Geno-Sen's

HBV 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.,

(For In Vitro Diagnostic Use)

PACK INSERT

Revised 2017

Genome Diagnostics Pvt. Ltd.

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

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1. Contents of the Kit:

Color Code	Contents	Cat. No. 9111004 100 rxns	Cat. No. 9111005 50 rxns	Cat. No. 9111006 25 rxns
R1 Blue	HBV 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
HBV-S1 Red	*HBV Standard 1* *1 X 10^5 IU/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
HBV-S2 Red	*HBV Standard 2* *1 X 10^4 IU/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
HBV-S3 Red	*HBV Standard 3* *1 X 10^3 IU/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
HBV-S4 Red	*HBV Standard 4* *1 X 10^2 IU/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
HBV-S5 Red	*HBV 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. HBV Information

Application

Hepatitis B virus, abbreviated HBV, is a species of the genus *Orthohepadnavirus*, which is likewise a part of the *Hepadnaviridae* family of viruses. This virus causes

the disease hepatitis B. In addition to causing hepatitis B, infection with HBV can lead to cirrhosis and hepatocellular carcinoma. It has also been suggested that it may increase the risk of pancreatic cancer.

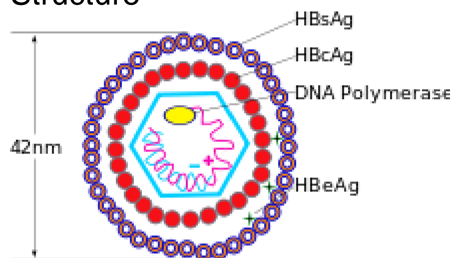
Classification

The hepatitis B virus is classified as the type species of the *Orthohepadnavirus*, which contains three other species: the *Ground squirrel hepatitis virus*, *Woodchuck hepatitis virus*, and the *Woolly monkey hepatitis B virus*. The genus is classified as part of the *Hepadnaviridae* family, which contains two other genera, the *Avihepadnavirus* and a second which has yet to be assigned. This family of viruses have not been assigned to a viral order. Viruses similar to hepatitis B have been found in all the Old World apes (orangutan, gibbons, gorillas and chimpanzees) and from a New World woolly monkey suggesting an ancient origin for this virus in primates.

The virus is divided into four major serotypes (adr, adw, ayr, ayw) based on antigenic epitopes present on its envelope proteins, and into eight genotypes (A-H) according to overall nucleotide sequence variation of the genome. The genotypes have a distinct geographical distribution and are used in tracing the evolution and transmission of the virus. Differences between genotypes affect the disease severity, course and likelihood of complications, and response to treatment and possibly vaccination.

Morphology

Structure



The structure of a Hepatitis B virus

Hepatitis B virus is a member of the Hepadnavirus family. The virus particle, (virion) consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of protein. The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity similar to retroviruses. The outer envelope contains embedded proteins which are involved in viral binding of, and entry into, susceptible cells. The virus is one of the smallest enveloped animal viruses with a virion diameter of 42 nm, but pleomorphic forms exist, including filamentous and spherical bodies lacking a core. These particles are not infectious and are composed of the lipid and protein that forms part of the surface of the virion, which is called the surface antigen (HBsAg), and is produced in excess during the life cycle of the virus.

Components of HBV

It consists of:

- HBsAg
- HBcAg (HBeAg is a splice variant)
- Hepatitis B virus DNA polymerase
- HBx. The function of this is not yet well known.
- Hepatitis D virus requires HBV envelope particles to become virulent.

Evolution

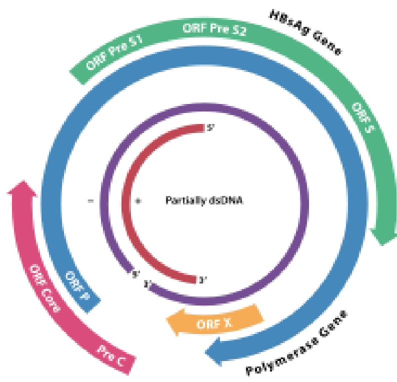
The early evolution of the Hepatitis B, like that of all viruses, is difficult to establish. The divergence of orthohepadnavirus and avihepadnavirus occurred ~125,000 years ago (95% interval 78,297–313,500). Both the Avihepadnavirus and Orthohepadna viruses began to diversify about 25,000 years ago. The branching at this time lead to the emergence of the Orthohepadna genotypes A-H. Human strains have a most recent common ancestor dating back to 7,000 (95% interval: 5,287–9,270) to 10,000 (95% interval: 6,305–16,681) years ago.

The Avihepadnavirus lack a X protein but a vestigial X reading frame is present in the genome of duck hepadnavirus. The X protein may have evolved from a DNA glycosylase.

The rate of nonsynonymous mutations in this virus has been estimated to be about 2×10^{-5} amino acid replacements per site per year. The mean number of nucleotide substitutions/site/year is ~7.9×10^{-5}.

A second estimate of the origin of this virus suggests a most recent common ancestor of the human strains evolved ~1500 years ago. The most recent common ancestor of the avian strains was placed at 6000 years ago. The mutation rate was estimated to be ~10^{-6} substitutions/site/year.

Genome



The genome organisation of HBV. The genes overlap.

Size of Genome

The genome of HBV is made of circular DNA, but it is unusual because the DNA is not fully double-stranded. One end of the full length strand is linked to the viral DNA polymerase. The genome is 3020–3320 nucleotides long (for the full length strand) and 1700–2800 nucleotides long (for the short length strand).

Encoding

The negative-sense, (non-coding), is complementary to the viral mRNA. The viral DNA is found in the nucleus soon after infection of the cell. The partially double-stranded DNA is rendered fully double-stranded by completion of the (+) sense strand and removal of a protein molecule from the (-) sense strand and a short sequence of RNA from the (+) sense strand. Non-coding bases are removed from the ends of the (-)sense strand and the ends are rejoined.

There are four known genes encoded by the genome called C, X, P, and S. The core protein is coded for by gene C (HBcAg), and its start codon is preceded by an upstream in-frame AUG start codon from which the pre-core protein is produced. HBeAg is produced by proteolytic processing of the pre-core protein. The DNA polymerase is encoded by gene P. Gene S is the gene that codes for the surface antigen (HBsAg). The HBsAg gene is one long open reading frame but contains three in frame "start" (ATG) codons that divide the gene into three sections, pre-S1, pre-S2, and S. Because of the multiple start codons, polypeptides of three different sizes called large, middle, and small (pre-S1 + pre-S2 + S, pre-S2 + S, or S) are produced. The function of the protein coded for by gene X is not fully understood.
Several non-coding RNA elements have been identified in the HBV genome. These include: HBV PREalpha, HBV PREbeta and HBV RNA encapsidation signal epsilon.

Genotypes

There are eight known genotypes labeled A through H. A possible new "I" genotype has been described, but acceptance of this notation is not universal. Different genotypes may respond to treatment in different ways.

The genotypes differ by at least 8% of the sequence and have distinct geographical distributions and this has been associated with anthropological history. Type F which

diverges from the other genomes by 14% is the most divergent type known. Type A is prevalent in Europe, Africa and South-east Asia, including the Philippines. Type B and C are predominant in Asia; type D is common in the Mediterranean area, the Middle East and India; type E is localized in sub-Saharan Africa; type F (or H) is restricted to Central and South America. Type G has been found in France and Germany. Genotypes A, D and F are predominant in Brazil and all genotypes occur in the United States with frequencies dependent on ethnicity. The E and F strains appear to have originated in aboriginal populations of Africa and the New World, respectively.

Within genotypes 24 subtypes have been described which differ by 4-8% of the genome.

- Type A has two subtypes: Aa (A1) in Africa/Asia and the Philippines and Ae (A2) in Europe/United States.

- Type B has two distinct geographical distributions: Bj/B1 ('j' — Japan) and Ba/B2 ('a' — Asia). Type Ba has been further subdivided into four clades (B2 — B4).

- Type C has two geographically subtypes: Cs (C1) in South-east Asia and Ce (C2) in East Asia. The C subtypes have been divided into five clades (C1 — C5). A sixth clade (C6) has been described in the Philippines but only in one isolate to date. Type C1 is associated with Vietnam, Myanmar and Thailand; type C2 with Japan, Korea and China; type C3 with New Caledonia and Polynesia; C4 with Australia; and C5 with the Philippines. A further subtype has been described in Papua, Indonesia.

- Type D has been divided into 7 subtypes (D1 — D7).

- Type F has been subdivided into 4 subtypes (F1 — F4). F1 has been further divided in to 1a and 1b. In Venezuela subtypes F1, F2, and F3 are found in East and West Amerindians. Among South Amerindians only F3 was found. Subtypes Ia, III, and IV exhibit a restricted geographic distribution (Central America, the North and the South of South America respectively) while clades Ib and II are found in all the Americas except in the Northern South America and North America respectively.

Hepatitis

The term 'hepatitis' simply means inflammation of the liver. Hepatitis may be caused by a virus or a toxin such as alcohol. Other viruses that can cause injury to liver cells include the hepatitis A and hepatitis C viruses. These viruses are not related to each other or to hepatitis B virus and differ in their structure, the ways they are spread among individuals, the severity of symptoms they can cause, the way they are treated, and the outcome of the infection.
Hepatitis B is an infection of the liver caused by the hepatitis B virus (HBV). It is estimated that 350 million individuals worldwide are infected with the virus, which

causes 620,000 deaths worldwide each year. According to the Centers for Disease Control (CDC), approximately 46,000 new cases of hepatitis B occurred in the United States in 2006.

In the United States, rates of new infection were highest among people aged 25 to 44 years (3.1 cases per 100,000 population) and lowest among those younger than 15 years of age (0.02 per 100,000). This reflects the major modes of transmission of hepatitis B (sexual transmission, illicit drug use, exposure to infected blood) and the effect of universal vaccination of infants. In the United States, there has been a 75% decrease in newly diagnosed cases of hepatitis B during the past decade. This decrease is attributed to increased vaccination and to heightened public awareness of HIV/AIDS and the resulting safer sexual practices.

When a person first gets hepatitis B, they are said to have an 'acute' infection. Most people are able to eliminate the virus and are cured of the infection. Some are not able to clear the virus and have 'chronic' infection with hepatitis B that is usually life-long (see below). In the United States an estimated 800,000 to 1.4 million people are chronically infected with hepatitis B. Hepatitis B is found throughout the world. Some countries have much higher rates of infection than the United States; for example, in Southeast Asia and Sub-Saharan Africa, as many as 15% to 20% of adults are chronically infected with hepatitis B.

The hepatitis B virus reproduces in liver cells, but the virus itself is not the direct cause of damage to the liver. Rather, the presence of the virus triggers an immune response from the body as the body tries to eliminate the virus and recover from the infection. This immune response causes inflammation and may seriously injure liver calls. Therefore, there is a balance between the protective and destructive effects of the immune response to the hepatitis B virus.

Hepatitis B is spread mainly by exposure to infected blood or body secretions. In infected individuals, the virus can be found in the blood, semen, vaginal discharge, breast milk, and saliva. Hepatitis B is not spread through food, water, or by casual contact.

In the United States, sexual contact is the most common means of transmission, followed by using contaminated needles for injecting illicit drugs, tattooing, body piercing, or acupuncture. Additionally, hepatitis B can be transmitted through sharing toothbrushes and razors contaminated with infected fluids or blood.

Hepatitis B also may be spread from infected mothers to their babies at birth (so-called 'vertical' transmission). This is the most prevalent means of transmission in regions of the world where hepatitis B rates are high. The rate of transmission of hepatitis B from mother to newborn is very high, and almost all infected infants will develop chronic hepatitis B. Fortunately, transmission can be significantly reduced through immunoprophylaxis (see below).

Rarely, hepatitis B can be transmitted through transfused blood products, donated livers and other organs. However, blood and organ donors are routinely screened for hepatitis which typically prevents this type of transmission.

Symptoms of hepatitis B

Acute hepatitis B is the period of illness that occurs during the first one to four months after acquiring the virus. Only 30% to 50% of adults develop significant symptoms during acute infection. Early symptoms may be non-specific, including fever, a flu-like illness, and joint pains. Symptoms of acute hepatitis may include:

- fatigue,
- loss of appetite,
- nausea,
- jaundice (yellowing of the skin and eyes), and
- pain in the upper right abdomen (due to the inflamed liver).

Rarely, acute hepatitis damages the liver so badly it can no longer function. This life-threatening condition is called "fulminant hepatitis." Patients with fulminant hepatitis are at risk of developing bleeding problems and coma resulting from the failure of the liver. Patients with fulminant hepatitis should be evaluated for liver transplantation. Small studies suggest that the drug lamivudine (Epivir), may be of limited assistance in these cases.

The body's immune response is the major determinant of the outcome in acute hepatitis B. Individuals who develop a strong immune response to the infection are more likely to clear the virus and recover. However, these patients also are more likely to develop more severe liver injury and symptoms due to the strong immune response that is trying to eliminate the virus. On the other hand, a weaker immune response results in less liver injury and fewer symptoms but a higher risk of developing chronic hepatitis B. People who recover and eliminate the virus will develop life-long immunity, that is, protection from subsequent infection from hepatitis B.

Most infants and children who acquire acute hepatitis B viral infection have no symptoms. In these individuals, the immune system fails to mount a vigorous response to the virus. Consequently, the risk of an infected infant developing chronic hepatitis B is greater than 95%. In contrast, only 5% of adults who have acute hepatitis B develop chronic hepatitis B.

Diagnosis

Infection with hepatitis B is suspected when the medical history and the physical examination reveal risk factors for the infection or symptoms and signs that are suggestive of hepatitis B. Abnormalities in the liver tests (blood tests) also can raise suspicion; however, abnormal liver tests can result from many conditions that affect the liver. The diagnosis of hepatitis B can be made only with specific hepatitis B virus blood tests. These tests are known as hepatitis 'markers' or 'serology.'

Markers found in the blood can confirm hepatitis B infection and differentiate acute from chronic infection. These markers are substances produced by the hepatitis B virus (antigens) and antibodies produced by the immune system to fight the virus. Hepatitis B virus has three antigens for which there are commonly-used tests - the surface antigen (HBsAg), the core antigen (HBcAg) and the e antigen (HBeAg).

HBsAg and anti-HBs

The presence of hepatitis B surface antigen (HBsAg) in the blood indicates that the patient is currently infected with the virus. HBsAg appears an average of four weeks after initial exposure to the virus. Individuals who recover from acute hepatitis B infections clear the blood of HBsAg within approximately four months after the onset of symptoms. These individuals develop antibodies to HBsAg (anti-HBs). Anti-HBs provides complete immunity to subsequent hepatitis B viral infection. Similarly, individuals who are successfully vaccinated against hepatitis B produce anti-HBs in the blood.

Patients who fail to clear the virus during an acute episode develop chronic hepatitis B. The diagnosis of chronic hepatitis B is made when the HBsAg is present in the blood for at least six months. In chronic hepatitis B, HBsAg can be detected for many years, and anti-HBs does not appear.

Anti-HBc

In acute hepatitis, a specific class of early antibodies (IgM) appears that is directed against the hepatitis B core antigen (anti-HBc IgM). Later, another class of antibody, anti-HBc IgG, develops and persists for life, regardless of whether the individual recovers or develops chronic infection. Only anti-HBc IgM can be used to diagnose an acute hepatitis B infection.

HBeAg, anti-HBe, and pre-core mutations

Hepatitis B e antigen (HBeAg) is present when the hepatitis B virus is actively multiplying, whereas the production of the antibody, anti-HBe, (also called HBeAg seroconversion) signifies a more inactive state of the virus and a lower risk of transmission.

