the rotor of the *RotorGene*™ instrument. The *RotorGene*™ software versions 5.0.53 and higher require a Locking Ring (accessory of the *RotorGene*™, Corbett Research) to be placed on top of the rotor to prevent accidental opening of the tubes during the run.

8.e. Programming of the instrument

The program for the detection of HCV can be divided into following steps:

A. Setting of general assay parameters & reaction volume
B. Thermal Profile & Calibration
C. Cycling profile/ cDNA synthesis & Initial activation of the Hot Start enzyme
D. Cycling for Amplification of cDNA
E. Adjustment of the sensitivity of the fluorescence channels
F. Starting of the *Rotor Gene*™ run

Program the *RotorGene*™ *2000/3000* for these 5 steps according to the parameters shown in Fig. 6-21. All specifications refer to the *RotorGene*™ software version 6.0.33. Please find further information on programming the *RotorGene*™ in the *RotorGene*™ *2000/3000 Operator's Manual*,. In the illustrations these settings are shown by arrows

g) Setting of general assay parameters & Reaction volume.

Please see to it that you are in advanced mode and then click Hydrolysis Probes.
On a double click the software will automatically go to the next function. If there is just a single click then click new after highlighting the Hydrolysis Probes.

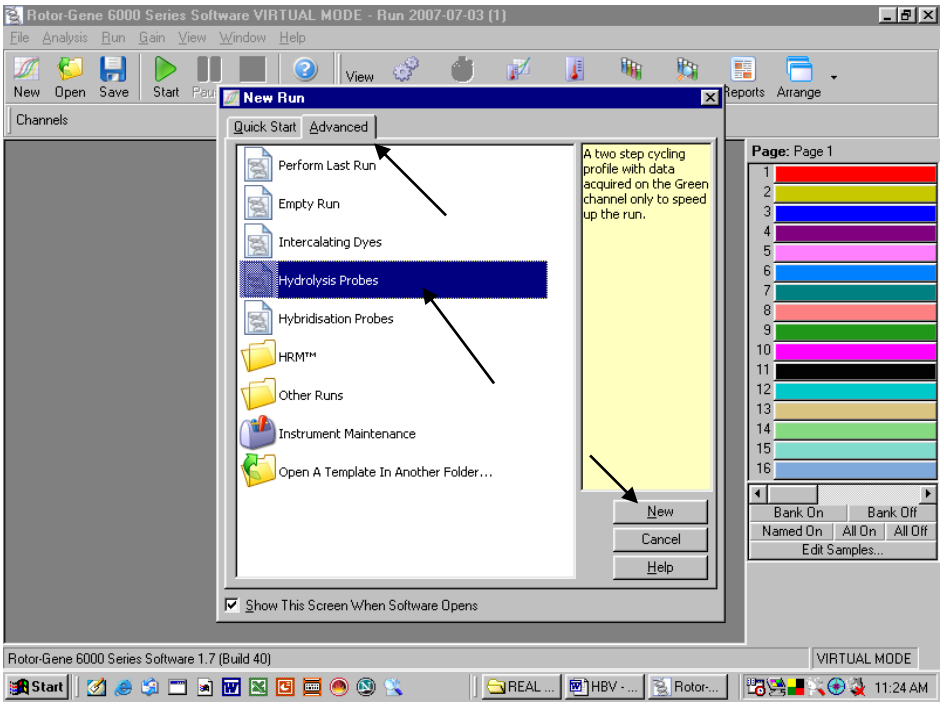


Fig. 22.

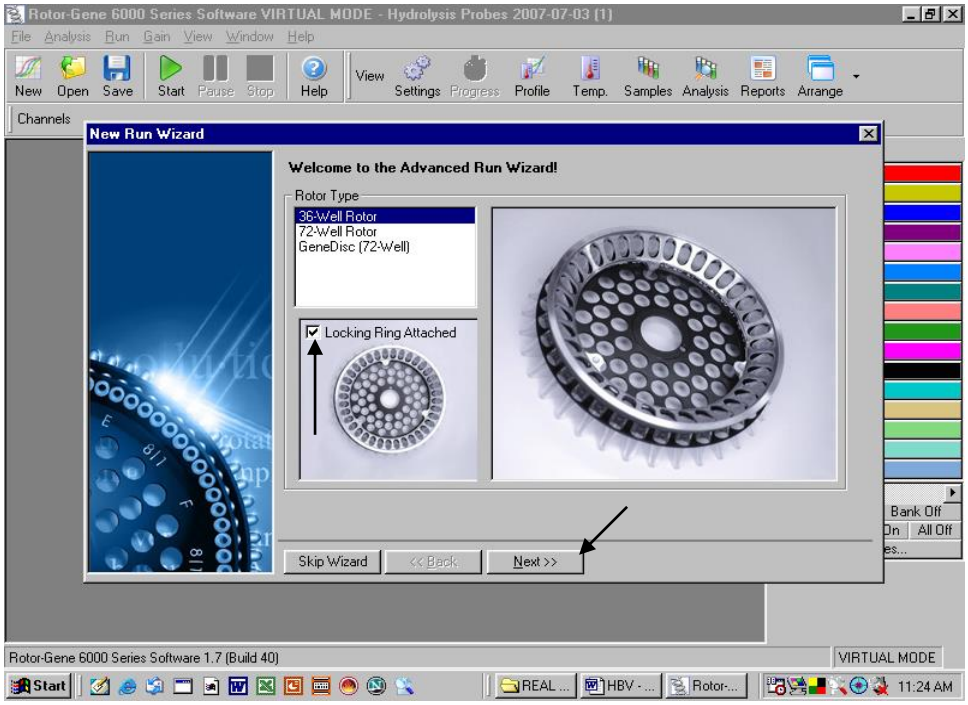


Fig. 23.

First, confirm by clicking in the box as shown in the figure above whether a locking ring has been attached. Then click next to proceed to the next step.

Confirmation of reaction Volume as follows.

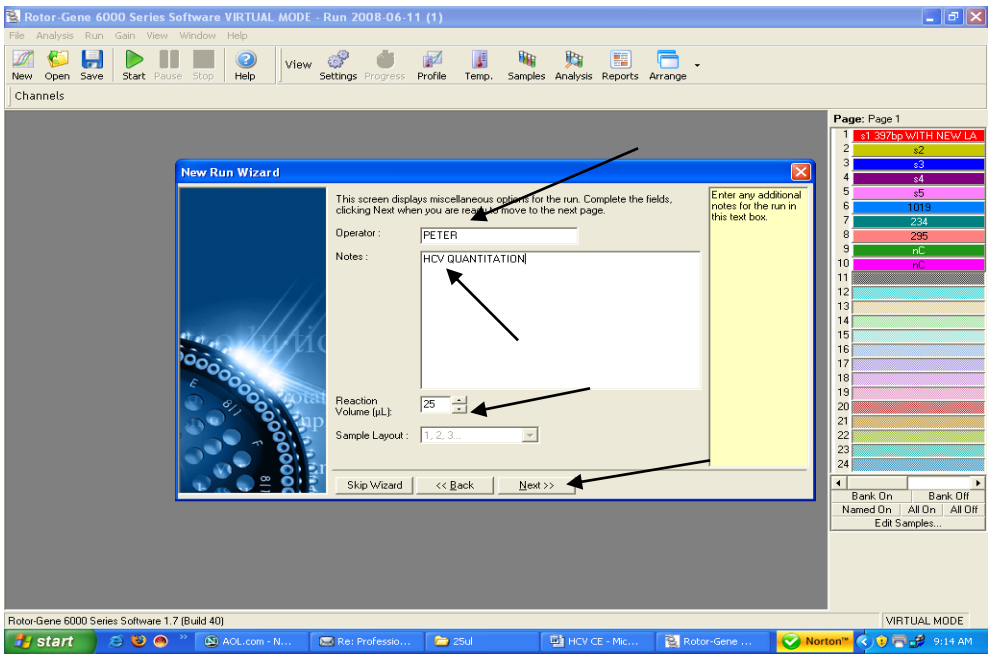


Fig. 24.

- Please click on the volume buttons to make sure that 25µl is reflected in the window as shown above.
- In case required, Operators Name can be Fed into the system.
- Any Notes which need to be either printed into the Report can be typed out in the Box of Notes.

- Then click next and a new window will open as shown below.

h) THERMAL PROFILE & CALIBRATION:

Here the thermal profile for the assay will be defined.

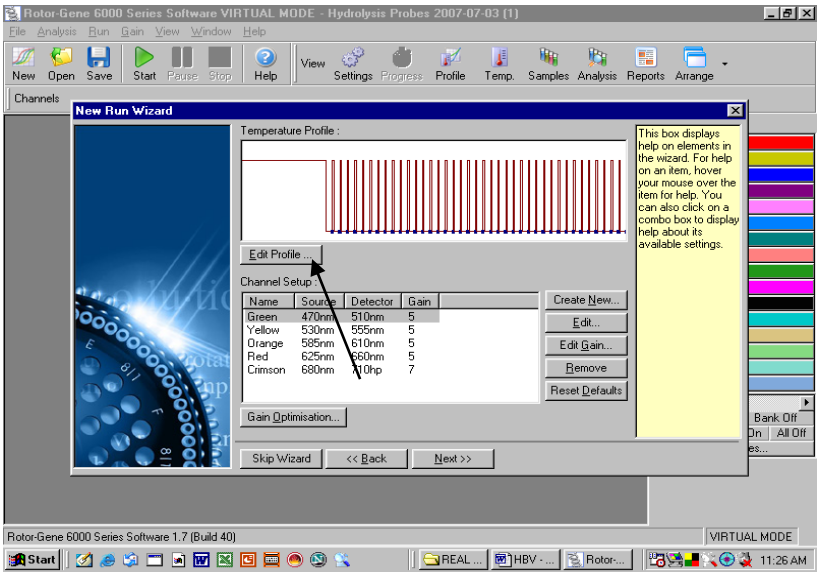


Fig. 25.

Programming the temperature profile is done by activating the button *Edit Profile* in the next *New RUN Wizard* menu window as shown above.

i) **CYCLING PROFILE: First hold** 50°C for 15 minutes i.e. cDNA synthesis step as below

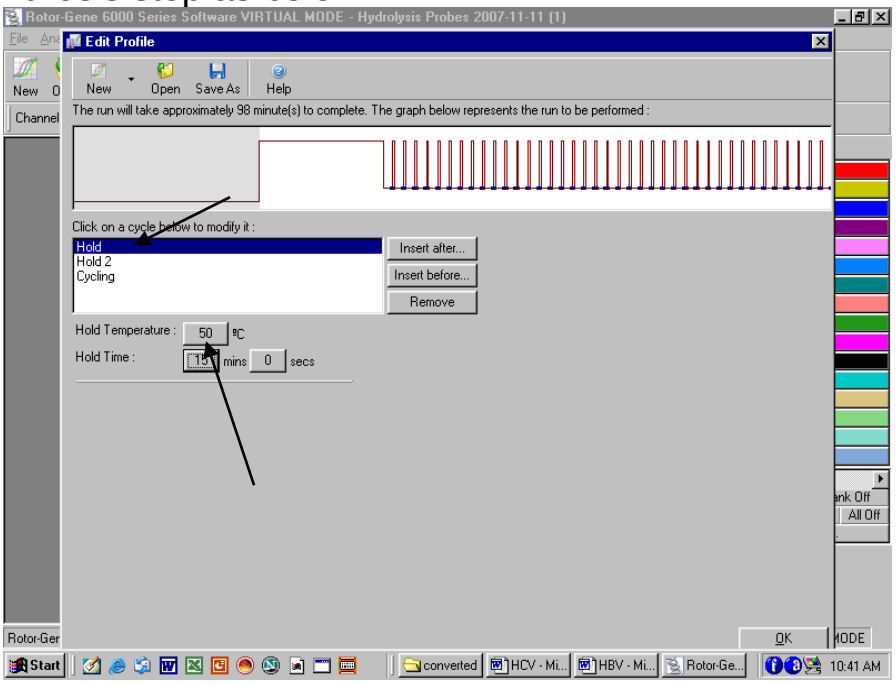


Fig. 26.

Second hold 95°C for 10 minutes as below

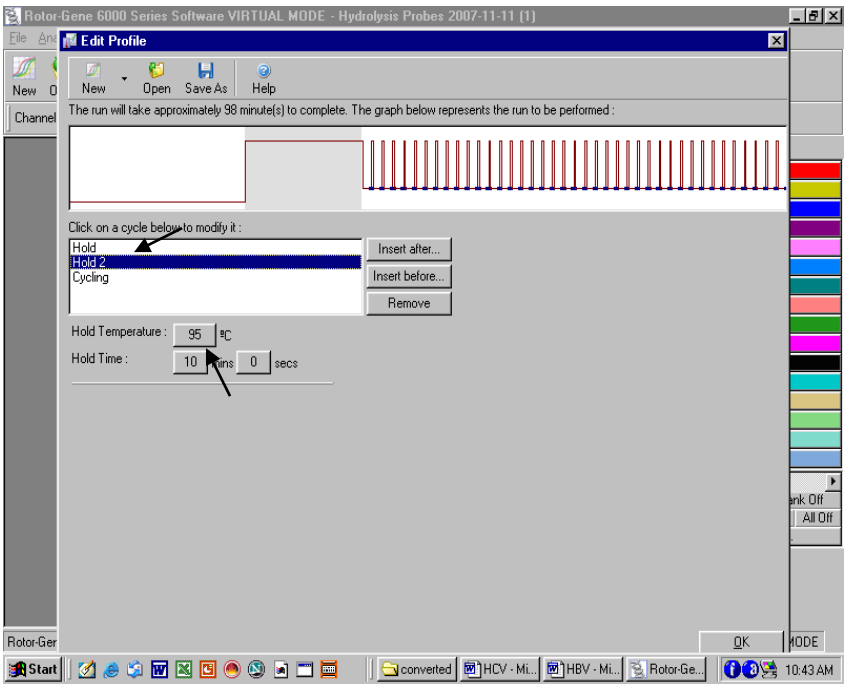


Fig. 27. Initial activation of the Hot Start enzyme. Generally the window will open with the Hold Temp as 95°C and the Hold Time as 10 minutes. In case it is different then set the same to as shown above.

j) Setting the Cycling and acquisition.

When clicked on Cycling the window will open as below.

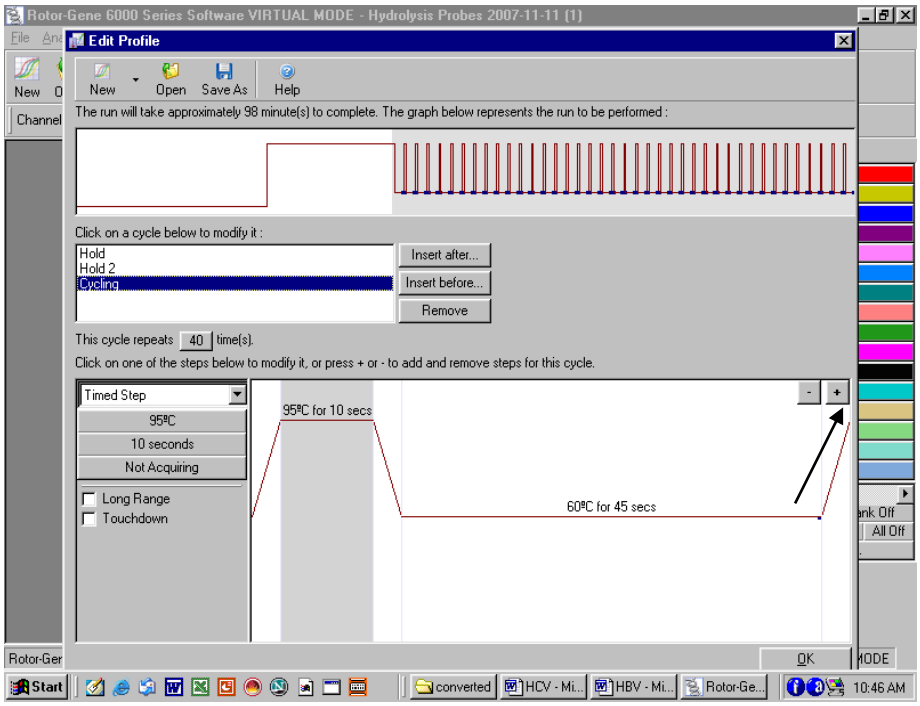


Fig. 28.

Click on the Plus sign at the right hand as shown in the figure to make the Cycling a three step process. A new window as shown below will be there.

Setting up of denaturation step in the cycling profile as depicted below i.e. 95°C for 15 seconds.