In some individuals infected with hepatitis B virus, the genetic material for the virus has undergone a structural change, called a pre-core mutation. This mutation results in an inability of the hepatitis B virus to produce HBeAg, even though the virus is actively reproducing. This means that even though no HBeAg is detected in the blood of people with the mutation, the hepatitis B virus is still active in these persons and they can infect others.

Hepatitis B virus DNA

The best marker of hepatitis B virus reproduction is the level of hepatitis B virus DNA in the blood. Detection of hepatitis B virus DNA in a blood sample signals that the virus is actively multiplying. In acute hepatitis, HBV DNA is present soon after infection, but is eliminated over time in patients' who clear the infection. In chronic hepatitis, levels of HBV DNA often continue to be elevated for many years and then decrease as the immune system controls the virus. HBV DNA levels are sometimes referred to as the 'viral load'.

Interpretation of the hepatitis B blood tests

The following table gives the usual interpretation for sets of results from hepatitis B blood (serological) tests.

Most Likely Status*	Tests	Results
Susceptible, not infected, not immune	HBsAg anti-HBc anti-HBs	negative negative negative
Immune due to natural infection	HBsAg anti-HBc anti-HBs	negative positive positive
Immune do to hepatitis B vaccination	HBsAg anti-HBc anti-HBS	negative negative positive
Acutely infected	HBsAg anti-HBc IgM anti-HBc anti-HBs	positive positive positive negative
Chronically infected	HBsAg anti-HBc IgM anti-HBc anti-HBs	positive positive negative negative

*Interpretation of the hepatitis B virus blood tests should always be made by an experienced clinician with knowledge of the patient's medical history, physical examination, and results of the standard liver blood tests.

Role of a liver biopsy in chronic hepatitis B

During a liver biopsy, a small sample of liver tissue is collected and examined under the microscope. This test is valuable because this sample reflects the health of the liver. It can show the amount of liver injury (inflammation or cirrhosis). Liver biopsy is not routinely needed to diagnose hepatitis B, but it is used for monitoring the progression of liver damage in people with chronic hepatitis and helping to choose or evaluate treatment options.

Natural course of chronic hepatitis B

The course of chronic hepatitis B is variable and depends on several factors. These factors are the patient's age at which the infection began, the extent of viral multiplication, and the immune system's ability to control the infection.

The infection can progress from an immune tolerant phase (in which the immune system ignores the virus)

Immune clearance phase (in which the immune system attempts to eliminate the virus)

Quiescent phase (in which the virus is less active)

For individuals infected at birth or at a young age, the immune system initially does not react to the hepatitis B virus. This phase of the infection is known as the immune tolerant phase. Despite high levels of virus in the body, there may be little evidence of inflammation and no symptoms. This phase typically lasts for years, even up to two or three decades. It is important to know that the immune tolerant phase is generally not seen in individuals who become infected during adulthood.

During the third to fourth decade of chronic hepatitis B acquired in childhood, the immune system may start to react to the virus. This is known as the immune clearance phase. In contrast, an infection acquired in adulthood usually begins with the immune clearance phase. In the immune clearance phase, the immune system attacks the hepatitis B virus-infected liver cells in an attempt to clear the virus. This causes inflammation, liver injury, and the development of scar tissue. Standard liver blood tests are abnormal, and the liver biopsy shows inflammation and/or formation of scar tissue (fibrosis). The severity of liver cell destruction, the degree of fibrosis, and the duration of the immune clearance phase determine the outcome of chronic hepatitis B. The more severe the destruction and fibrosis and the longer the phase, the more likely it is that cirrhosis will develop.

Quiescent phase

Following the immune clearance phase, the viral infection may enter a less active phase known as the quiescent phase. During this phase, there are no symptoms, the levels of hepatitis B virus become very low, and the standard liver blood tests become normal or nearly normal. Advanced scaring or cirrhosis that may have developed earlier, however, remains. Occasionally, during the quiescent phase, the virus becomes active again. This is known as a "flare," and often is associated with symptoms, abnormal liver blood tests, and further injury to the liver. The flares are caused by reactivation of the immune system against the virus. Flares can be very severe and result in further scarring of the liver. The disease in many of these individuals will progress to cirrhosis and eventually to advanced or end-stage cirrhosis with its associated complications, including liver cancer.

Infected individuals who experience a mild immune clearance phase and move into the quiescent phase are known as healthy carriers of hepatitis B virus. These individuals usually have normal liver tests and do not have symptoms; however, they can still transmit the hepatitis B viral infection to others. The risk of hepatitis B virus carriers developing cirrhosis and liver cell cancer is small although the risk is higher as compared to people without chronic hepatitis B.

Medications used to treat Hepatitis B

Acute infection

Acute infection with hepatitis B usually does not require treatment. In rare cases, however, the infection may cause life-threatening liver failure. Patients with liver failure due to acute hepatitis B should be evaluated for liver transplantation. Small studies suggest that the drug lamivudine (Epivir) may be effective in this setting.

Chronic infection

If a person is chronically infected with hepatitis B and has few signs or symptoms of complications, medications usually are not used. These patients are watched carefully and given periodic blood tests. One test measures the 'viral load,' that is, the amount of viral DNA in the blood. Doctors will recommend treatment if there are signs that the virus is beginning to cause damage or if the viral load is high. Another reason to prescribe medication is if the patient has a positive test for the Hepatitis B e-antigen (HBeAg) in the blood. HBeAg is associated with an increased risk of progression of liver disease and its complications.

In chronic hepatitis B, the goal of treatment is to reduce the risk of complications including cirrhosis and liver failure. However, it takes decades for complications to occur, which makes it difficult to study the effect of medications. As a substitute for waiting years to find out what happens, scientists have used tests like the viral load or liver function tests to evaluate if medicines are working. This is logical because it is known that people who have large amounts of the virus in their blood are at highest risk to get cirrhosis. Up to one-third of people with very high viral loads (more than one million viral copies per milliliter of blood) will develop cirrhosis over a decade, compared to only 4.5% of those with low viral loads (fewer than 300 viral copies per milliliter).

Medications can reduce the number of viruses in the body and may be able to eliminate the virus from the bloodstream. Logically, this should lead to them having a low rate of progression to cirrhosis (<1% per year), although large, long-term studies have not been done. Even in people who clear the virus from their blood, low numbers of viruses still live in the liver and other cells. Thus, the medications do not cure the disease, but they can prevent or delay complications and symptoms. People who have a good response to treatment can still transmit the virus. Doctors follow blood tests that measure viral load and liver function and they may recommend liver biopsies to evaluate if the medications are working.

The medications in current use for chronic hepatitis B include the interferons and nucleoside/nucleotide analogues. New agents are being developed although they are still under investigation and considered experimental. There are no accepted guidelines that tell how every patient should be treated. As a result, treatment is individualized.

Interferon

Interferon-alpha has been used to treat hepatitis B for more than 20 years. Interferon-alpha is a naturally occurring protein that is made in the body by white blood cells to combat viral infections. In addition to its direct anti-viral effects, interferon works against the hepatitis B virus by stimulating the body's immune system to clear the virus. Compared to older interferon alpha agents, pegylated interferon alpha, marketed as Pegasys or Pegintron, has a more convenient dosing schedule, may be slightly more effective and suppresses the virus for a longer period of time. Pegylated interferon alpha is given once a week for 48 weeks.

A significant reduction in the viral load or elimination of detectable viral DNA from the blood occurs in two-thirds of persons during treatment.

Blood tests for liver functions normalize in approximately 40% people treated with interferon.

People who have significant abnormalities in liver function before therapy are more likely to respond to treatment.

Those who have normal liver blood tests before treatment are less likely to respond to interferon therapy.

Liver biopsy results show improvement in about one-third of patients.
Only 27%-32% of persons who have Hepatitis B e-antigen (HBeAg) in their blood will be able to eliminate HBeAg and produce antibodies against the HBe antigen after treatment with interferon. Relapse may occur after treatment is stopped.

Sustained response (undetectable viral load in the blood, normal liver function tests) occurs in approximately 15% to 30% of patients after the drug is stopped. Although this is not a cure (some virus still lives in the liver and elsewhere), people with sustained response are at low risk for complications of liver disease. If the responder's immune system is compromised, for example through the use of steroids or acquiring HIV, the disease can recur. Periodic monitoring of blood tests can help confirm that the response continues to be sustained.

Interferon side effects

Fatigue, generalized muscle aches, fever, chills and loss of appetite. These flu-like symptoms occur in approximately 80% of treated patients; mood swings, depression, anxiety and other neuropsychiatric effects may occur; and thyroid gland abnormalities resulting in hypothyroidism (too little thyroid hormone); significant suppression of the bone marrow and production of blood cells; infection; or hair loss may occur.

The side effects may be severe enough that the patient is unable to continue treatment. During treatment, the normal immune response to the virus is stimulated and may cause worsening inflammation in the liver. This is normally a good sign showing that the interferon is working, but more extreme responses may in rare cases cause liver failure. Thus, physicians will monitor blood tests closely during therapy. Persons with unstable liver disease due to cirrhosis usually should not take interferon because of the increased risk of liver failure.

Nucleoside/nucleotide analogues

Nucleoside/nucleotide analogues (NAs) are man-made chemicals that mimic the nucleosides and nucleotides that are used for making DNA. When the virus tries to use the analogues to make its own DNA, it is unable to make the DNA and, therefore, cannot reproduce. Examples of these agents include adefovir (Hepsera), entecavir (Baraclude), lamivudine (Epivir-HBV, Heptovir, Heptodin), telbivudine (Tyzeka) and tenofovir (Viread).

In patients who have HBeAg in their blood, NAs reduce the viral load, causing the virus to become undetectable in 21% to 67% of patients.

Normalization of blood liver tests occurs in 40% to 77%, and loss of HBeAg occurs in approximately 12% to 22% of cases after one year of treatment.

Results are better in patients who do not have HBeAg in their blood, with 50% to 90% having non-detectable virus and 60% to 80% having normalization of liver function tests.

In a 2004 study in people who already had cirrhosis from hepatitis B, treatment with lamivudine cut the risk of liver cancer and progressive liver failure by more than 50%. Newer NAs such as entecavir (Baraclude) and telbivudine (Tyzeka) appear to have higher response rates than older agents such as lamivudine (Epivir-HBV, Heptovir, Heptodin), but there is less experience with these NAs.

Unfortunately, the hepatitis B virus may become resistant to NAs over time (see below). Adefovir may be effective against strains of virus that have become resistant to lamivudine and may be added to lamivudine when resistance appears. Simply switching from one NA to another is not recommended because this leads to virus strains that are resistant to multiple medications.

Currently, the optimal duration of treatment with nucleoside/nucleotide analogues is uncertain. Persons with HBeAg may be treated until six months after the HBeAg disappears from the blood and is replaced by antibodies (anti-HBe), if this occurs. In persons without HBeAg, the endpoints are less clear. Some experts advocate treating until the viral load (viral DNA) is undetectable and the surface antigen (HbsAg) has been cleared from the blood. Others suggest continuing medications for prolonged periods to suppress the virus. All of these strategies are hampered by the risk of the virus becoming resistant to the medications. Patients who discontinue treatment with NAs should be monitored carefully for recurrent hepatitis, which may be severe.

Resistance to nucleoside/nucleotide analogues

The major challenge associated with long-term therapy with NAs is the development of viral resistance to the NAs. This resistance results from a change (mutation) in the genetic material of the virus.

For lamivudine (Epivir-HBV, Heptovir, Heptodin), the incidence of resistance is 25% after one year and as high as 50% after three years of treatment.

With telbivudine (Tyzeka), resistance rates are 5% to 11% after one year.
Therefore, some guidelines do not recommended lamivudine or telbivudine alone as the first treatment for chronic hepatitis B.

For other NAs such as adefovir (Hepsera), resistance is less common after one year of therapy but rises to 30% after five years. Early results with entecavir (Baraclude)

suggest that resistance may be uncommon with this agent. When resistance occurs, the viral load may rise or blood liver tests may become abnormal.

There are no clear guidelines to recommend which agent to use first in treating chronic hepatitis B. Interferon is given for a defined period of time and may have a more prolonged response after the medication is discontinued than NAs. However, interferon is given as an injection, and side effects often are troublesome. NAs are given as a pill and have few side effects, but the duration of treatment is unclear, and prolonged therapy may be required. NAs may be preferred in patients with unstable disease and cirrhosis because they are thought to be less likely to cause serious flares of hepatitis with more severe liver disease.

Effects of alcohol on hepatitis B virus

Agents that damage the liver are particularly harmful in patients who already have hepatitis B. For this reason, it is recommended that persons with hepatitis B avoid drinking alcohol.

Effects of immunosuppressive medications on hepatitis B virus

Even in people with chronic hepatitis B, the immune system is working to suppress the virus. Medications that suppress the immune system allow the virus to reproduce in large numbers and may cause the hepatitis to flare.

Examples of medications that suppress the immune system are:

prednisone: used to treat many diseases, including asthma, inflammatory bowel disease, and certain types of skin disease and arthritis
methotrexate (Rheumatrex, Trexall): used to treat certain types of skin disease, arthritis, and cancer;
cyclophosphamide (Cytoxan): used to treat some cancers.
If an immunosuppressant drug is stopped, the body's immune system's activity may rebound and cause severe inflammation of the liver.

Delta hepatitis

Delta hepatitis is caused by a virus that only infects people who already have hepatitis B. The delta hepatitis virus (also known as hepatitis D or HDV) is an RNA virus, meaning that its genetic material is made up of ribonucleic acid. It is spread through exposure to contaminated blood, especially with illicit, intravenous drug use, and by sexual contact. Delta hepatitis can be acquired at the same time as acute hepatitis B. When this happens, infected people are quite sick but more than 95% are eventually able to eliminate the viruses from their bodies. People who already have chronic hepatitis B can acquire delta hepatitis as well. This often causes severe inflammation of the liver, and the viruses are less likely to be cleared.
Delta hepatitis makes chronic hepatitis B much worse. It increases the risk of complications, especially cirrhosis, which occurs in up to two-thirds of patients.
There is no vaccine against delta hepatitis. Interferon treatment may cause improvement in the hepatitis, but relapse is common after therapy is stopped. Prevention includes avoiding contaminated needles and practicing safer sex

(abstaining or limiting the number of partners, using barrier methods of contraception). Universal vaccination of newborns with hepatitis B vaccine effectively prevents delta hepatitis because the delta hepatitis virus only causes disease in the presence of hepatitis B virus.

Co-infection with hepatitis B virus and hepatitis C virus

Hepatitis C is caused by a virus that is spread through contaminated needles or blood products and, less commonly, through sexual intercourse. About 10% of patients with chronic hepatitis B also are co-infected chronically with hepatitis C virus (HCV). The two viruses interfere with each other and one usually predominates. If hepatitis C is the predominant infection, treatment is directed against the hepatitis C. Patients infected with both viruses are at higher risk for complications of liver disease. There is no effective vaccine against hepatitis C. Persons with hepatitis C should be vaccinated against hepatitis B to prevent co-infection.

Co-infection with hepatitis B virus and HIV

The human immunodeficiency virus (HIV) and hepatitis B virus are transmitted in similar ways, and it is not uncommon for an individual to have both infections. Persons with HIV who acquire hepatitis B are more likely to become chronically infected with hepatitis B than persons who do not have HIV. The reason for this is thought to be that HIV suppresses the immune system and impairs the ability of the body to eliminate the hepatitis B virus. Some nucleoside/nucleotide analogues (a class of antiretroviral drugs) are used to treat both HIV and hepatitis B, although dosages may vary in the two different infections. Stopping one of these agents when the HIV regimen is adjusted may cause hepatitis to flare.