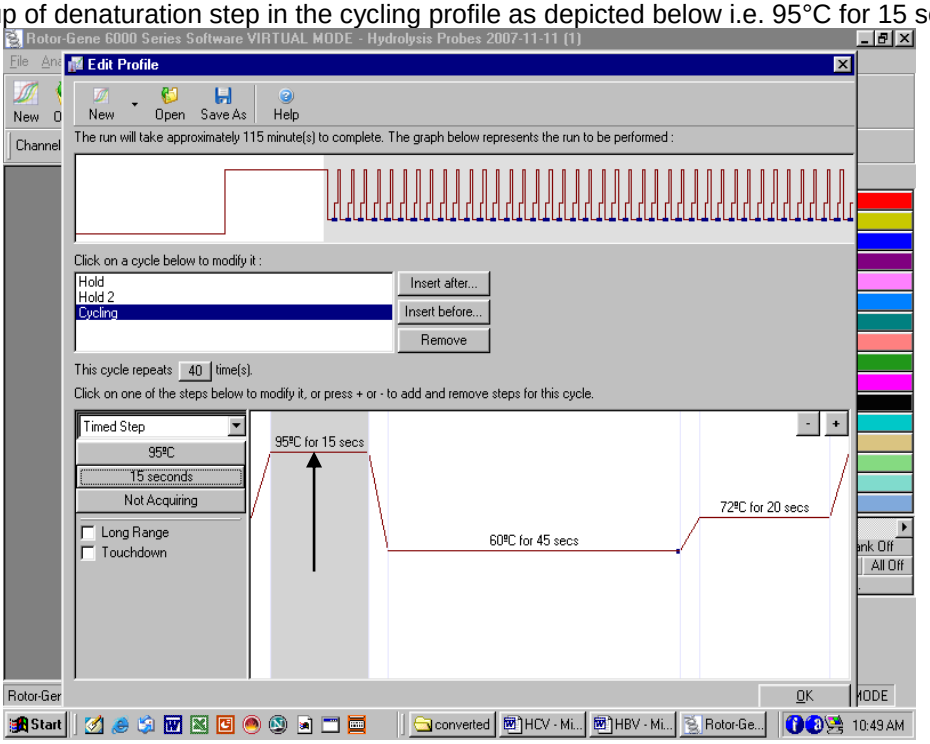


Fig. 29.

Setting up of Anneling step in the cycling profile as depicted below i.e. 55°C for 20 Seconds

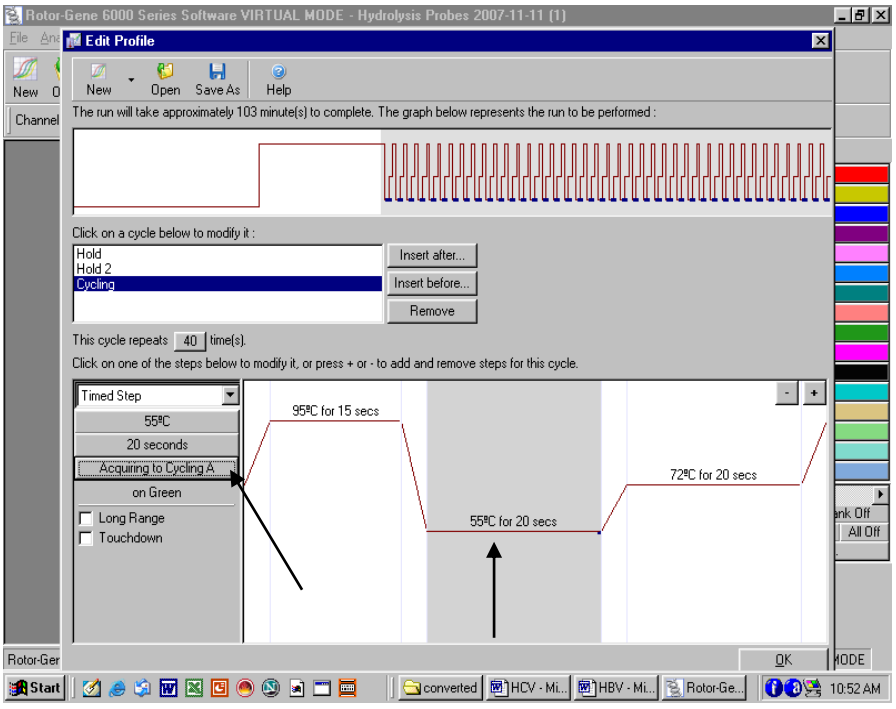


Fig. 30.

After setting the Anneling temperature and the time for anneling click on the "Acquiring to Cycling A" as shown by arrow. A New window will open as shown below.

Defining the Data acquiring channel i.e Green (FAM) & Yellow (JOE)

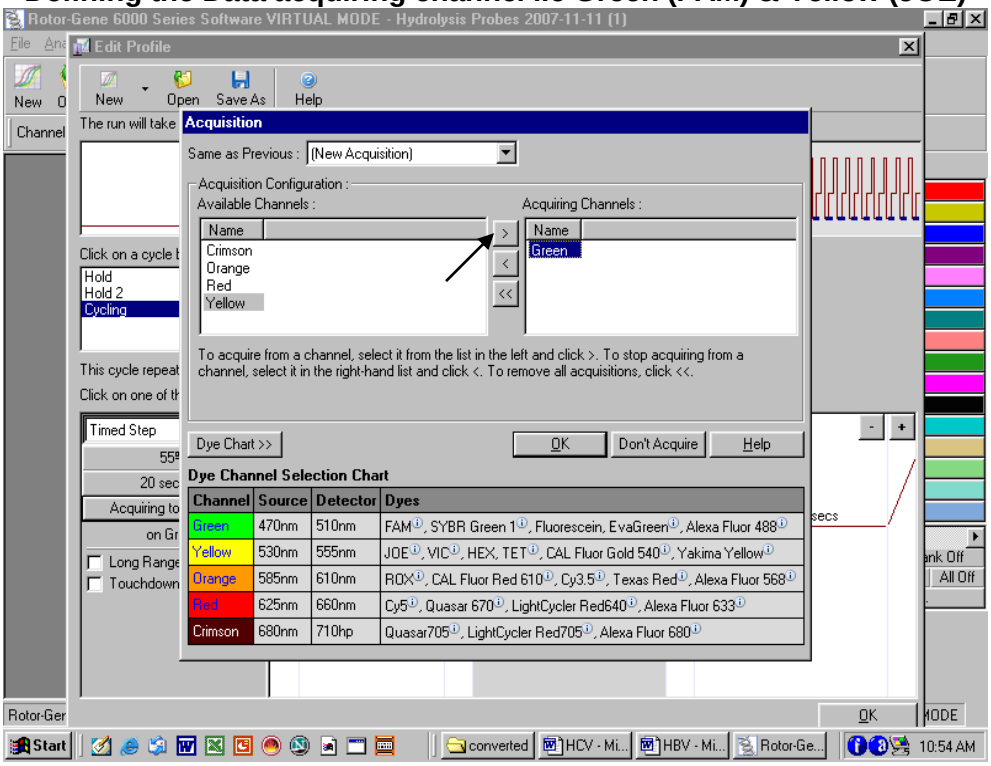


Fig. 31.

Highlight the Yellow and then press the right arrow. Just see before shifting the yellow to right that there is no other channel in the right except for Green. In case any other Channel appears besides Green on the right then the same be shifted to the left.

Confirmation of Channels as shown below.

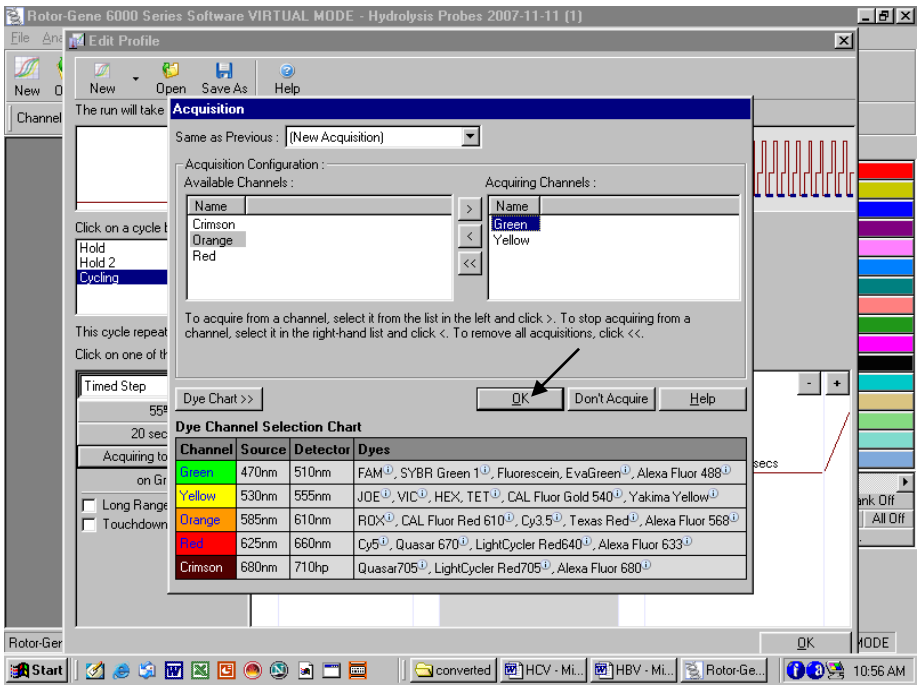


Fig. 32.