Liver transplantation in hepatitis B infection

Liver transplantation has been successful in patients who have irreversible, life-threatening complications of hepatitis B. This includes patients with liver failure due to end-stage cirrhosis or unusually severe (fulminant) hepatitis. Liver transplantation does not cure hepatitis B, and hepatitis may occur in the new liver. The incidence of recurrent hepatitis has been reduced to less than 10% through use of lamivudine and HBIG in transplant recipients. Use of these agents has also improved long-term survival, with 75% to 85% of patients alive after five years.

Prevention from hepatitis B

Hepatitis B is a preventable disease. Vaccination and post-exposure prophylaxis have significantly reduced rates of infection. Risk can also be reduced by avoiding unprotected sex, contaminated needles, and other sources of infection.
Vaccination for hepatitis B

The hepatitis B vaccine contains a protein (antigen) that stimulates the body to make protective antibodies. Examples of hepatitis B vaccines available in the United States include hepatitis b vaccine-injection (Engerix-B, Recombivax-HB). Three doses (given at 0, 1, and 6 months) are necessary to assure protection. There are also

combination vaccines on the market that provide protection against hepatitis B and other diseases.

Examples include:

Hepatitis-b-hepatitis-a vaccine - injection (Twinrix), which provides protection against both hepatitis A and hepatitis B.
Haemophilus B/hepatitis B vaccine - injection (Comvax) provides protection against hepatitis B and Haemophilus influenzae type b (a cause of meningitis).
Pediarix provides protection against hepatitis B, tetanus, pertussis (whooping cough), and polio.

Hepatitis B vaccines are effective and safe. Up to 95% of vaccinated individuals form effective antibodies when they get the vaccine and are protected from hepatitis B. In healthcare workers, high-risk public safety workers, dialysis patients, and sexual partners of infected persons, a blood test for antibodies is recommended after vaccination to ensure that the person produced antibodies. For the few who do not form antibodies, revaccination may improve response, especially in infants. However, a small proportion of individuals will never respond to hepatitis B vaccination. Side effects from the vaccine are usually mild and include soreness at the site of injection. The risk of serious allergic reactions (anaphylaxis) is less than one per million doses. Vaccination has reduced the number of new cases of hepatitis B by more than 75% in the United States.

In the United States, hepatitis B vaccination is recommended for all infants at birth. Older children and adolescents should receive the vaccine if they did not do so at birth.

Adults in high risk situations also are advised to receive hepatitis B vaccine.

This includes:

- health care workers
- dentists
- intimate and household contacts of patients with chronic hepatitis B infection
- public safety workers who may be exposed to blood
- men who have sex with men
- individuals with multiple sexual partners
- dialysis patients
- injection drug users
- persons with chronic liver disease
- residents and staff in institutions that care for persons with developmental disabilities
- persons infected with HIV
- persons who require repeated transfusions or blood products.

Centers that serve high-risk individuals are encouraged to provide the vaccine to their clients. Such centers include dialysis units, drug treatment facilities, sexually transmitted diseases clinics and correctional facilities. Some countries have a high prevalence of hepatitis B in their population. Travelers who visit these countries for a

prolonged period of time (usually six months) and those who may be exposed to blood or semen should consider vaccination.

Effectiveness of hepatitis B immune globulin (HBIG) in preventing hepatitis B?
HBIG is a product that contains antibodies against hepatitis B. When injected, it provides temporary protection against hepatitis B. HBIG is used when people have had significant exposure to the virus. An example would be an accidental needle stick in an unvaccinated health care worker from a needle contaminated with blood from a person with hepatitis B. HBIG should be given as soon as possible after exposure, preferably within seven days. Persons who need HBIG should also receive hepatitis B vaccine. HBIG also is given to patients with hepatitis B following liver transplantation to suppress the hepatitis B virus in the transplanted liver.

Unvaccinated individuals who are exposed to a known case of hepatitis B or to a person at high risk for hepatitis B should be evaluated by a physician. Examples of such exposures include needle stick injuries in health care workers or sexual intercourse with an infected person. If the exposure is significant, the physician will recommend vaccination and also may recommend an injection of hepatitis B immune globulin (HBIG). HBIG is prepared from the plasma of blood donors and contains antibodies to hepatitis B. Vaccination and HBIG can substantially reduce the risk of disease in persons exposed to hepatitis B if given within one week of a needle stick or two weeks of sexual intercourse.

Vaccination provides long-term immunity in people who respond to the vaccine. There is no need for HBIG if an exposure occurs to a vaccinated person who is known to respond to the vaccine; however, a blood test might be drawn to verify that the person did respond to the vaccine.

Transmission of hepatitis B virus from mother to newborn infant

Infected mothers can pass hepatitis B to their newborn infants. All pregnant women should have blood tested to determine if they are infected. Infants born to infected mothers should receive HBIG and hepatitis B vaccine at birth. This is 85% to 95% effective in eliminating the risk of hepatitis B in the infant.

Hepatitis B At A Glance

The hepatitis B virus is a DNA virus belonging to the Hepadnaviridae family of viruses. Hepatitis B virus is not related to the hepatitis A virus or the hepatitis C virus.

Some people with hepatitis B never clear the virus and are chronically infected. Approximately 350 million individuals in the world and one million in the United States are chronically infected with hepatitis B. Many of these people appear healthy but can spread the virus to others.

Hepatitis B infection is transmitted through sexual contact, contact with contaminated blood (for example, through shared needles used for illicit, intravenous drugs), and from mother to child. Hepatitis B is not spread through food, water, or casual contact.

Serologic (blood) markers specifically for hepatitis B virus are used to diagnose hepatitis B viral infection. The blood tests can also identify people who are at highest risk for complications.

Injury to the liver by hepatitis B virus is caused by the body's immune response as the body attempts to eliminate the virus.

In the United States, 95% of adults who get hepatitis B are able to clear the virus and cure themselves of infection. The remaining 5% of adults with acute hepatitis B go on to develop chronic hepatitis B. Those who acquire the infection in childhood are much more likely to have chronic infection. Chronic hepatitis B may lead to cirrhosis or liver failure. Approximately 15% to 25% of persons with chronic infection will die prematurely as a result of the infection.

Progression of chronic hepatitis B viral infection occurs insidiously (subtly and gradually), usually over several decades. The course is determined primarily by the age at which the hepatitis B viral infection is acquired and the interaction between the virus and the body's immune system.

Treatment with interferons or nucleoside/nucleotide analogues suppresses viral reproduction in about 40% to 90% of patients with chronic hepatitis B. The medications are also effective in reducing inflammation and improving blood tests. This can delay or reduce complications such as cirrhosis. However, most people do not have a permanent response and relapse is common. The medications do not cure the infection.

Liver transplantation should be considered for patients with impending liver failure due to acute (initial) infection or advanced cirrhosis.

Hepatitis B is preventable through vaccination. All children should receive the vaccine. In addition, adults at high risk for hepatitis B should be vaccinated.

Unvaccinated people who are exposed to hepatitis B should be evaluated by a physician to determine if they need specific immune globulin (HBIG).

Geno Sen's® HBV Real Time PCR RG Kit is helpful in monitoring the viral loads of the patient when he/she is on anti viral therapy & confirmation of Active infections.

Samples which can be used for Extraction: Serum, plasma, whole blood, Liver Biopsy 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

- DNA isolation kit (see **8.a. DNA 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
- *Rotor Gene™ 2000,3000* or *Rotor Gene™ 6000*, Corbett Research (The Real time PCR Instrument)

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.

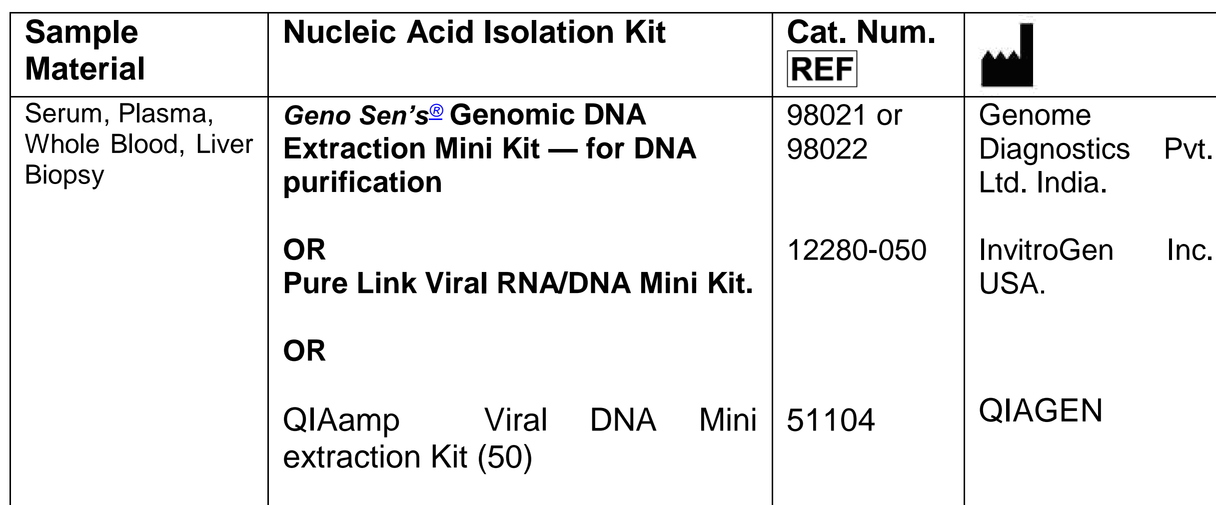
7. Description Of the Product.

The ***Geno-Sen's HBV*** *PCR* Reagents constitute a ready to use system for detection and quantification of HBV 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 HBV 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 (HBV 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 DNA Extraction

DNA Extraction kits are available from various manufacturers. Sample volumes for the DNA Extraction procedure depend on the protocol used. Please carry out the DNA 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, Liver Biopsy	***Geno Sen's®*** **Genomic DNA Extraction Mini Kit — for DNA purification** **OR** **Pure Link Viral RNA/DNA Mini Kit.** **OR** QIAamp Viral DNA Mini extraction Kit (50)	98021 or 98022 12280-050 51104	Genome Diagnostics Pvt. Ltd. India. InvitroGen Inc. USA. QIAGEN

Note::

All the Above Kits were evaluated in conjuction with the *Geno Sen's® HBV Real Time PCR RG Kit on the Rotor Gene 3000/6000. The performance of* Pure Link Viral RNA/DNA Mini Kit manufactured by InvitroGen Inc. USA was found to be almost equivalent to the *Geno Sen's®* Genomic DNA Extraction Mini Kit — for DNA purification. However the sample size was limited for this study hence for all practical purposes all the validations were conducted with the *Geno Sen's®* Genomic DNA Extraction Mini Kit — for DNA purification. The Qiagen DNA extraction Kit consistently had a Ct value difference of 1.0 or more. Which clearly means that the yield by Qiagen Kit was low, hence preferably *Geno Sen's®* Genomic DNA Extraction Mini Kit or the Pure Link Viral RNA/DNA Mini Kit by InvitroGen Inc. USA is strongly recommended.

However the customer can use their own extraction systems depending on how good the yield is. Always use an extraction kit with a higher DNA yield.

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 *HBV 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 MBD 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 *(HBV S 1-5)* are treated in the same way as extracted samples and the same volume is used i.e. (10µl) 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 copies/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.

If the starting volume of the sample while using the ***Geno Sen's®*** Genomic DNA Extraction Mini Kit is 200µl & 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 =	30000000 IU/ml
S2: 10^4 IU /µl =	3000000 IU/ml
S3: 10^3 IU /µl =	300000 IU/ml
S4: 10^2 IU /µl =	30000 IU/ml
S5: 10^1 IU /µl =	3000 IU/ml

If the starting volume of the sample while using the **Pure Link Viral RNA/DNA Mini Kit.** Or Qiagen QIAamp DNA Mini extraction kit is 200µl & 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 =	25000000 IU/ml
S2: 10^4 IU/µl =	2500000 IU/ml
S3: 10^3 IU/µl =	250000 IU/ml
S4: 10^2 IU/µl =	25000 IU/ml
S5: 10^1 IU/µl =	2500 IU/ml

8.d Preparation for PCR

8. e. 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 *(HBV 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

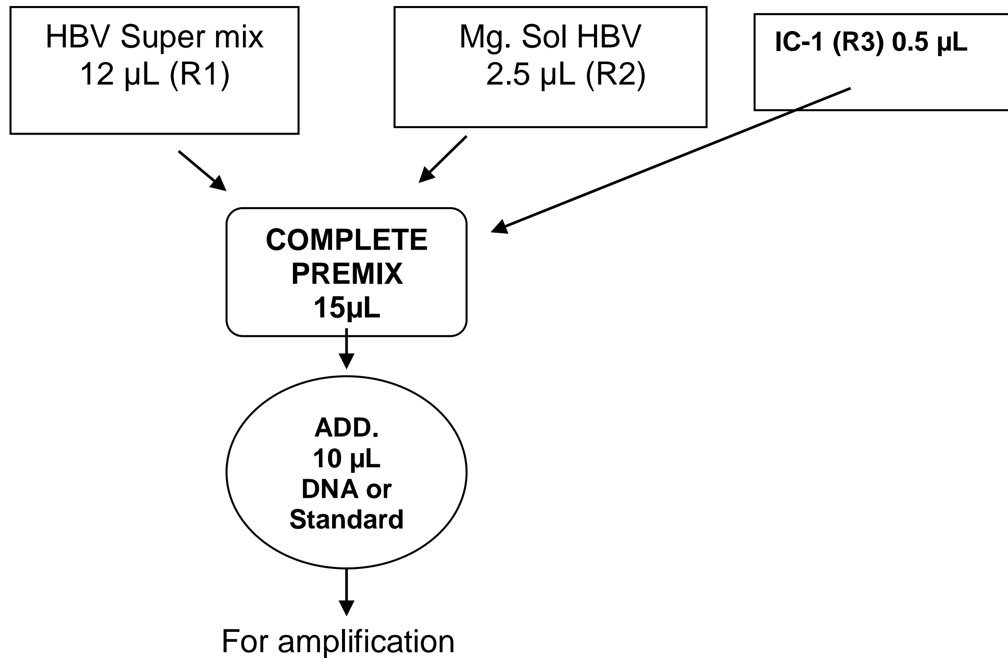


Fig. 4.

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

HBV MASTER MIX	1 rxns.	10 rxns.
HBV Super Mix (R1)	12 µL	120 µL
HBV Mg Sol. (R2)	2.5 µL	25 µL
IC-1 (R3)	0.5 µL	5 µL
Total	15µL	150µL

Fig. 5.

Pipette 15 µl of the Master Mix into each labelled PCR tube. Then add 10 µl of the earlier extracted DNA to each sample tube and mix well by pipeting up and down. Correspondingly, 10 µl of the Standards (HBV *S1-5*) must be used as a positive control and 10 µl of water (*Water, PCR grade*) as a

negative control. Close the PCR tubes and transfer the HBV tubes 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.f. Programming the *Rotor Gene*™ 2000/3000

The *RotorGene*™ *2000/3000* PCR program for the detection of HBV can be divided into following steps:

A. Setting of general assay parameters & reaction volume
B. Thermal Profile & Calibration
C. Cycling profile/ & Initial activation of the Hot Start enzyme
D. Cycling for Amplification of DNA
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 Figures 6-20 below 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

a) Setting of general assay parameters & Reaction volume.

First, confirm whether the PCR tubes used are No Domed PCR tubes by clicking in the box as shown in the figure. Please note that tubes with domes cannot be used in Rotor gene hence click in the box as shown below.