Once the Yellow and Green Channels are on the Right side then press OK as shown by the arrow.

Setting up of Extension step in the cycling profile as depicted below i.e. 72°C for 15 Seconds

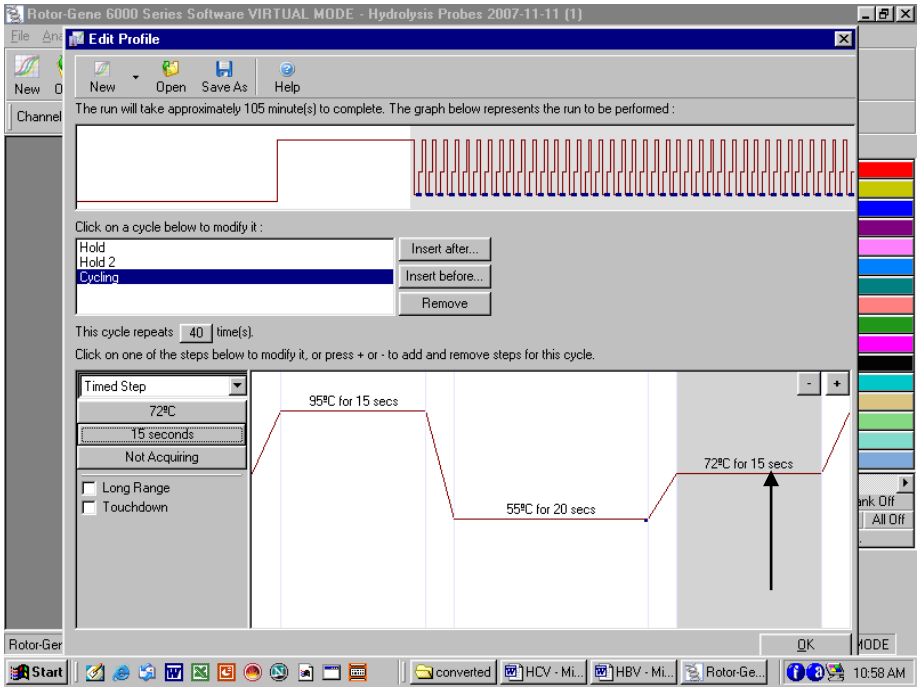


Fig. 33.

Setting up of Number of Cycles to 45 cycles in the cycling profile as depicted below.

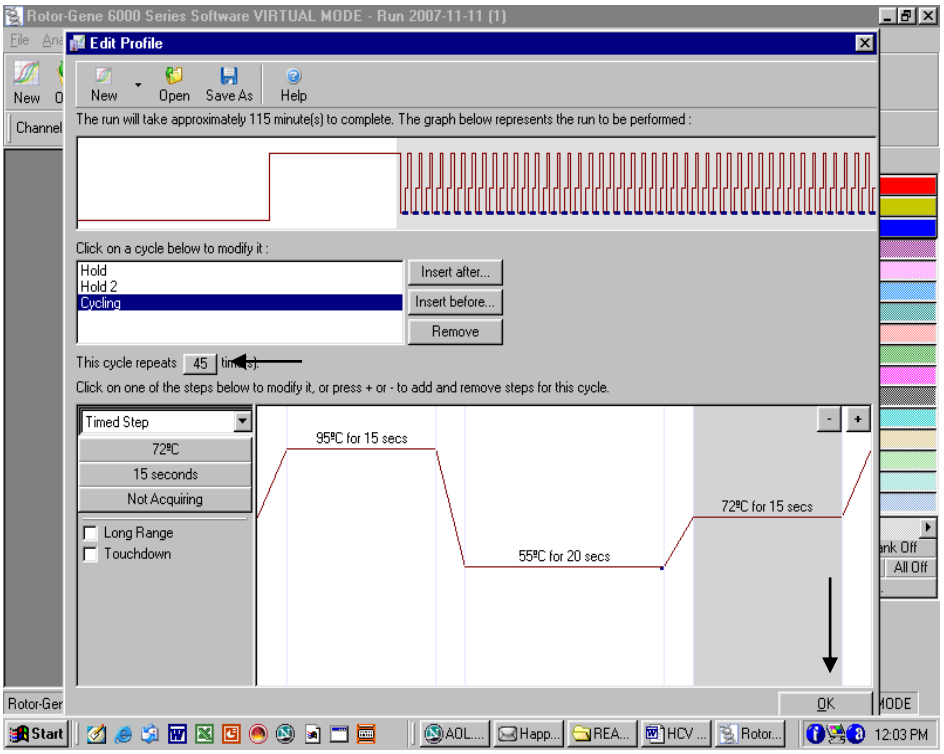


Fig. 34.

After setting the number of Cycles Press OK.

k) Setting the gains for the acquiring channel by clicking at the Gain Optimisation button as shown below.

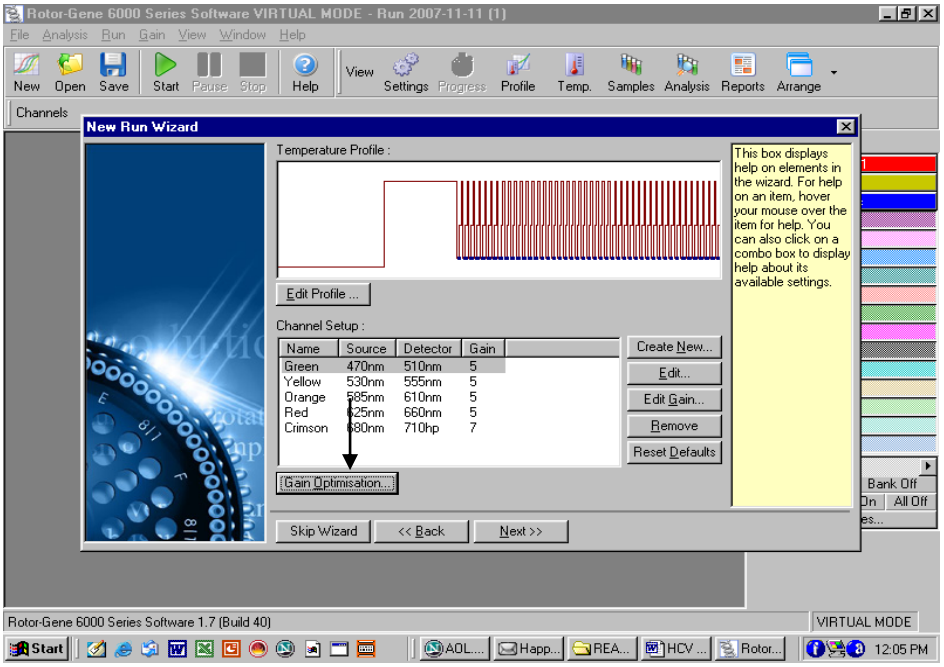


Fig. 35

The detection range of the fluorescence channels has to be determined according to the fluorescence intensities in the PCR tubes. This adjustment is done in the menu window *Auto Gain optimization Setup* (activation in menu window *New Experiment Wizard* under Gain Optimization). Please set the temperature to the annealing temperature of the amplification program (compare Fig.36 .