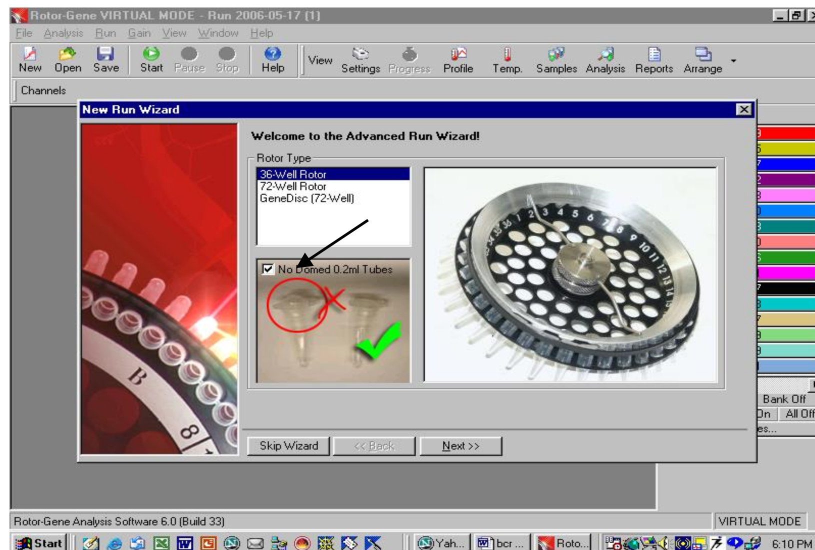


Fig. 6.

Confirmation of Reaction Volume as follows.

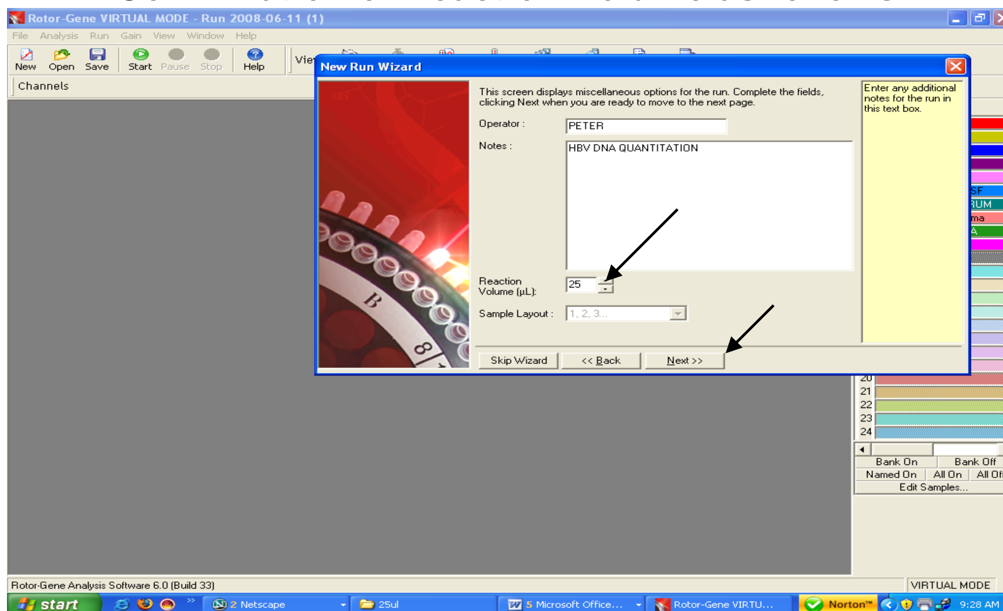


Fig. 7.   Setting of general assay parameters.

- Please click on the volume buttons to make sure that 25µl is reflected in the window as shown above.
- Then click next and a new window will open as shown below.

b) THERMAL PROFILE & CALIBRATION:

Here the thermal profile for the assay will be defined.

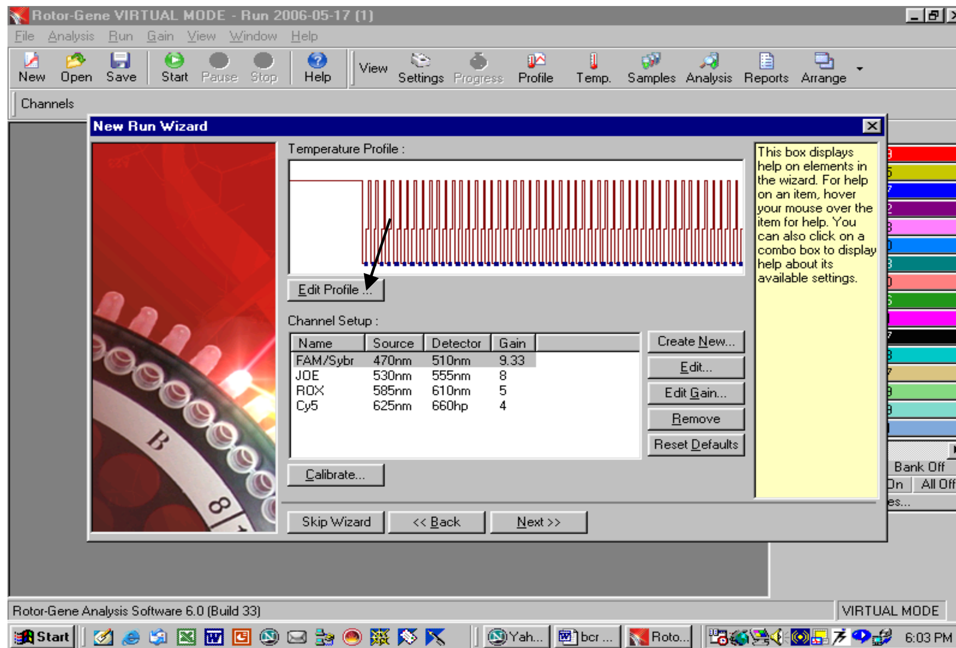


Fig. 8.

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

c) CYCLING PROFILE: First hold 95°C for 10 minutes as below

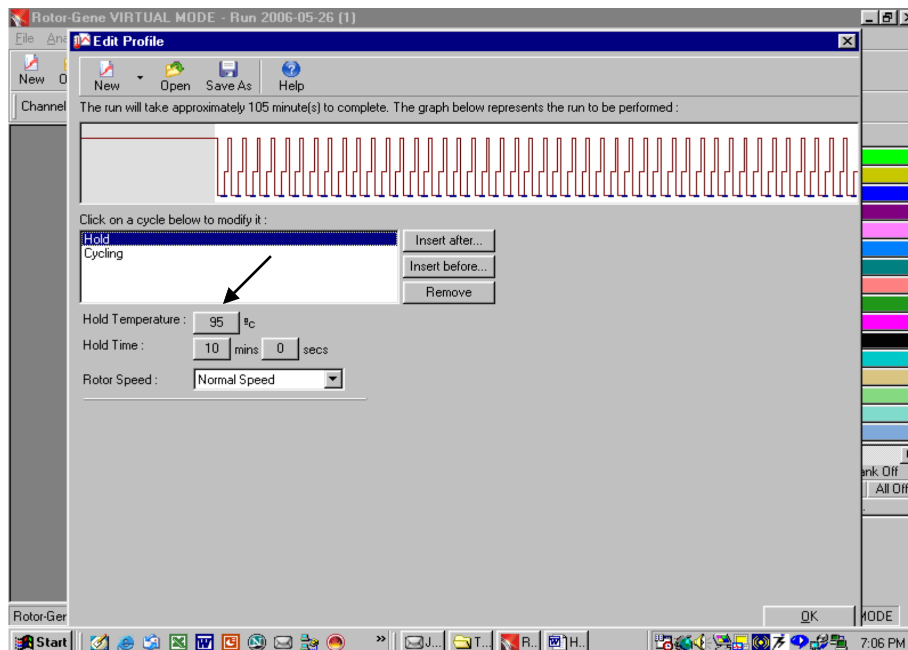


Fig. 9. Initial activation of the Hot Start enzyme.

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

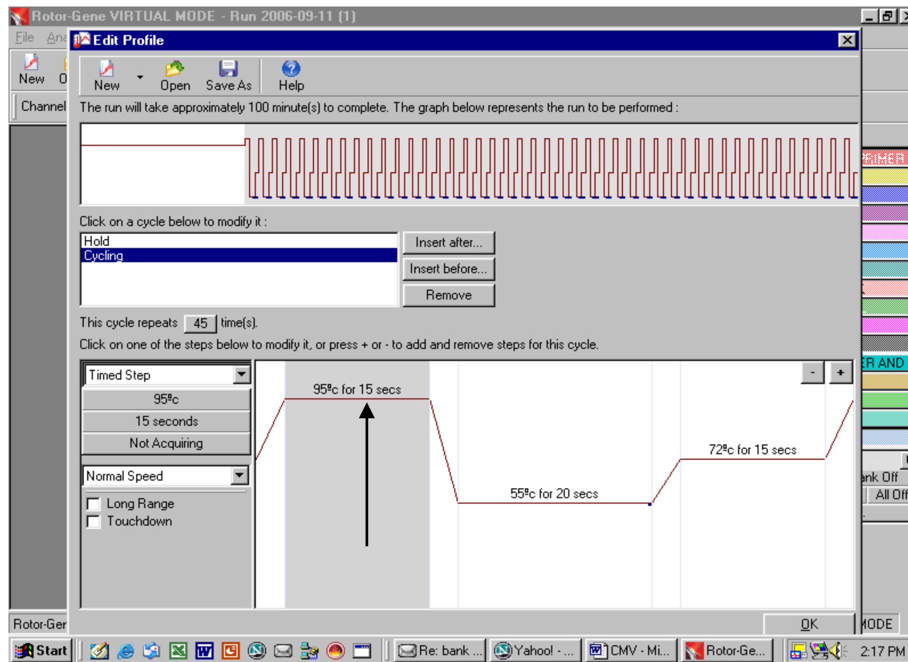


Fig. 10.

Setting up of Anneling step in the cycling profile as depicted below i.e. 55°C for 20 **Seconds and defining the Data acquiring channel i.e FAM & JOE**

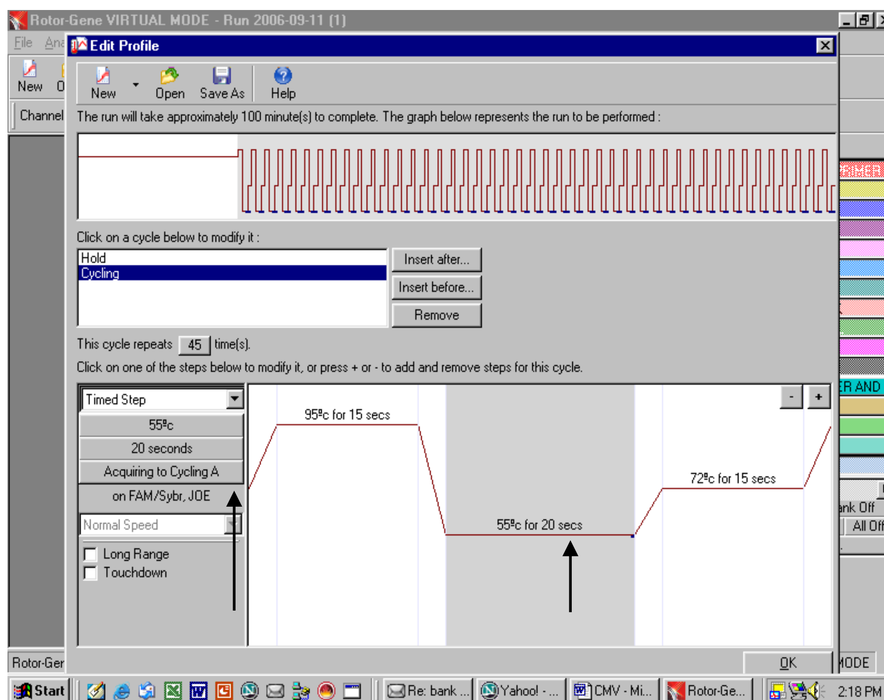


Fig. 11.

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

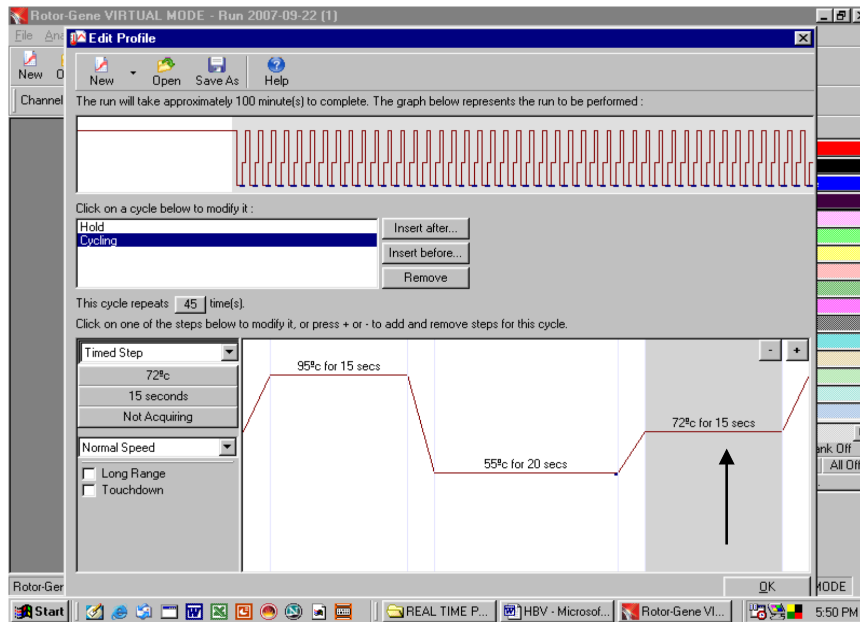


Fig. 12.

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

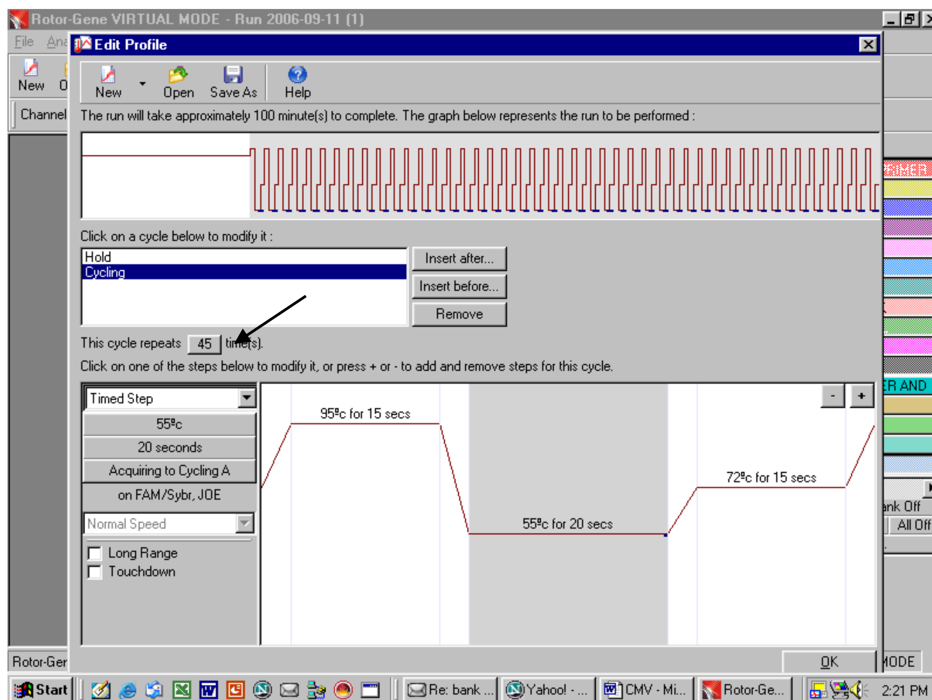


Fig. 13.