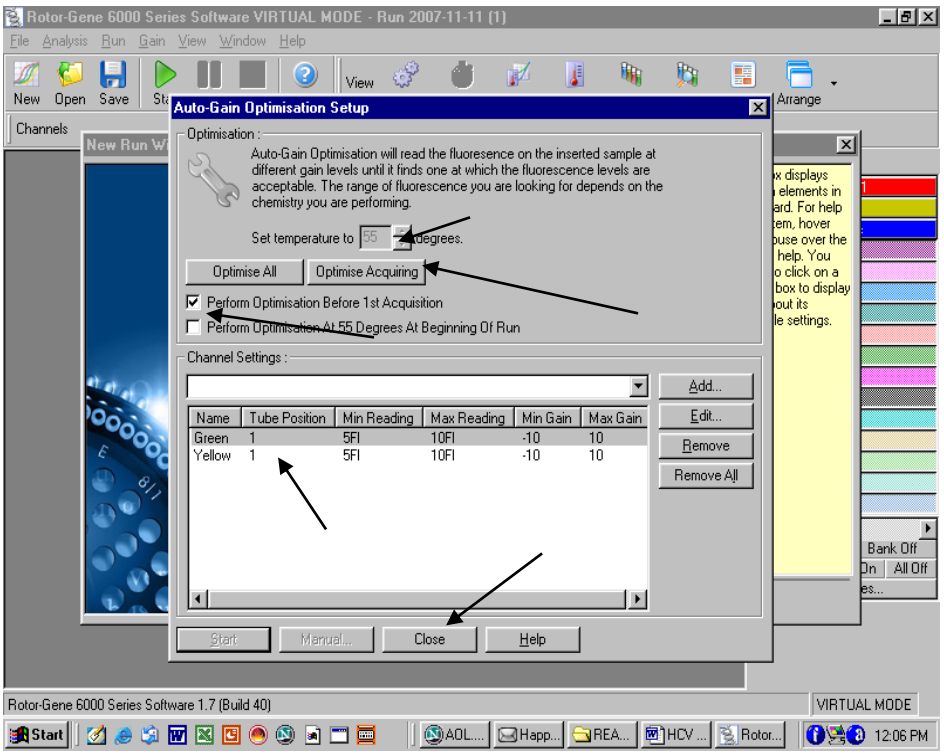


Fig. 36.

The following needs to be done.

- Click on the Optimise Acquiring
- Click on Set temperature To Adjust the Temperature to 55°C.
- Click on the Box Perform Optimisation before 1st Acquisition.
- Just see that below the channel settings there appear only two channels i.e. Green and Yellow. In case it is different then the channels have not been set properly. Close the window and reset the channels.
- Then Press Close.
- The press Next as shown below.

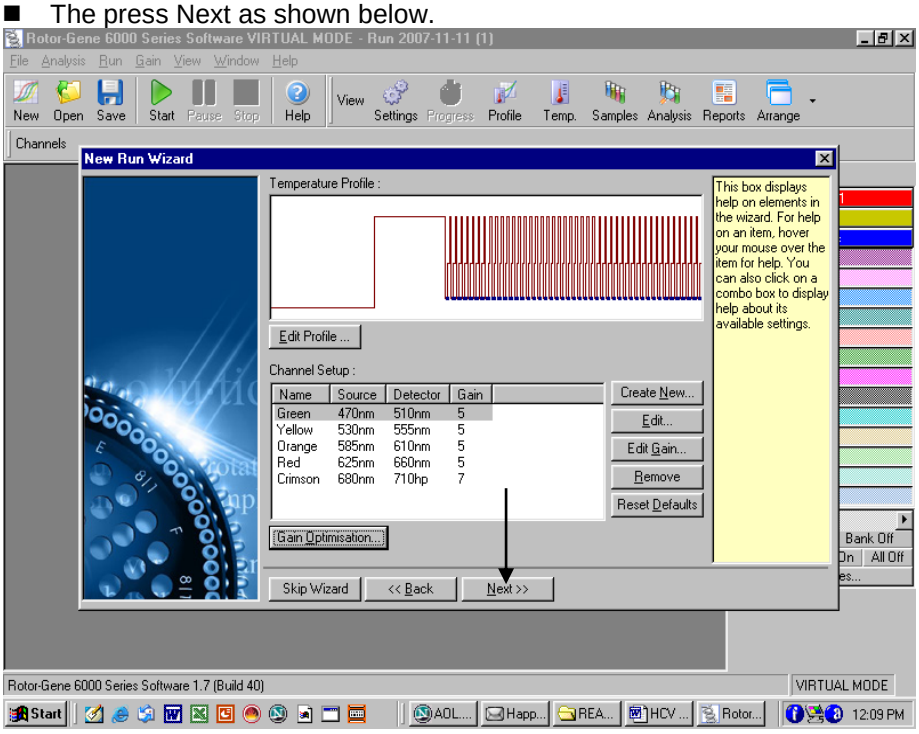


Fig. 37.

L) PRESS Start RUN

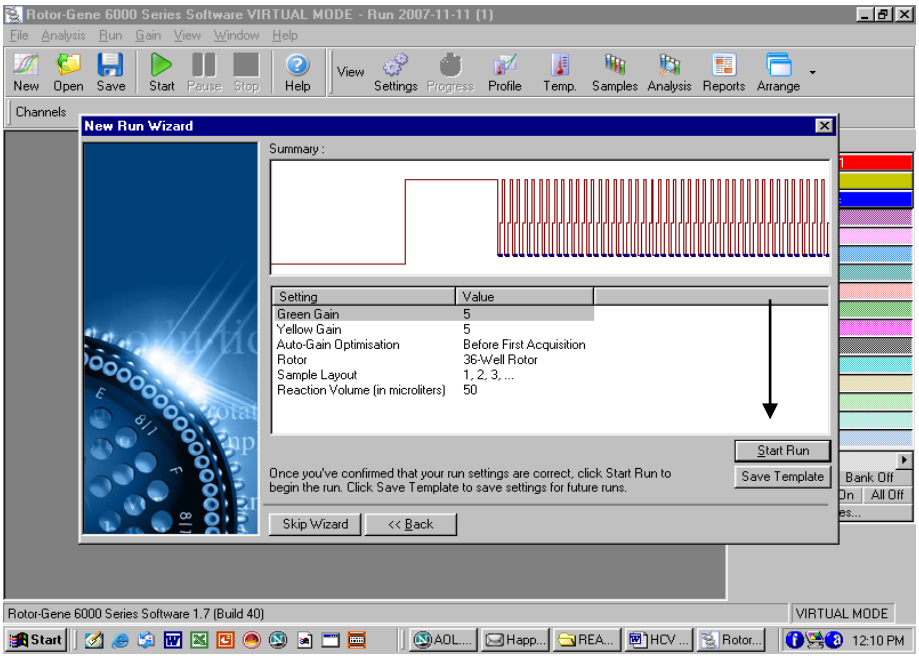


Fig. 38.

Saving the RUN File.

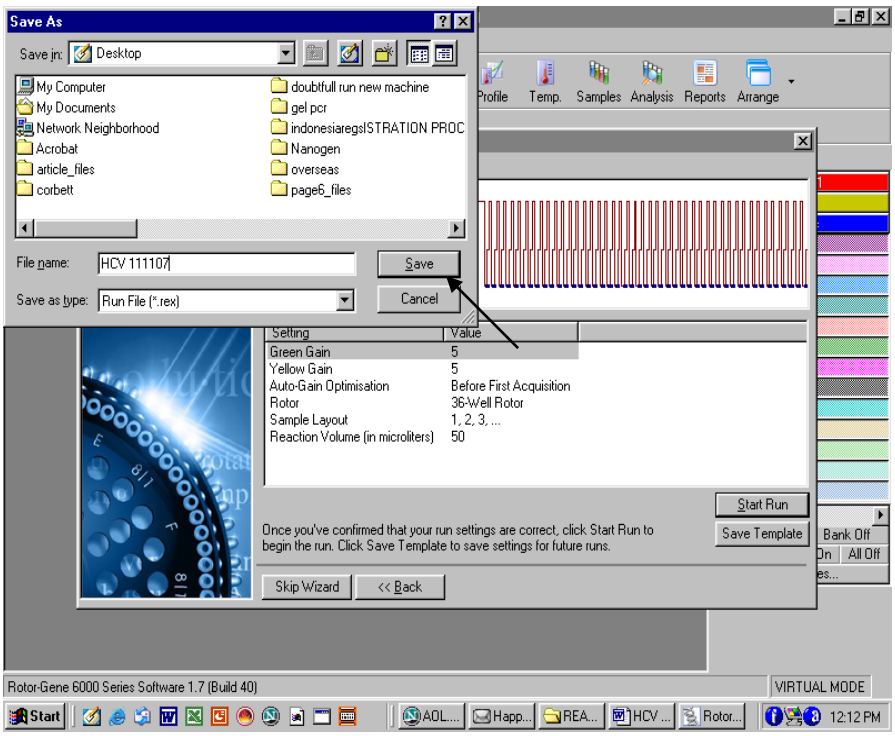


Fig. 39.

Store the run file either in my documents or a designated folder as shown above.

The moment save button is clicked after the file name, machine will start.

9. Generated Data Interpretation & Analysis

a) For Rotor Gene 6000

Data analysis is performed with the *RotorGene*™ software according to the manufacturer's instructions (*RotorGene*™ 6000 *Operator's* Manual).

The following results are possible:

A signal is detected in fluorescence channel Cycling A.FAM.

The result of the analysis is positive: The sample contains HCV RNA.

In fluorescence channel Cycling A.FAM no signal is detected.

No HCV RNA detectable. It can be considered negative if there is amplification in the Joe Channel. Please refer for details on PCR inhibition in the section PCR inhibition.

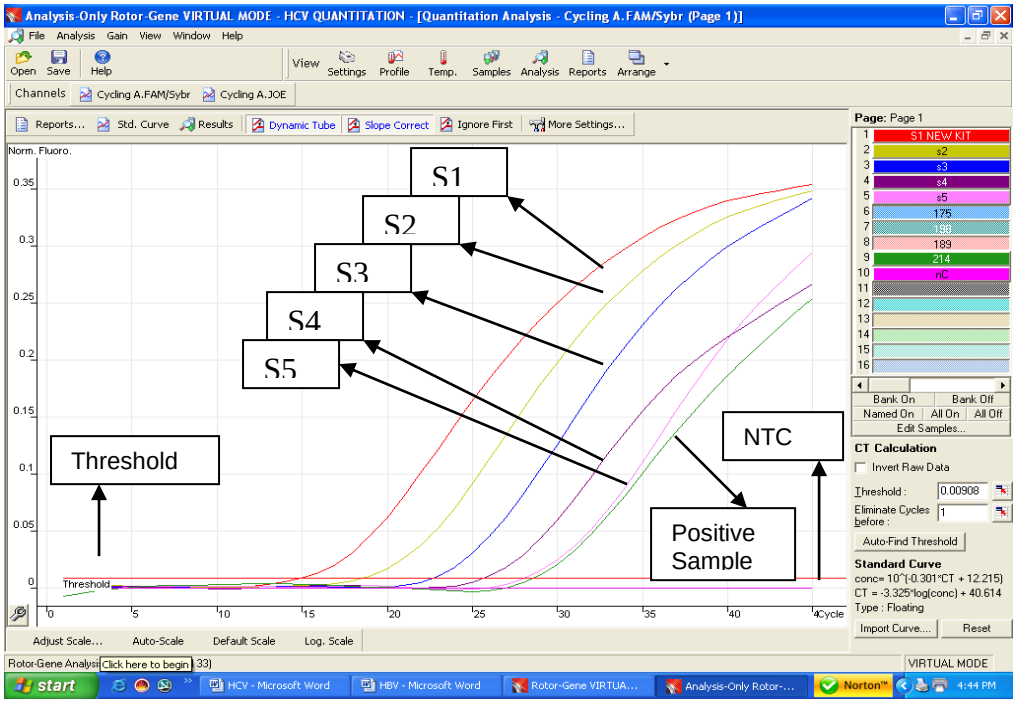


Fig. 40: Detection of the quantitation standards *(HCV S 1-5)* in fluorescence channel Cycling A.FAM. NTC: non-template control.

Examples of positive and negative PCR reactions are given in the above figure.

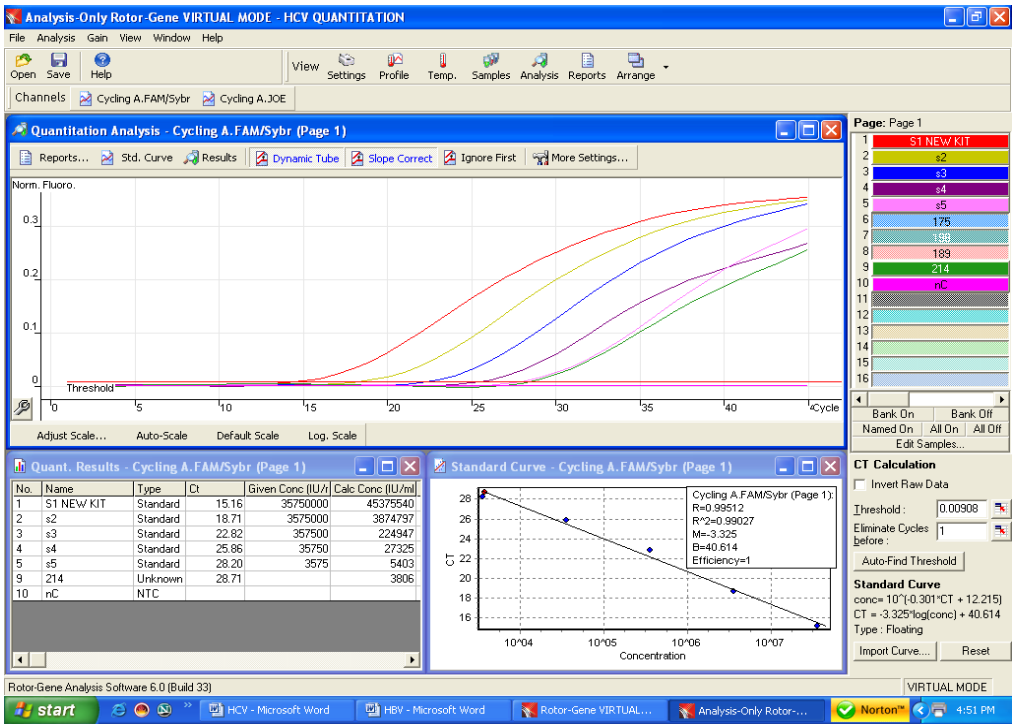


Fig. 41.

Example of analysed data for HCV where all the amplification curves can be seen as well as The standard curve, reaction efficiency, M value etc. can be seen and the quantitative data for the samples can be seen.

Inhibition Control gene amplification.

Analyze the Joe Channel

PCR Inhibition

A signal is detected in fluorescence channel Cycling A. Joe: **No PCR inhibition**

In fluorescence channel Cycling A. Joe no signal is detected.
But signal detected in FAM: The sample is positive for HCV RNA.

In fluorescence channel Cycling A. Joe no signal is detected.
No signal detected in FAM as well: A possible PCR inhibition has occurred.

9. Generated Data Interpretation & Analysis

b) For Rotor Gene 6000

Data analysis is performed with the *RotorGene*™ software according to the manufacturer's instructions (*RotorGene*™ 6*000 Operator's* Manual).

The following results are possible:

A signal is detected in fluorescence channel Cycling A.Green.

The result of the analysis is positive: The sample contains HCV RNA.
In fluorescence channel Cycling A.Green no signal is detected.
No HCV RNA detectable. It can be considered negative if there is amplification in the Yellow Channel. Please refer for details on PCR inhibition in the section PCR inhibition.

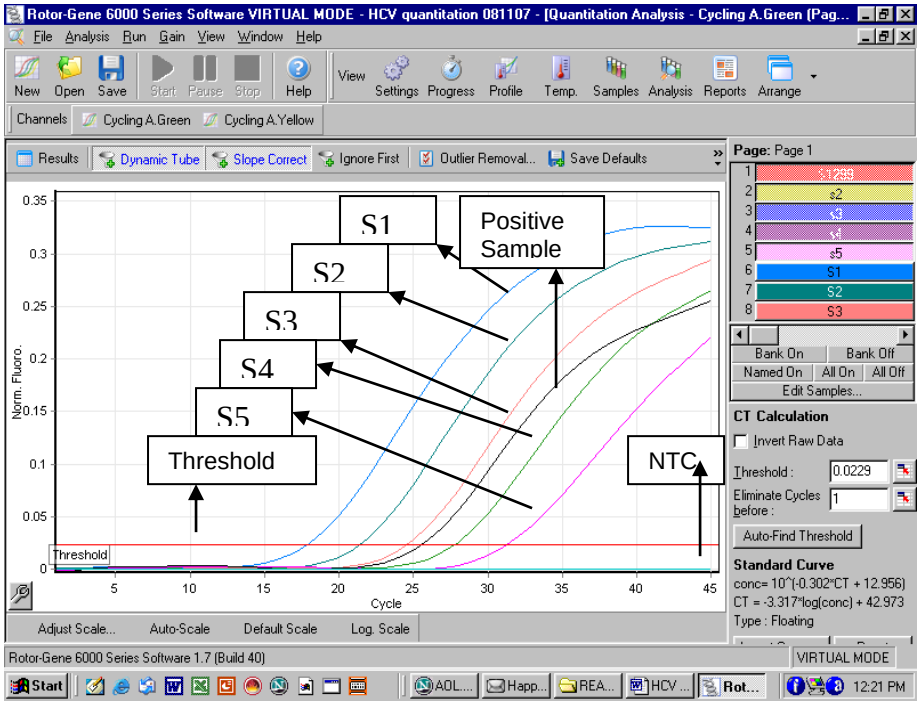


Fig. 42: Detection of the quantitation standards *(HCV S 1-5)* in fluorescence channel Cycling A.Green. NTC: non-template control.