Final Confirmation of the Thermal profile by pressing OK button as shown below.

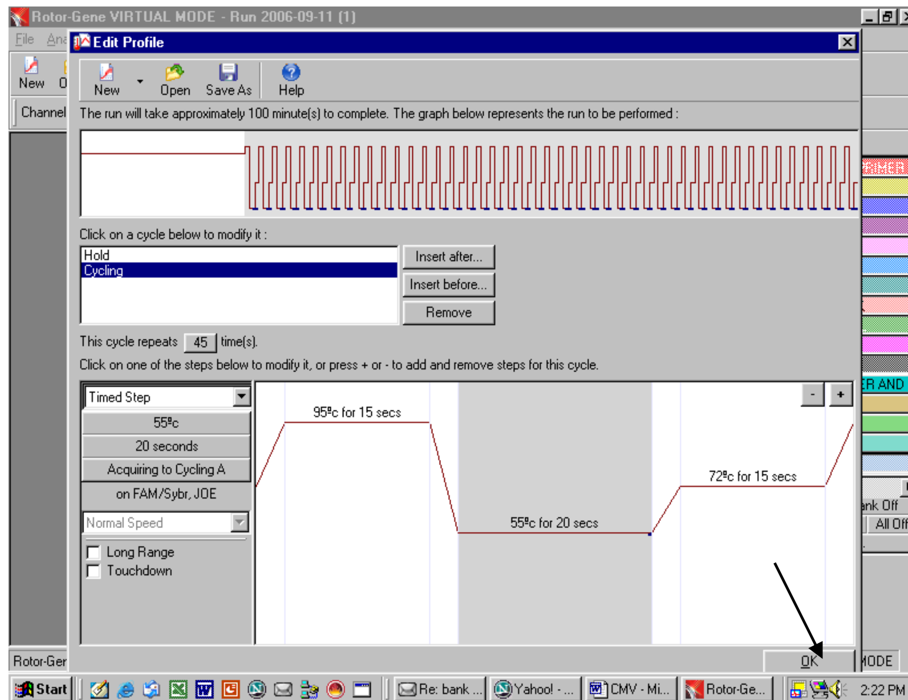


Fig. 14.

e) Setting the gains for the acquiring channel by clicking at the Calibrate button as shown below.

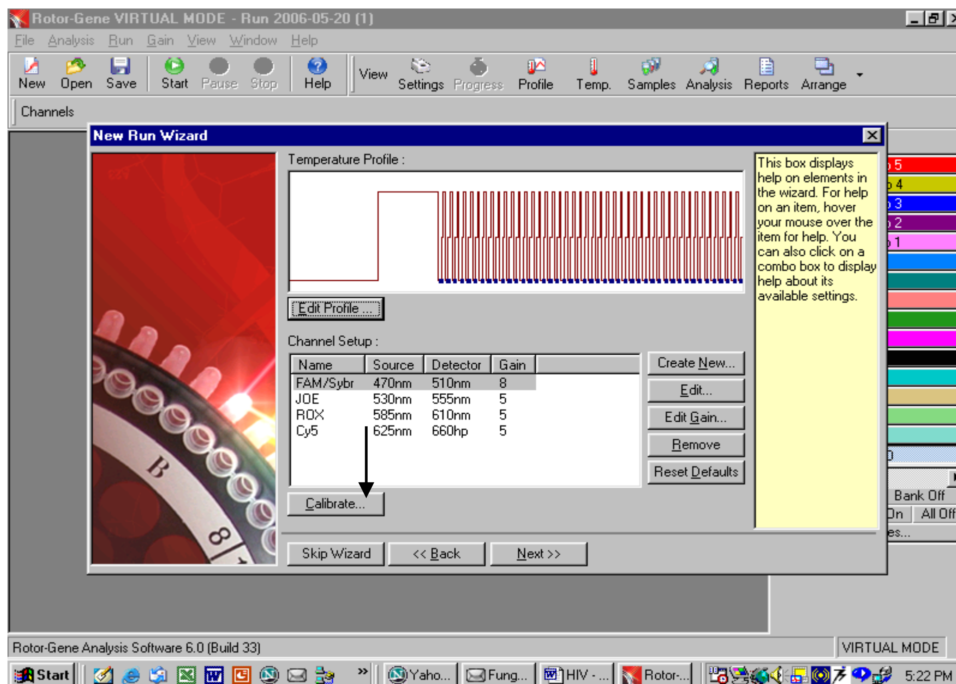


Fig. 15.

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 Calibration*

Setup (activation in menu window *New Experiment Wizard* under *Calibrate*). Please set the calibration temperature to the annealing temperature of the amplification program (compare Fig. 16).

Adjustment of the fluorescence channel sensitivity as shown below.

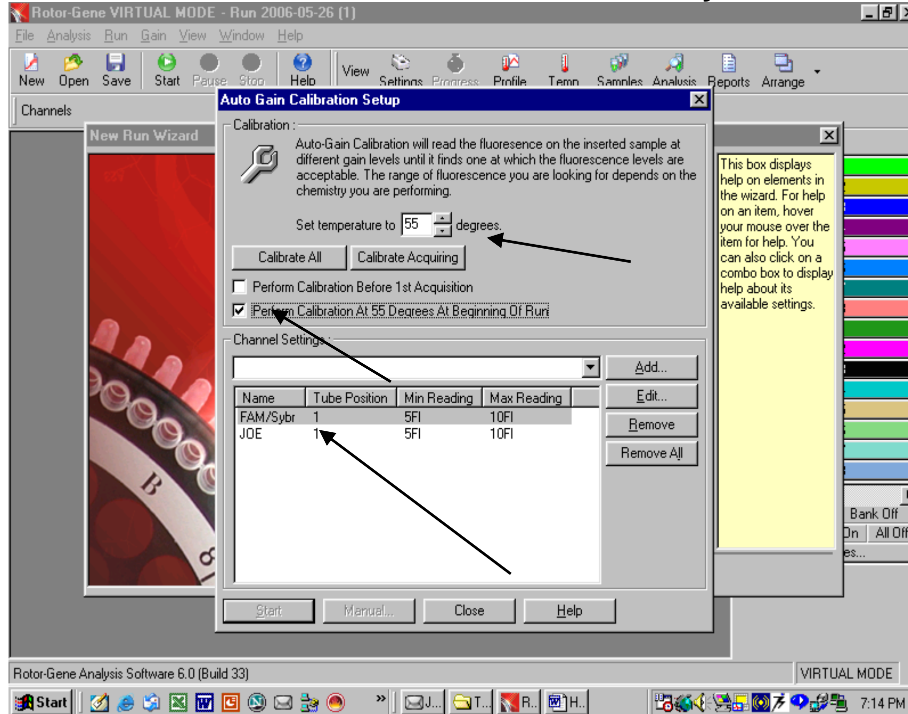


Fig. 16.

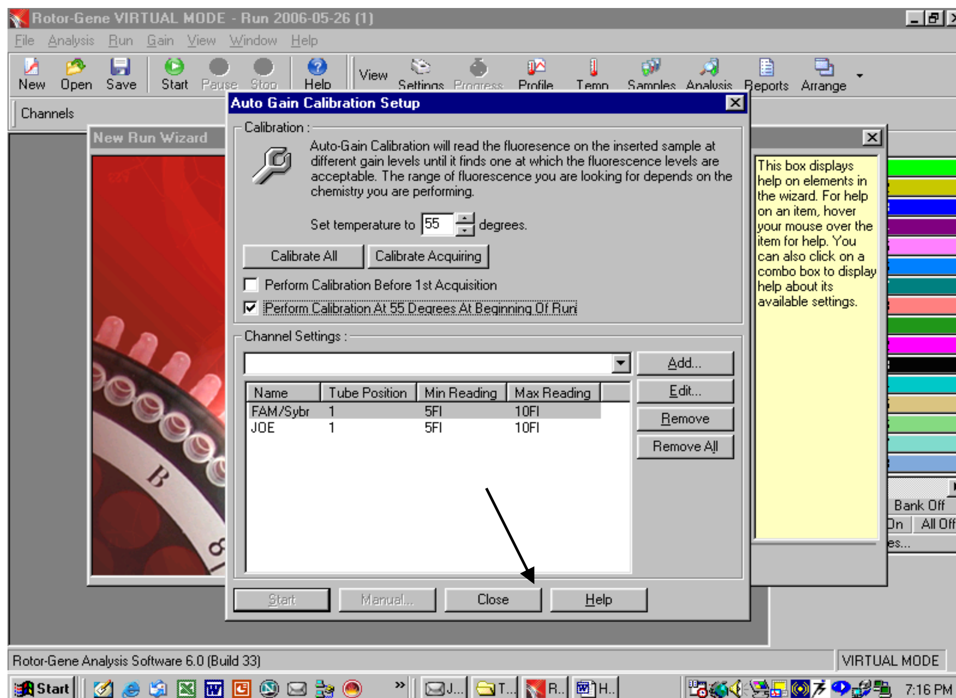


Fig. 17.

Please do not forget to click on the box against " Perform calibration at 55°C at beginning of the run." After that press Close and a new window will open as shown below.

The gain values determined by the channel calibration are saved automatically and are listed in the last menu window of the programming procedure.

PRESS NEXT

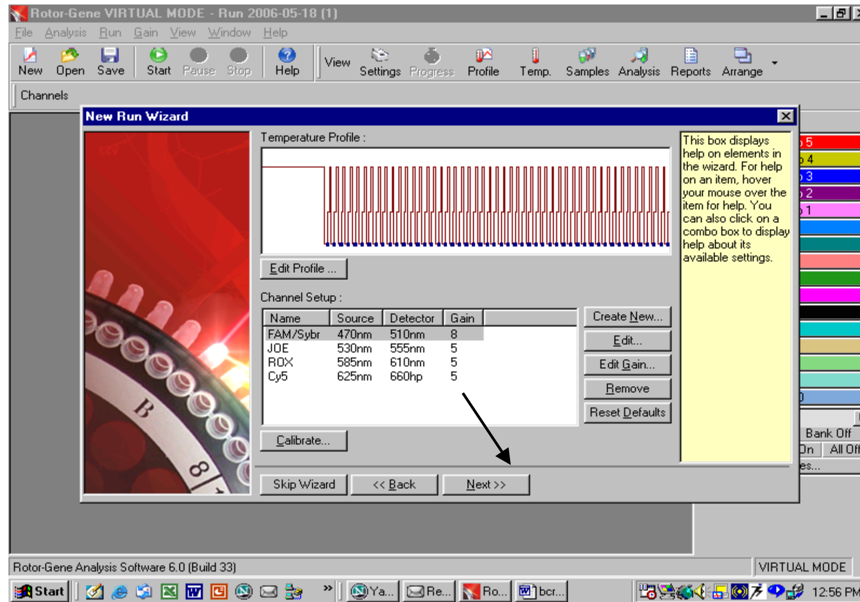


Fig. 18.

f) Starting of the *Rotor Gene*™ run.

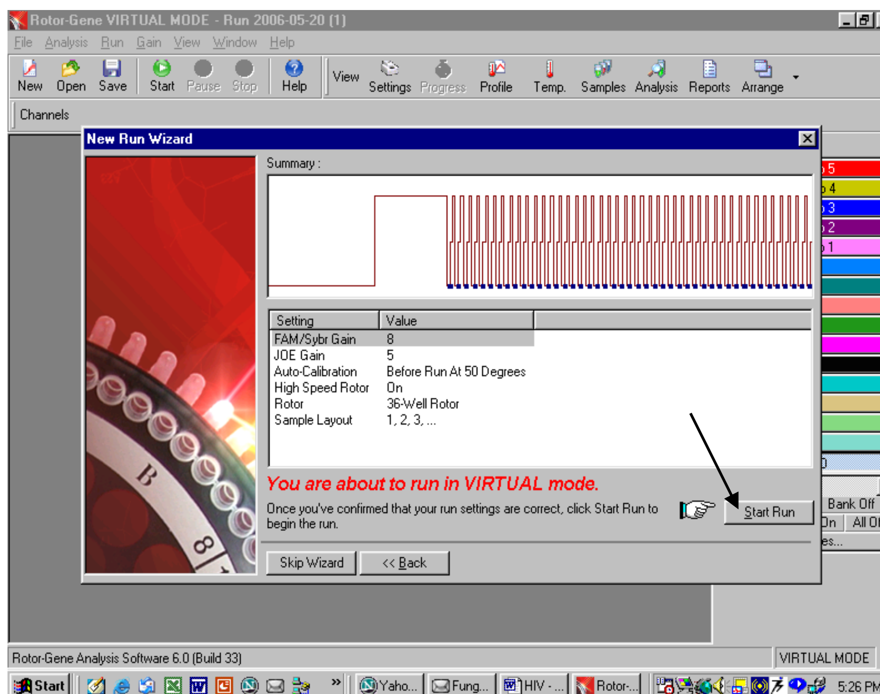


Fig. 19.

Press Start Run Button.

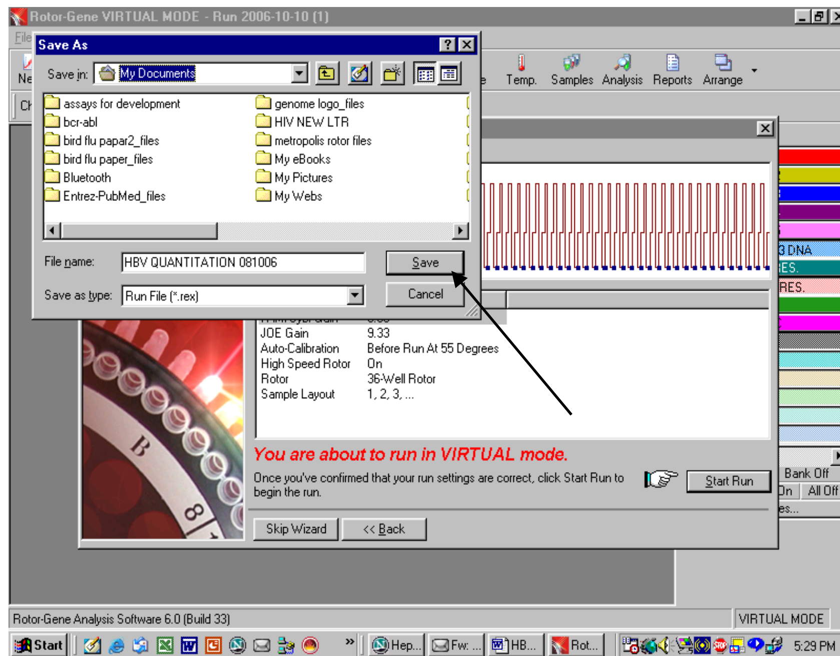


Fig. 20.

SAVING THE RUN FILE AS ABOVE

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.

8.g. Programming the *RotorGene*™ 6000

The *RotorGene*™ 6*000* PCR program for the detection of HBV can be divided into following steps:

G. Setting of general assay parameters & reaction volume
H. Thermal Profile & Calibration
I. Cycling profile/ & Initial activation of the Hot Start enzyme
J. Cycling for Amplification of DNA
K. Adjustment of the sensitivity of the fluorescence channels
L. Starting of the *Rotor Gene*™ run

Program the *RotorGene*™ 6000 for these 5 steps according to the parameters shown in Figures 21-37 below All specifications refer to the *RotorGene*™ 6000 software version 1.7 Please find further information on programming the *RotorGene*™ in the *RotorGene*™ 6000 *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 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.

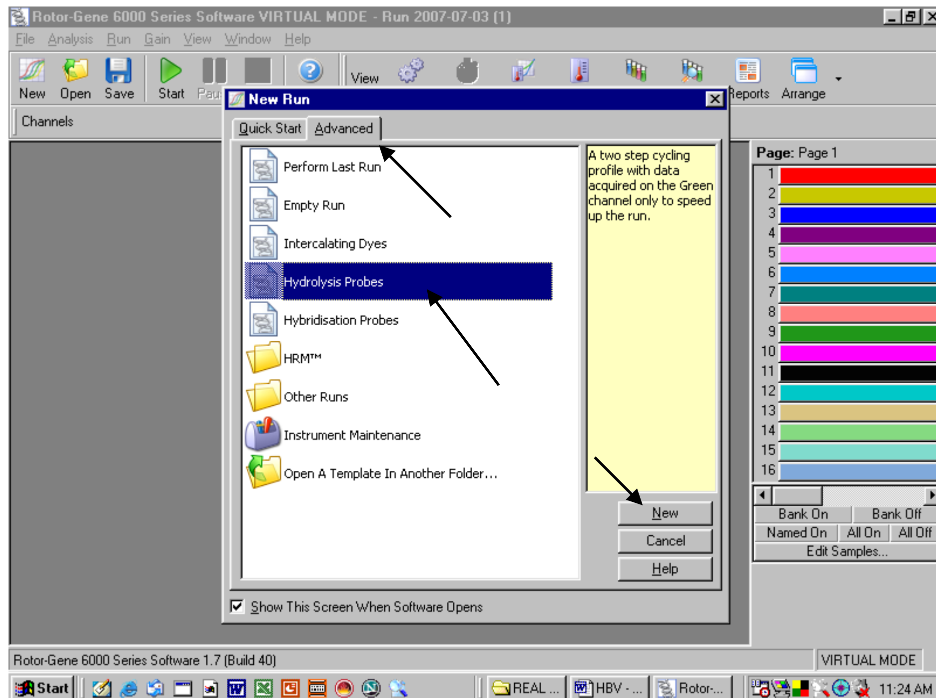


Fig. 21.

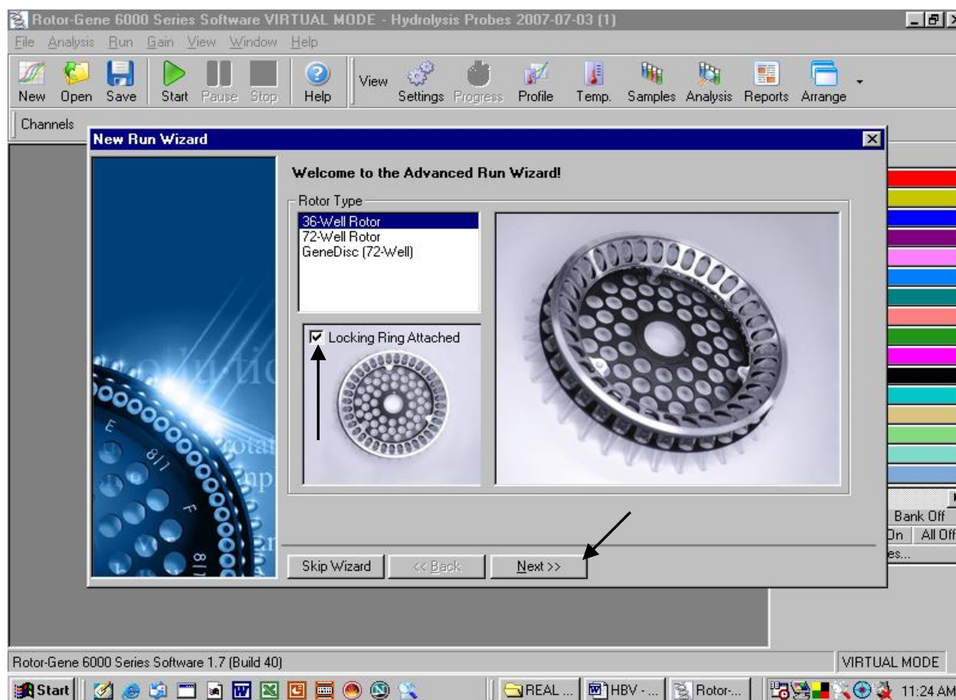


Fig. 22.

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.

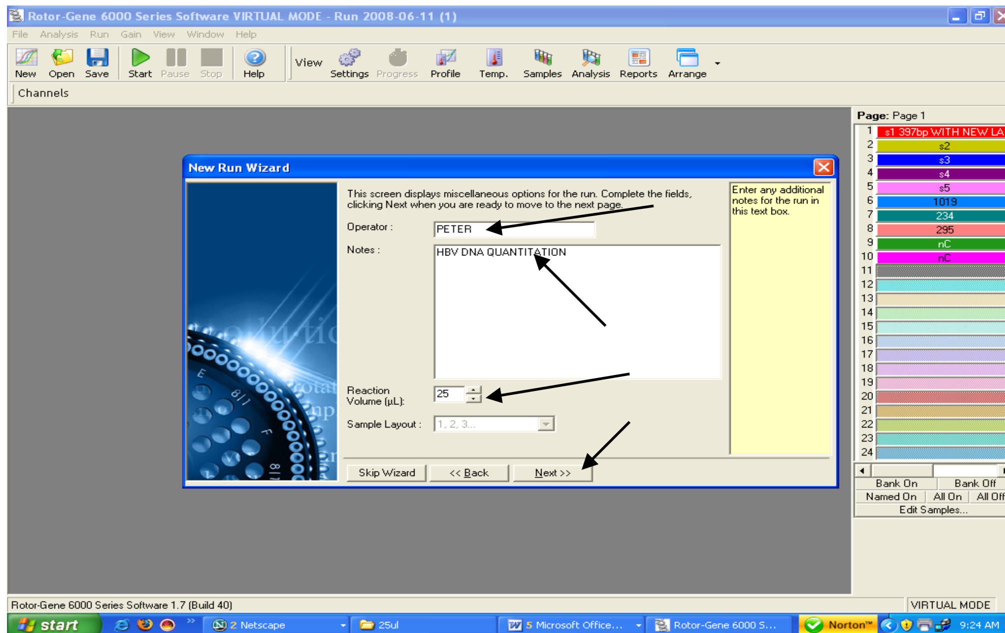


Fig. 23.

- 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.

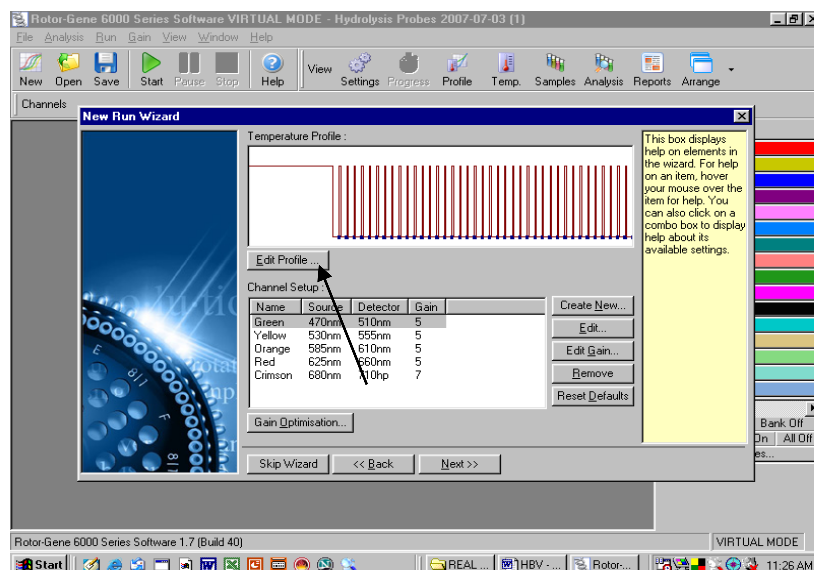


Fig. 24.

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 95°C for 10 minutes as below

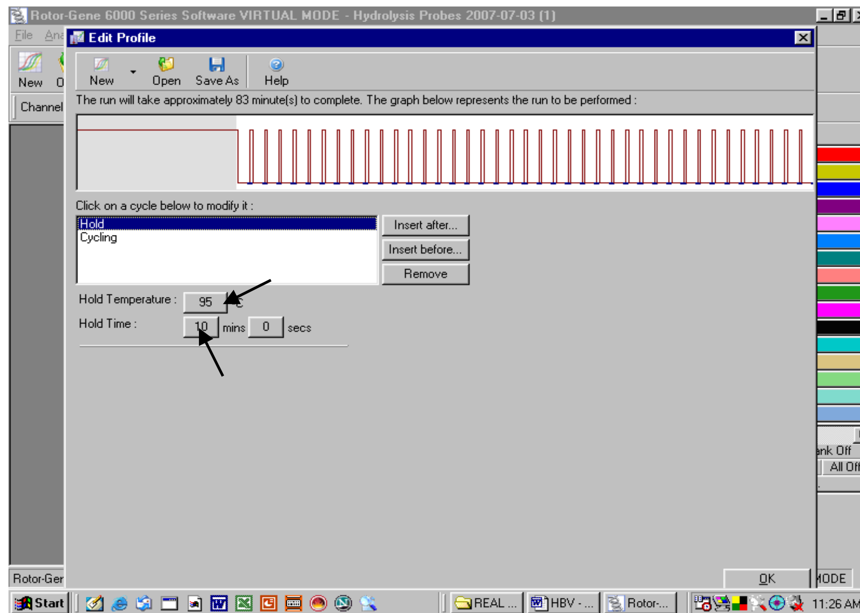


Fig. 25. Initial activation of the Hot Start enzyme. Generally the window will open with the Hold Temp as 95°C and the Hold Time as 10 minutes. In case it is different then set the same to as shown above.

j) Setting the Cycling and acquisition.

When clicked on Cycling the window will open as below.

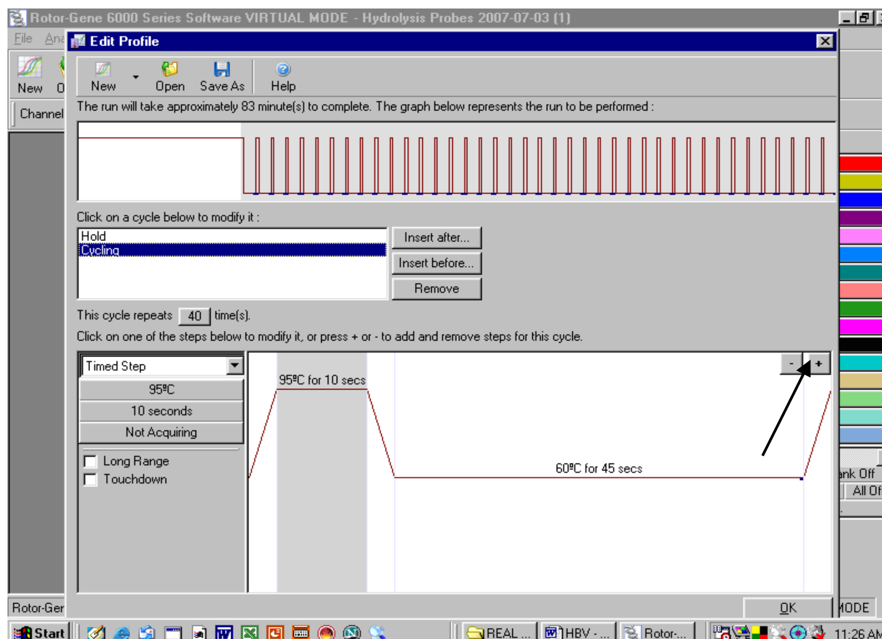


Fig. 26.

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.

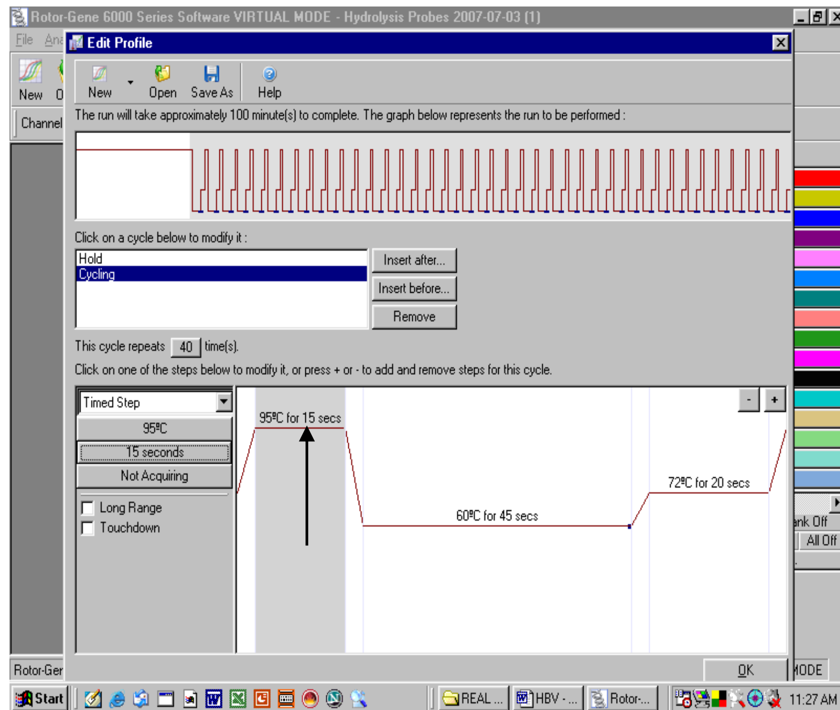


Fig. 27.

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

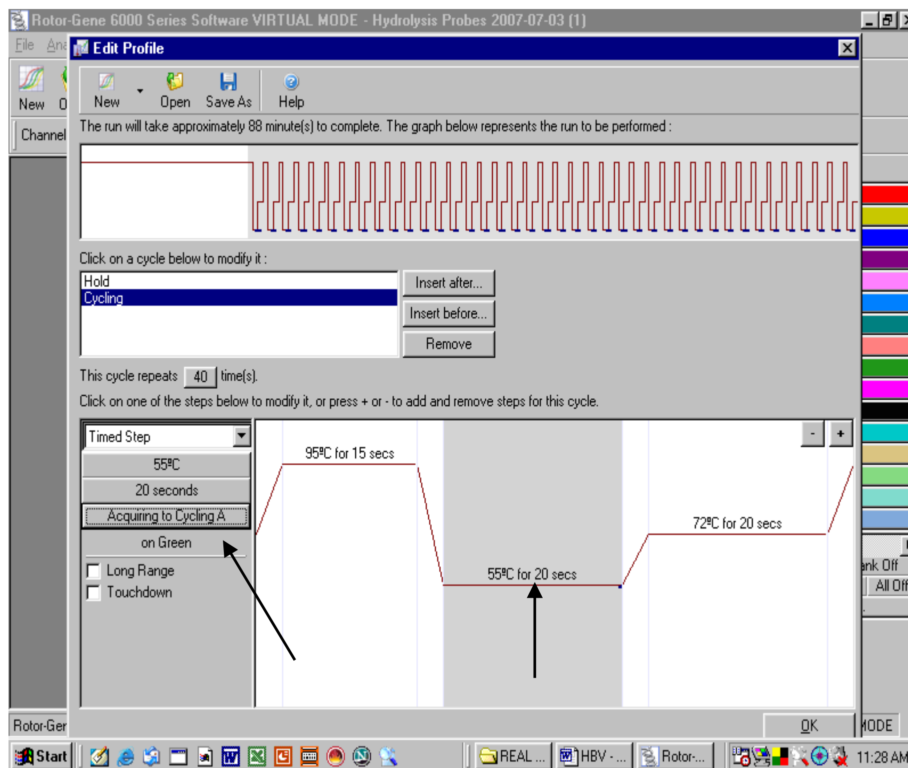


Fig. 28.