Examples of positive and negative PCR reactions are given in the above figure.

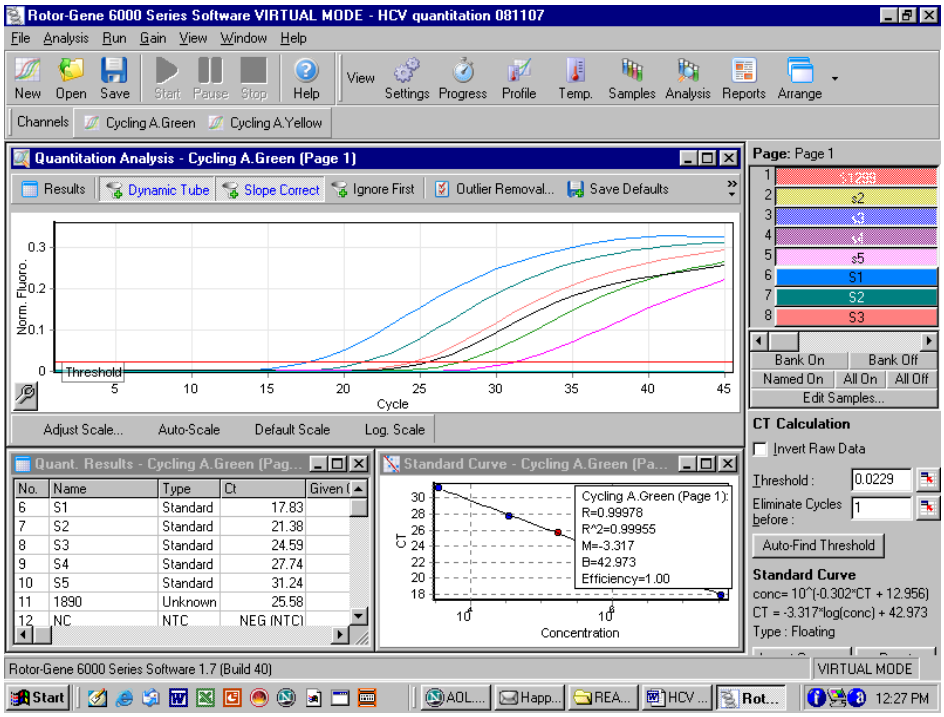


Fig. 43.

Example of analysed data for HCV where all the amplification curves can be seen as well as The standard curve, reaction efficiency, M value etc. can be seen and the quantitative data for the samples can be seen.

Inhibition Control gene amplification.

Analyze the Yellow Channel

PCR Inhibition

A signal is detected in fluorescence channel Cycling A. Yellow: **No PCR inhibition**

In fluorescence channel Cycling A. Yellow no signal is detected.
But signal detected in Green: The sample is positive for HCV RNA.

In fluorescence channel Cycling A. Yellow no signal is detected.
No signal detected in Green as well: A possible PCR inhibition has occurred.

10. Troubleshooting

For Rotor Gene 6000

1. No signal with positive Standards (HCV *S 1-5*) in fluorescence channel Cycling A.Green.

- Incorrect programming of the *Rotor-Gene*™ 6*000.*

  ➔ Repeat the PCR with corrected settings.

2. Weak or no signal in fluorescence channel Cycling A. Green:

- The PCR conditions do not comply with the protocol.

  ➔ Repeat the PCR with corrected settings.

- The HCV Super Mix *R1* has been thawed and frozen too often.
- The HCV Super Mix R1 has been kept at +4°C for longer than 5 hours.

  ➔ Please mind the storage conditions given in the **Storage.**
  ➔ Repeat the assay using a new HCV super mix (R1).

- **The PCR was inhibited.**

➔ Make sure that you use a recommended extraction method (see **8.a. RNA extraction**) and stick closely to the manufacturer's instructions.

11. Specifications

11.a Sensitivity and Reproducibility

In order to determine the sensitivity of the ***Geno-Sen's®*** HCV *Real Time PCR RG Kit*, a dilution series has been set up from 10^7 IU/ul down to 10^0 IU/µl of HCV In-Vitro Transcription and analyzed with the ***Geno-Sen's®*** HCV Real Time PCR RG kit ***using*** *Rotor Gene 3000/6000 systems. T*he assays were carried out in triplicate except for the last dilution run in 5 tubes. The results were determined by a probit analysis. The detection limit as determined for ***Geno-Sen's®*** HCV *Real Time PCR RGKit* is consistently 30 IU/ML. This means that there is 95% probability that 30 IU/ml will be detected.

Analytical Sensitivity in Conjunction with the ***Geno-Sen's®*** **Viral RNA Extraction Mini Kit — for RNA purification (Cat. No. 98001) of the** ***Geno-Sen's®*** *HCV Real Time PCR RG Kit on Rotor Gene™ was determined by Spiking a known negative Serum to a nominal 35 IU/ml. This was subjected to extraction using the* ***Geno-Sen's®*** **Viral RNA Extraction Mini Kit — for RNA purification (Cat . No. 98001) eight times with starting volume of 150**µl & elution volume of 60µl.

All the Eight extractions were then analyzed with the ***Geno-Sen's®*** HCV *Real Time PCR RG Kit along with the standards & the NTC.*

All the eight extractions were amplified correctly & the mean value obtained for the eight replicates was 36 IU/ml.

Hence Analytical Sensitivity in Conjunction with the ***Geno-Sen's®*** **Viral RNA Extraction Mini Kit — for RNA purification (Cat . No. 98001) of the** ***Geno-Sen's®*** HCV *Real Time PCR RG Kit on Rotor Gene 3000/6000 was determined to be 35 IU/ml.*

11.b Specificity

The specificity of the ***Geno-Sen's®*** HCV *Real Time PCR RG Kit is ensured by selection of very specific Primers & probes. This is also ensured by stringent reaction conditions. The primers & probes were blasted for possible homologies to all sequences published in the GeneBank. It was found that the primers & probes are very specific to all the subtypes of HCV.*

To further Validate the stringent data In order to check the specificity of the ***Geno-Sen's®*** HCV *Real Time PCR RG Kit*, different RNA & DNA listed below were analyzed with

Geno-Sen's® HCV *Real Time PCR RG Kit*. None of these led to a positive signal with the ***Geno-Sen's***® HCV *Real Time PCR RG Kit*.

Vericella Zoster Virus	HIV 2	N. Meningitis
Human Herpes Virus 6	Parvovirus B 19	S. Pneumonia
Human Herpes Virus 7	TTV	Chlamydia pneumonia
Cytomagalovirus	West Nile Virus	MTb DNA
Hepatitis B Virus	EnteroVirus	B.pseudomallie
Hepatitis A Virus	Hantaan Virus	Staphylococcus aureus
HCV	JEV	Salmonella enteritidis
Epstein barr Virus	Chikungunya	Scrub typhus
Hepatitis E Virus	H. Influenza	Leptospira interrogans.
Dengue	Malaria	Leprosy
HSV 1 DNA	HSV 2 DNA	

In order to validate the Specificity further with wet lab testing the following Known clinical samples were analyzed using the ***Geno-Sen's***® HCV Real Time PCR RG kit on Rotor Gene 3000/6000. The extraction was carried out with the ***Geno-Sen's***® **Viral RNA Extraction Mini Kit — for RNA purification (Cat . No. 98001)**

The run was carried out with the known set of standards in order to quantiate the HCV RNA Gene.

Sample Type	Serum	Plasma	Liver Biopsy
High +ve's	6	2	3
Medium +ve's	15	10	4
Low +ve's	12	6	2
Extremely low +ve's	9	8	
Negative samples.	6	6	
	48	32	9

All the above samples were correctly identified by the ***Geno-Sen's***® HCV Real Time PCR RG kit & all the 17 extremely low samples were accurately detected by the ***Geno-Sen's***® HCV Real Time PCR RG kit & exhibited copies around 35 IU /ml or less than 35 IU/ml.