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)

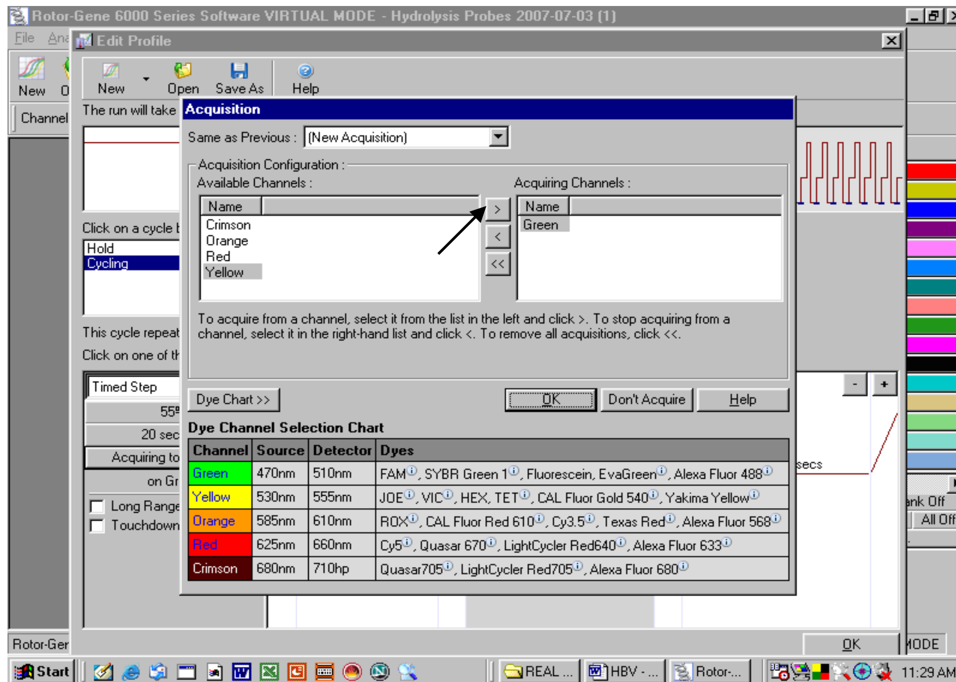


Fig. 29.

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.

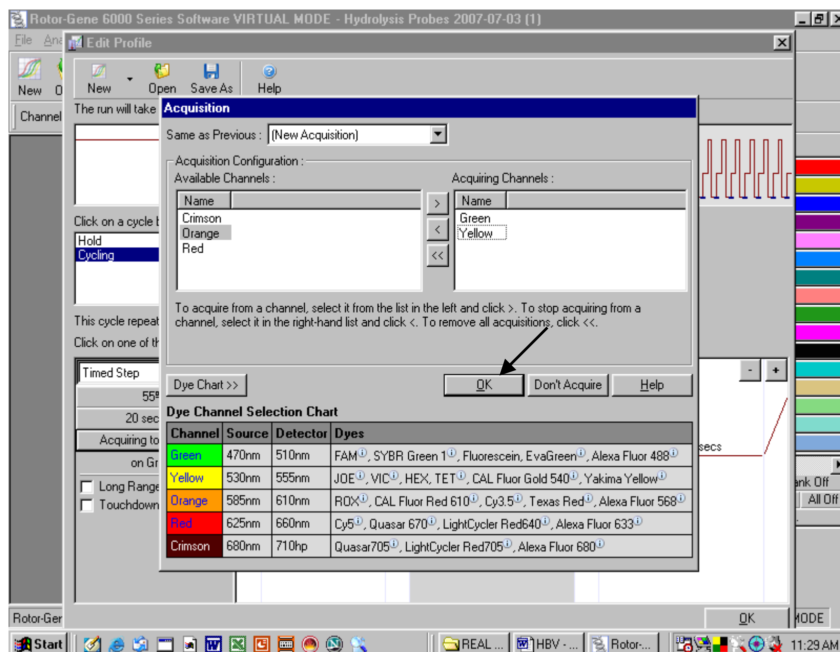


Fig. 30.

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

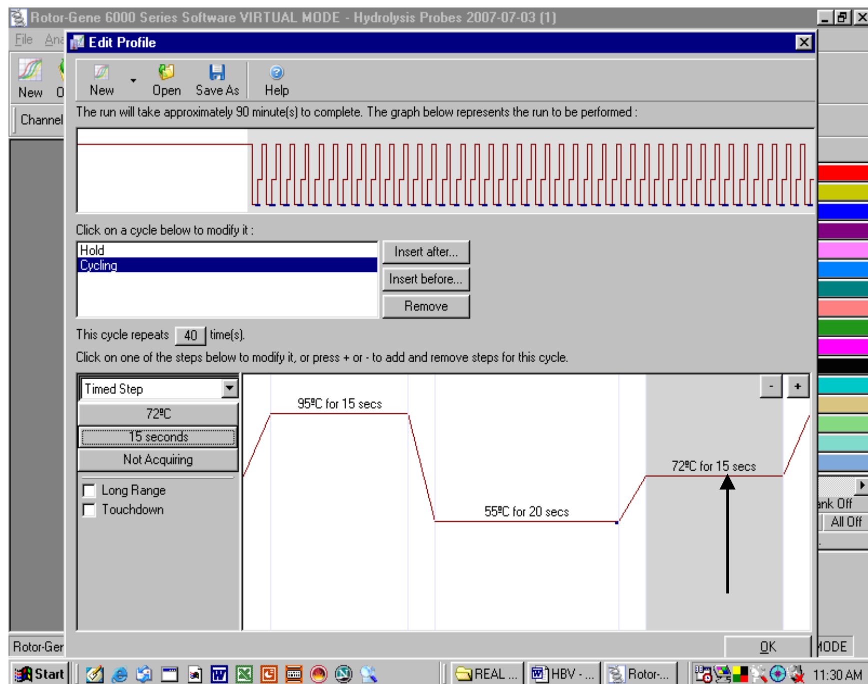


Fig. 31.

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

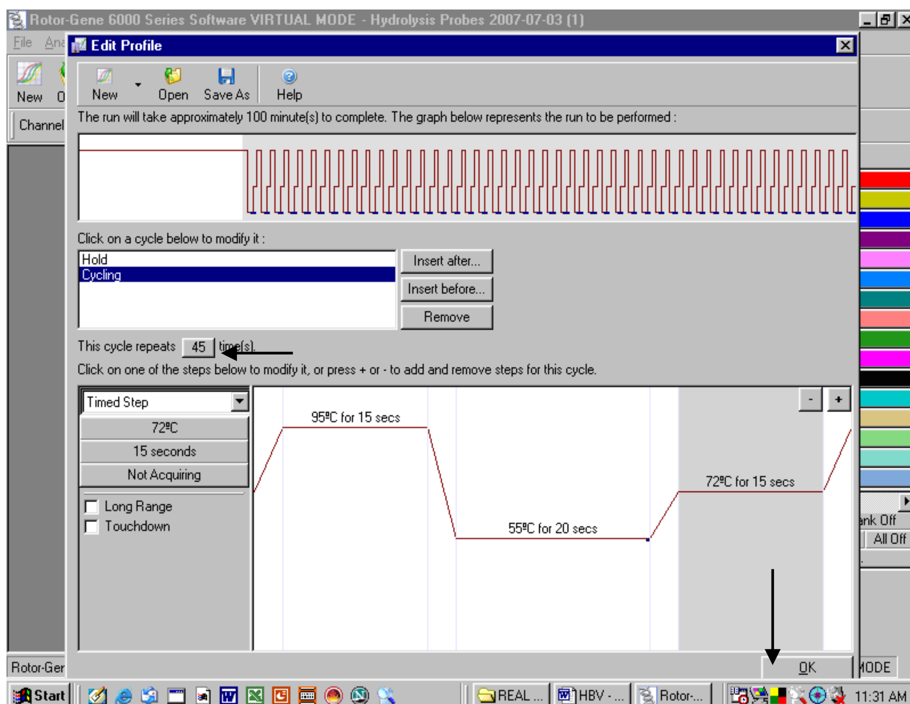


Fig. 32.

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.

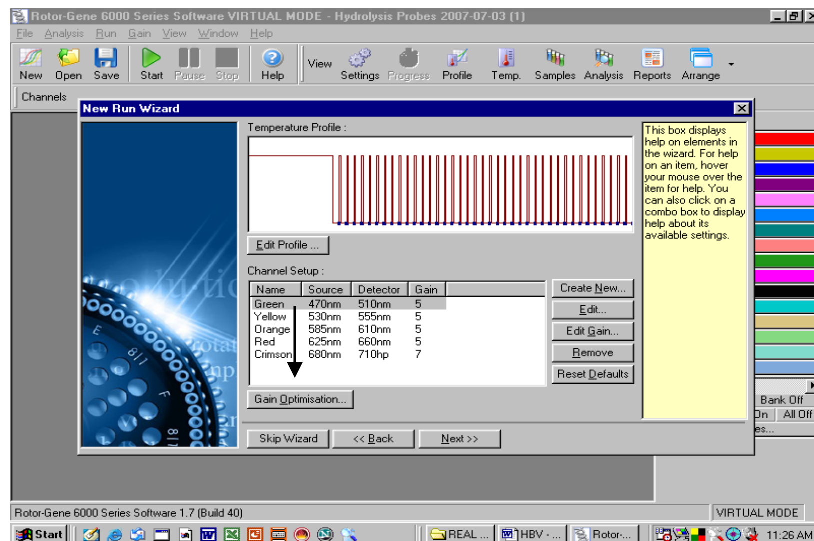


Fig. 33

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.34 .

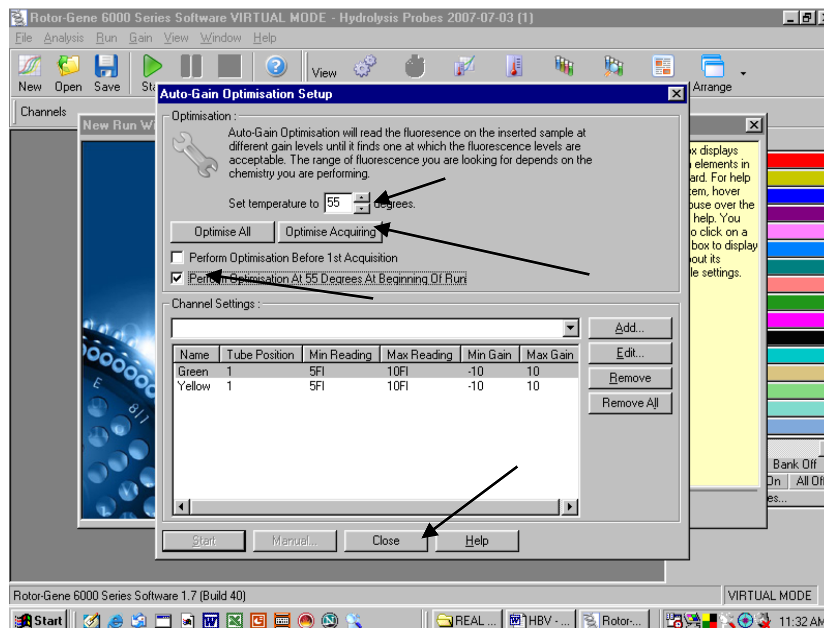


Fig. 34.

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 At 55 degree At beginning of Run.
- 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.

L) PRESS Start RUN

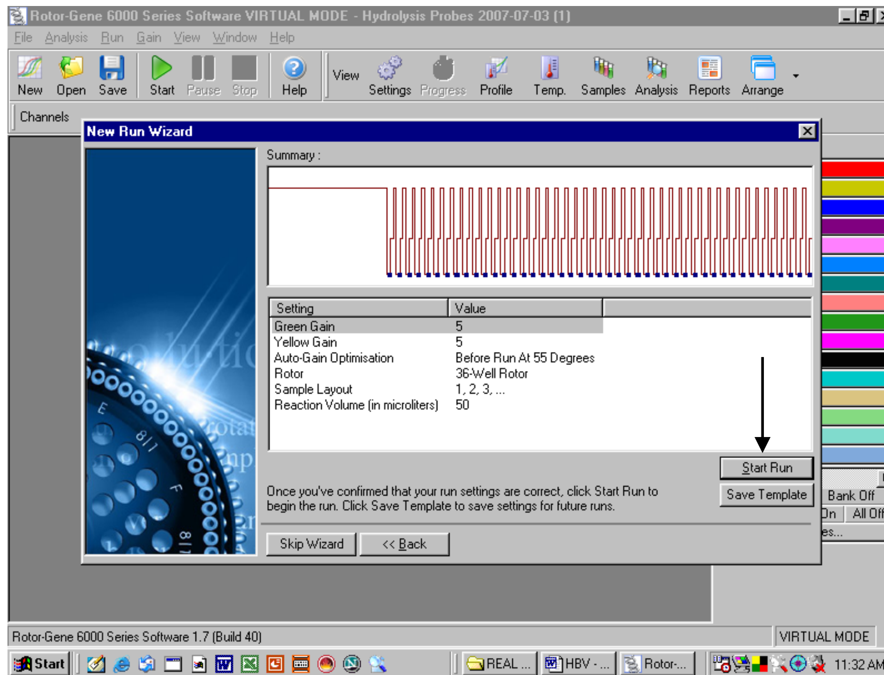


Fig. 35.

Saving the RUN File.

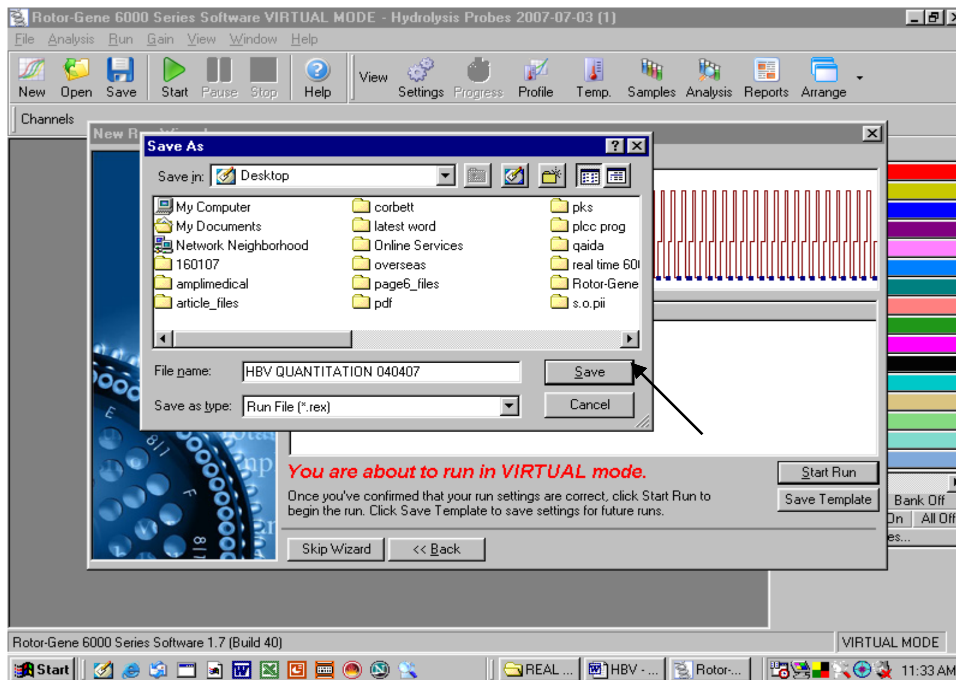


Fig. 36.

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. (a) Generated Data Interpretation & Analysis

a) For Rotor Gene 2000/3000:

Data analysis is performed with the *RotorGene*™ software according to the manufacturer's instructions (*RotorGene*™ *3000 Operator's* Manual).

The following results are possible:

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

The result of the analysis is positive: The sample contains HBV DNA.

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

No HBV DNA 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.

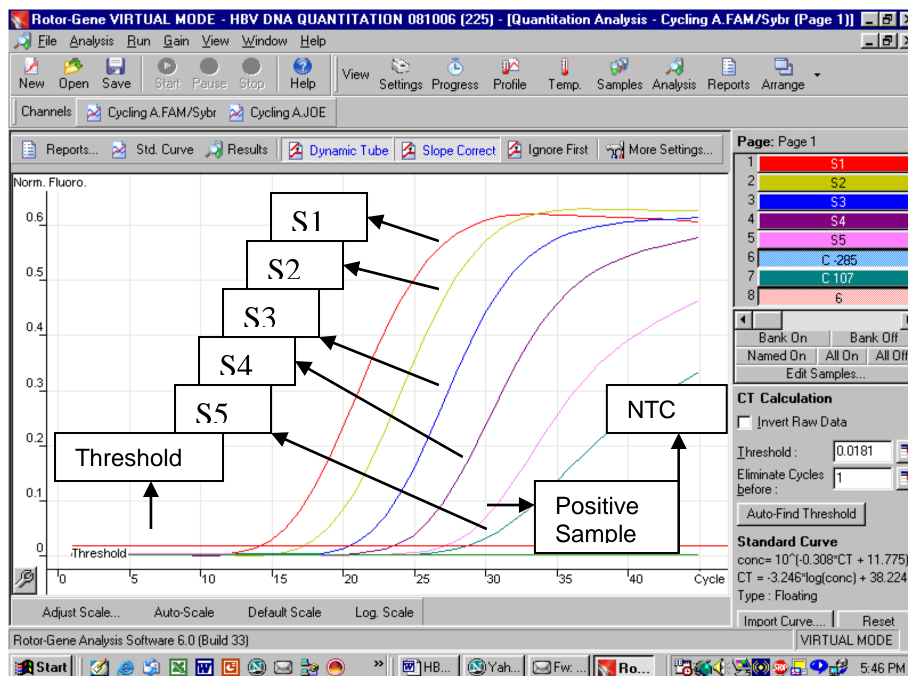


Fig. 37: Detection of the quantitation standards *(HBV S 1-5)* in fluorescence channel Cycling A.FAM. NTC: non-template control.

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

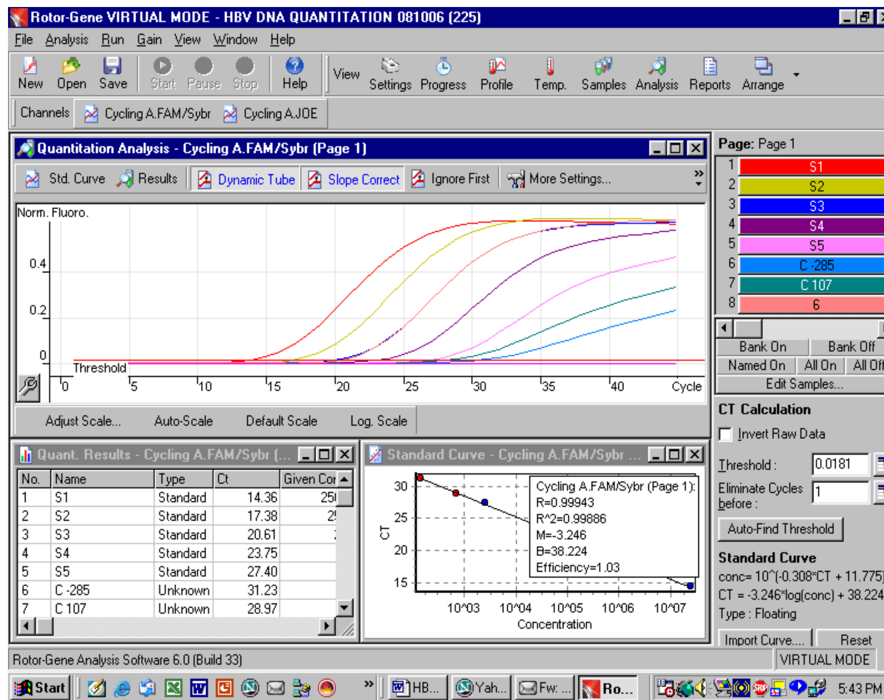


Fig. 38.

Example of analysed data for HBV 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 HBV DNA.

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*™ *6000 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 HBV DNA.

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

No HBV DNA 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.

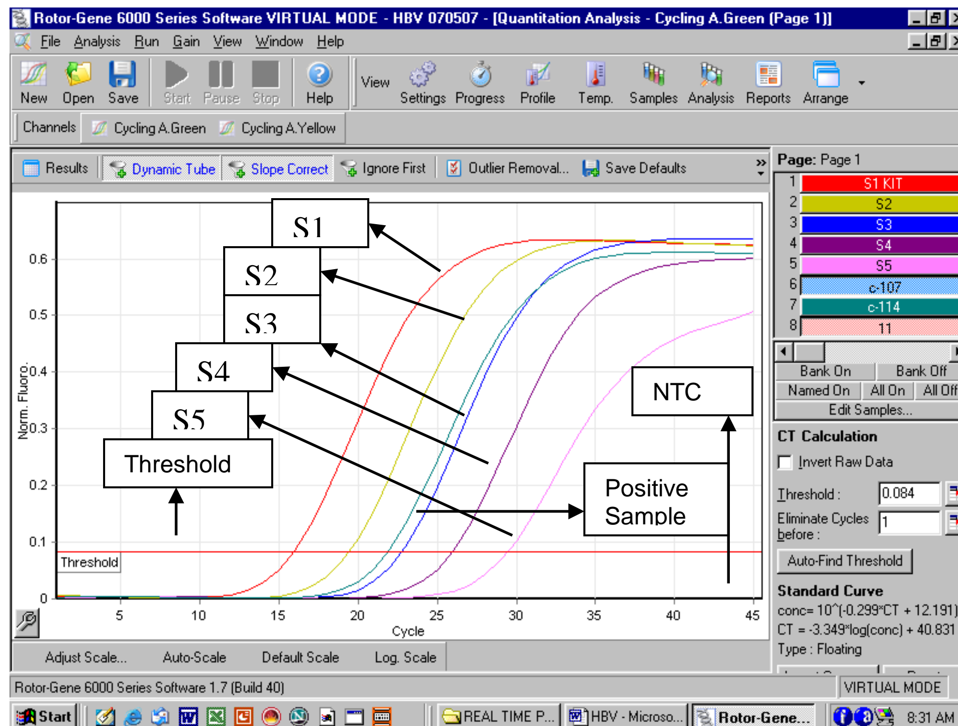


Fig. 39: Detection of the quantitation standards *(HBV S 1-5)* in fluorescence channel Cycling A.Green. NTC: non-template control.

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

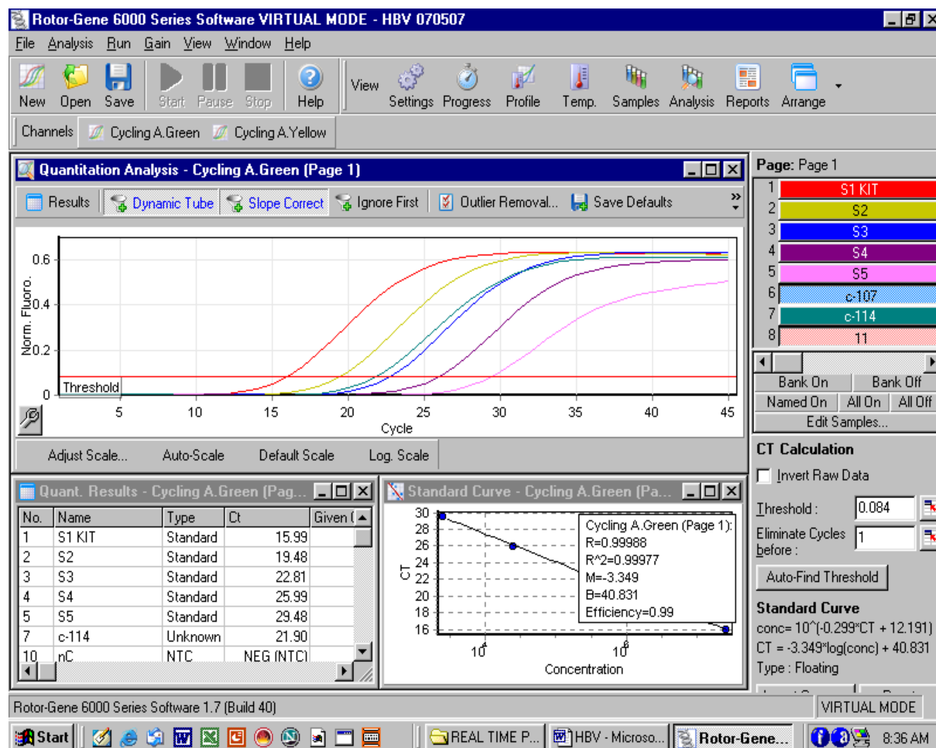


Fig. 40.

Example of analysed data for HBV 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 HBV DNA.

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

a) For Rotor Gene 2000/3000:

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

- Incorrect programming of the *Rotor-Gene*™ 2000/300*0.*

  ➔ Repeat the PCR with corrected settings.

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

- The PCR conditions do not comply with the protocol.

  ➔ Repeat the PCR with corrected settings.

- The HBV Super Mix *R1* has been thawed and frozen too often.
- The HBV 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 HBV super mix (R1).

- **The PCR was inhibited.**

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

b) For Rotor Gene 6000:

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

- Incorrect programming of the *Rotor-Gene*™ *6000.*

  ➔ 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 HBV Super Mix *R1* has been thawed and frozen too often.
- The HBV 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 HBV super mix (R1).

- **The PCR was inhibited.**

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

11. Specifications

11.a Analytical Sensitivity and Reproducibility

In order to determine the sensitivity of the ***Geno Sen's®*** HBV *Real Time PCR RG Kit*, a dilution series has been set up from 10^7 IU/ul down to 10^0 IU/µl of HBV In-Vitro Transcription and analyzed with the ***Geno Sen's®*** HBV Real Time PCR RG kit ***using*** *Rotor Gene 3000/6000. 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® HB***V *Real Time PCR RG Kit* is consistently 12 IU/ml. This means that there is 95% probability that 12 IU/ml will be detected.

Analytical Sensitivity in Conjunction with the ***Geno Sen's®*** **Genomic DNA Extraction Mini Kit — for DNA purification (Cat . No. 98021) of the** ***Geno Sen's®*** HBV *Real Time PCR RG Kit on Rotor Gene 3000/6000 was determined by Spiking a known negative Serum to a nominal 12 IU/ml. This was subjected to extraction using the* ***Geno Sen's®*** **Genomic DNA Extraction Mini Kit — for DNA purification (Cat . No. 98021) eight times with starting volume of 200**µl & elution volume of 60µl.

All the Eight extractions were then analyzed with the ***Geno Sen's®*** HBV *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 12.1 IU/ml.

Hence Analytical Sensitivity in Conjunction with the ***Geno Sen's®*** **Genomic DNA Extraction Mini Kit — for DNA purification (Cat . No. 98021) of the** ***Geno Sen's®*** HBV *Real Time PCR RG Kit on Rotor Gene 3000/6000 was determined to be 12 IU/ml.*

11.b Specificity

The specificity of the ***Geno Sen's®*** HBV *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 the HBV sequences.*

To further Validate the stringent data In order to check the specificity of the ***Geno Sen's®*** HBV *Real Time PCR RG Kit*, different RNA & DNA listed below were analyzed with ***Geno Sen's®*** HBV *Real Time PCR RG Kit*. None of these led to a positive signal with the ***Geno Sen's®*** HBV *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	Dengue RNA
MTb DNA	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.
HIV-1	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®*** HBV Real Time PCR RG kit on Rotor Gene 3000/6000. The extraction was carried out with the ***Geno Sen's®*** **Genomic DNA Extraction Mini Kit — for DNA purification (Cat . No. 98021)**

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

Sample Type	Serum	Plasma	Whole Blood	Liver Biopsy
High +ve's	9	4	1	
Medium +ve's	13	7	3	2
Low +ve's	11	4	2	1
Extremely low +ve's	6	3		
Negative samples.	8	4	2	1
	47	22	8	4

All the above samples were correctly identified by the ***Geno Sen's®*** ***HBV Real Time*** PCR RG kit & all the 9 extremely low samples were accurately detected by the ***Geno Sen's®*** HBV Real Time PCR RG kit & exhibited copies around 12 IU /ml or less than 12 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®*** HBV Real Time PCR RG kit was determined by analyzing a dilution series of the HBV In-Vitro Transcription ranging from 10^7 IU/µl down to 10^0 IUµl of HBV In-Vitro Transcription and analyzed with the ***Geno Sen's®*** HBV *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®*** HBV *Real Time PCR RG* kit *on Rotor Gene 3000/6000 systems.*

The Linear Range of the ***Geno Sen's®*** HBV *Real Time PCR RG Kit* has been determined to cover concentrations from 10^7 IU /µl down to 10^0 IU/µl.

In terms of IU per ml this translates to 2.5 x 10^9 IU/ml down to 12 IU/ml.

Conversion of IU to Genome Copies/ml

As per the Latest WHO report on Standardization of the NAT assays One IU of HBV is equal to 5.3 Genome Copies.

Hence for conversion of Patient results to genome copies/ml the results obtained in IU/ml should be multiplied by 5.3 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.com or at pbpl@vsnl.net).
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 DNA 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.com OR pbpl@vsnl.net.

14. PUBLICATION

1.Comparison between RT-PCR and ELISA for the detection of HBV in blood donors

Al Shaban Z O1 , Abdel-Hamid A Z 1 , Hock T T1 , Hassan R2 1 Infectious Disease Cluster (IDC), Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia (USM), Penang, Malaysia.
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(Received October 29th, 2009 Revised February 21st, 2010 Accepted April 28th, 2010 Published online June 8th, 2010.) Correspondence: Zahrah Omar Al Shaban Email: saqiil@hotmail.com

2.Evaluation of adefovir & lamivudine in chronic hepatitis B: Correlation with HBV viral kinetic, hepatic-necro inflammation & fibrosis

Kumar S Pradeep, Subhash Medhi, Mohammad Asim, Bhudev C Das,* Ranjana Gondal,** and Premashis Kar

3.Immune Molecular Diagnosis of HIV/AIDS Infections and antimicrobial studies for AIDS associated Opportunistic Pathogen—by M.Suresh

P.G. and Rresearch Department of Botany And Microbiology
A.V.V.M.Sri Pushpam Collage(Affilated to Bharthidarsan University)Poondi-613503-Tanjavur Dist.TamilNadu India year May 2010

4.Real Time-PCR HBV-DNA Analysis : Significance and First Experience in Armed Forces

Col GS Chopra, SM* , Lt Col PK Gupta+, Col AC Anand, VSM# , Col PP Varma, VSM**, Col V Nair, VSM++, Lt Gen Ramji Rai, AVSM, VSM, PHS

15. 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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