External Evaluation results:

Currently the external evaluations are being pursued & the results of the same will be incorporated soon.

11.C Linear Range:

The Linear range of the ***Geno-Sen's***® HCV Real Time PCR RG kit was determined by analyzing a dilution series of the HCV In-Vitro Transcription ranging from 10^7 IU/µl down to 10^7 IU/µl of HCV In-Vitro Transcription and analyzed with the ***Geno-Sen's***® HCV *Real Time PCR RG Kit*.

All the Dilutions were run in triplicate except the lowest dilution which was run in 5 wells using the ***Geno-Sen's***® HCV *Real Time PCR RG* kit & Rotor Gene 3000/6000.

The Linear Range of the ***Geno-Sen's***® HCV *Real Time PCR RG Kit* has been determined to cover concentrations from 10^7 IU/µl down to 10^1 IU/µl.

In terms of IU per ml this translates to 4 x 10^9 IU/ml down to 30 IU/ml.

Conversion of IU to Genome Copies/ml

As per the Latest WHO report on Standardization of the NAT assays One IU of HCV is equal to 2.7 Genome Copies.

Hence for conversion of Patient results to genome copies/ml the results obtained in IU/ml should be multiplied by 2.7 to obtain GEQ.

Further studies are underway on this aspect.

12. Warranty::

Products are guaranteed to confirm to the quality and content indicated on each vial and external labels during their shelf life. Genome Diagnostics Pvt. Ltd. obligation and purchaser's rights under the warranty are limited to the replacement by Genome Diagnostics Pvt. Ltd of any product that is shown defective in fabrication, and that must be returned to Genome Diagnostics Pvt. Ltd Freight prepaid, or at Genome Diagnostics Pvt. Ltd. option, replacement of the purchasing price. Any complaint on damaged goods during transport must be directed to the handling or transport agent.

In Vitro Diagnostic Medical device, as per the Directive 98/79/EC specifications. This product must be used by qualified professionals only. It is the responsibility of the user to ascertain that a given product is adequate for a given application. Any product not fulfilling the specifications included in the product sheet will be replaced. This warranty limits our responsibility to the replacement of the product. No other warranties, of any kind, express or implied, including without limitation, implicit warranties of commercialization ability or adequacy for a given purpose, are provided by Genome Diagnostics Pvt. Ltd. Genome Diagnostics Pvt. Ltd will not be responsible for any direct, indirect, consequential or incidental damage resulting of the use, misuses, results of the use or inability to use any product.

13. Limitations of product use:

a.) All reagents may exclusively be used for *in vitro* diagnostics.
b.) The product is to be used by personnel specially instructed and trained in for the *in-vitro* diagnostics procedures only.
c.) It is important to pipet the indicated quantities, and mix well after each reagent addition. Check pipettes regularly.
d.) Instructions must be followed Correctly in order to obtain correct results. If the user has any questions, please contact our Technical Dept. (dharam.vsnl@gmail.com or at genome24@rediffmail.com).
e.) This test has been validated for use with the reagents provided in the kit. The use of other Reagents or methods, or the use of equipment not fulfilling the

specifications, may render equivocal results. User is responsible for any modifications done in any of the indicated parameters. Compliance with the kit protocol is required.

f.) Detection of Viral RNA depends on the number of Viruses present in the sample, and can be affected by sample collection methods, patient-related factors (e.g. age, symptoms), or for infection stage and sample size.

g.) False negative results may be obtained due to polymerase inhibition. It is recommended to perform control reactions to distinguish between inhibition and true negatives. This is achieved by the inhibition Control included in the kit.

h.) Cross contamination between samples and exogenous DNA can only be avoided by following good laboratory practice. Instructions in this document must be strictly followed.

i.) Attention should be paid to expiration dates printed on the kit box and labels of all components. Do not use expired components.

If you have any further questions or problems, please contact our technical support at (dharam.vsnl@gmail.com or at genome24@rediffmail.com).

14. PUBLICATION

1. Viral Load Analysis of Hepatitis C Virus in Huh7.5 Cell Culture System

2015 May; 8(5): e19279.
Published online 2015 May 31. doi: 10.5812/jjm.8(5)2015.19279
Roghayeh Teimourpour,[1] Zahra Meshkat,[1,*] Aida Gholoubi,[2] Hosein Nomani,[1] and Sina Rostami[3] IRAN

2. The Relationship between Distribution of HCV-RNA and ALT-AST Levels with Genotypes of Hepatitis C Virus Infected Patients

Viral Hepat J 2013; 19: 67-70
10.4274 / Vhd.57966
Ahmet ÇALIŞKAN Sümeyra ALKIŞ KOÇTÜRK Pınar ERDOĞMUŞ Mustafa GÜL Assistant Professor

3. Correlation Study Between HCV Genotypes Distribution Pattern and Viral Load in a Tertiary Care Hospital in Kolkata, India

Debojyoti Bhattacharjee[1], Kheya Mukherjee[2], Goutam Chakroborti[3], Ranadeep Ghosh[4], Nabarun Mandal[5], Mohua Bose[6]

4.Significance of RT-PCR over ELISA Technique for the Detection of HCV in Blood Donor

Zinab OA1 , Hock TT1 , AbdelHamid AZ1 , Rozline Hassan2 .
1 Infectious Disease Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia,2 Transfusion Medicine Unit, Hospital University Science Malaysia, Kelantan, Malaysia. (Received October 25th, 2009. Accepted December 24th 2009. Published Online December 31st, 2009.) Correspondence: Tang Thean Hock Email: tangth.amdi@gmail.com

5.Hepatitis C Virus (RNA)A 2012 EQA Programme Final Report QAV994112 (HCVRNA12A)

Professor Jacques Izopet Scientific Expert on behalf of QCMD Report authorised by the QCMD Executive in July 2012

14. LIST OF GENO-SEN'S RANGE OF REAL TIME PCR KITS

S.NO.	PRODUCT
1	HIV-1 RG quantitative Real time PCR kit.
2	HBV RG quantitative Real time PCR kit.
3	HCV RG quantitative Real time PCR kit.
4	HCV Genotyping 1/2/3/4 RG qualitative Real time PCR kit.
5	HEV RG quantitative Real time PCR kit.
6	HAV RG quantitative Real time PCR kit.
7	JEV RG quantitative Real time PCR kit.
8	ENTEROVIRUS RG quantitative Real time PCR kit.
9	DENGUE RG quantitative Real time PCR KIT
10	HSV 1 & 2 RG quantitative Real time PCR kit.
11	CMV RG quantitative Real time PCR kit.
12	Hanta Virus RG quantitative Real time PCR kit.
13	Measles Virus RG quantitative Real time PCR kit.
14	West Nile Virus RG quantitative Real time PCR kit.
15	H5 N1 (Bird Flu) RG quantitative Real time PCR kit.
16	Chikungunya RG quantitative Real time PCR kit.

17	TTV RG quantitative Real time PCR kit.
18	SARS RG quantitative Real time PCR kit.
19	JC/BK Virus RG quantitative Real time PCR kit.
20	MTb Complex RG quantitative Real time PCR kit.
21	MTb Complex /MOTT RG quanlitative Real time PCR kit.
22	Chlamydia pneumonia RG quantitative Real time PCR kit.
23	Streptococcous pneumonia RG quantitative Real time PCR kit.
24	N. Meningitis RG quantitative Real time PCR kit.
25	H. Influenza RG quantitative Real time PCR kit.
26	Leprosy RG quantitative Real time PCR kit.
27	Helicobacter Pylori RG quantitative Real time PCR kit.
28	Scrub Typhus RG quantitative Real time PCR kit.
29	B. Pseudomalie RG quantitative Real time PCR kit.
30	Filaria RG quantitative Real time PCR kit.
31	Leptospira(pathogenic) RG quantitative Real time PCR kit.
32	CCL3-L1 RG quantitative Real time PCR kit.
33	Malaria (P. Vivax) RG quantitative Real time PCR kit.
34	Bcr/abl Major RG quantitative Real time PCR kit.
35	Bcr/abl Minor RG quantitative Real time PCR kit.
36	PML/RARA RG quantitative Real time PCR kit.
37	RARA/PML RG quantitative Real time PCR kit.
38	GAPDH RG quantitative Real time PCR kit.
39	β-Actin RG quantitative Real time PCR kit.
40	β-Globin RG quantitative Real time PCR kit.

41	**Abl gene RG quantitative Real time PCR kit.**
42	**Rabies RG quantitative Real time PCR kit.**
43	**Factor V Leiden detection RG Real time PCR kit.**

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