Geno-Sen's

EnteroVirus

Real Time PCR Kit

Quantitative

IVD

PACK INSERT

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Genome Diagnostics Pvt. Ltd.

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

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Geno-Sen's EnteroVirus Real Time PCR Kit

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EnteroVirus Geno-Sen's Real Time PCR Kit

1. Contents of the Kit:

Color Code	Contents	REF 9111019 100 rxns	REF 9111020 50 rxns	REF 9111021 25 rxns
R1 Blue	*EnteroVirus* 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
EV-S1 Red	*EnteroVirus Standard 1* *1 X 10^5 copies/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
EV-S2 Red	*EnteroVirus Standard 2* *1 X 10^4 copies/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
EV-S3 Red	*EnteroVirus Standard 3* *1 X 10^3 copies/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
EV-S4 Red	*EnteroVirus Standard 4* *1 X 10^2 copies/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
EV-S5 Red	*EnteroVirus Standard 5* *1 X 10^1 copies/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. ENTEROVIRUS Information

Application

Enteroviruses are a genus of positive-sense single-stranded RNA viruses associated with several human and mammalian diseases. Serologic studies have distinguished 66 human enterovirus serotypes on the basis of antibody neutralization tests. Additional antigenic variants have been defined within several of the serotypes on the basis of reduced or nonreciprocal cross-neutralization between variant strains. On the basis of their pathogenesis in humans and animals, the enteroviruses were originally classified into four groups, polioviruses, Coxsackie A viruses (CA), Coxsackie B viruses (CB), and echoviruses, but it was quickly realized that there were significant overlaps in the biological properties of viruses in the different groups. Enteroviruses isolated more recently are named with a system of consecutive numbers: EV68, EV69, EV70, and EV71, etc.

Enteroviruses affect millions of people worldwide each year, and are often found in the respiratory secretions (e.g., saliva, sputum, or nasal mucus) and stool of an infected person. Historically, poliomyelitis was the most significant disease caused by an enterovirus, poliovirus. There are 62 non-polio enteroviruses that can cause disease in humans: 23 Coxsackie A viruses, 6 Coxsackie B viruses, 28 echoviruses, and 5 other enteroviruses. Poliovirus, as well as coxsackie and echovirus are spread through the fecal-oral route. Infection can result in a wide variety of symptoms ranging from mild respiratory illness (common cold), hand, foot and mouth disease, acute hemorrhagic conjunctivitis, aseptic meningitis, myocarditis, severe neonatal sepsis-like disease, and acute flaccid paralysis.

Species and genetics

Enteroviruses are members of the picornavirus family, a large and diverse group of small RNA viruses characterized by a single positive-strand genomic RNA. All enteroviruses contain a genome of approximately 7,500 bases and are known to have a high mutation rate due to low-fidelity replication and frequent recombination. After infection of the host cell, the genome is translated in a cap-independent manner into a single polyprotein, which is subsequently processed by virus-encoded proteases into the structural capsid proteins and the nonstructural proteins, which are mainly involved in the replication of the virus.

The enterovirus genus includes the following ten species:

- Bovine enterovirus
- Human enterovirus A
- Human enterovirus B
- Human enterovirus C
- Human enterovirus D
- Human rhinovirus A

- Human rhinovirus B
- Human rhinovirus C
- Porcine enterovirus B
- Simian enterovirus A

Within these ten species are the serotypes:

- Coxsackievirus

  serotypes CV-A2, CV-A3, CV-A4, CV-A5, CV-A6, CV-A7, CV-A8, CV-A10, CV-A12, CV-A14, & CV-A16 found under the species: Human enterovirus A.
  serotypes CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, CV-B6, CV-A9, & CV-A23 found under the species: Human enterovirus B.
  serotypes CV-A1, CV-A11, CV-A13, CV-A17, CV-A19, CV-A20, CV-A21, CV-A22, & CV-A24 found under the species: Human enterovirus C.

- Echovirus

  serotypes E-1, E-2, E-3, E-4, E-5, E-6, E-7, E-8, E-9, E-11, E-12, E-13, E-14, E-15, E-16, E-17, E-18, E-19, E20, E-21, E-24, E-25,
  E-26, E-27, E-29, E-30, E-31, E-32, & E-33 found under the species: Human enterovirus B.

- Enterovirus

  serotypes EV-71, EV-76, EV-89, EV-90, EV-91, & EV-92 found under the species: Human enterovirus A.
  serotypes EV-69, EV-73, EV-74, EV-75, EV-77, EV-78, EV-79, EV-80, EV-81, EV-82, EV-83, EV-84, EV-85, EV-86, EV-87, EV-88,
  EV-93, EV-97, EV-98, EV-100, EV-101, EV-106, & EV-107 found under the species: Human enterovirus B.
  serotypes EV-95, EV-96, EV-99, EV-102, EV-104, EV-105, EV-109 & EV-116 found under the species: Human enterovirus C.
  serotypes EV-68, EV-70, & EV-94 found under the species: Human enterovirus D.

- Human rhinovirus

  serotypes HRV-1, HRV-2, HRV-7, HRV-8, HRV-9, HRV-10, HRV-11, HRV-12, HRV-13, HRV-15, HRV-16, HRV-18, HRV-19, HRV-20,
  HRV-21, HRV-22, HRV-23, HRV-24, HRV-25, HRV-28, HRV-29, HRV-30, HRV-31, HRV-32, HRV-33, HRV-34, HRV-36,
  HRV-38, HRV-39, HRV-40, HRV-41, HRV-43, HRV-44, HRV-45, HRV-46, HRV-47, HRV-49, HRV-50, HRV-51, HRV-53,
  HRV-54, HRV-55, HRV-56, HRV-57, HRV-58, HRV-59, HRV-60, HRV-61, HRV-62, HRV-63, HRV-64, HRV-65, HRV-66,
  HRV-67, HRV-68, HRV-71, HRV-73, HRV-74, HRV-75, HRV-76, HRV-77, HRV-78, HRV-80, HRV-81, HRV-82, HRV-85,
  HRV-88, HRV-89, HRV-90, HRV-94, HRV-95, HRV-96, HRV-98, & HRV-100 found under the species: Human rhinovirus A.

serotypes HRV-3, HRV-4, HRV-5, HRV-6, HRV-14, HRV-17, HRV-26, HRV-27, HRV-35, HRV-37, HRV-42, HRV-48, HRV-52, HRV-69, HRV-70, HRV-72, HRV-79, HRV-83, HRV-84, HRV-86, HRV-91, HRV-92, HRV-93, HRV-97, & HRV-99 found under the species: Human rhinovirus B.

- Poliovirus

  serotypes PV-1, PV-2, & PV-3 found under the species: Human enterovirus C.

Coxsackie and echovirus

Coxsackie viruses are a non-phylogenetic group.[7] Coxsackie A viruses are mainly associated with human hand, foot and mouth disease. Coxsackie B viruses can cause signs and symptoms, similar to a "cold", but these viruses also can lead to more serious diseases, including myocarditis (inflammation of the heart); pericarditis (inflammation of the sac lining the heart); meningitis (inflammation of the membranes that line the brain and spinal cord); and pancreatitis (inflammation of the pancreas).

Echoviruses are a cause of many of the nonspecific viral infections. It is mainly found in the intestine, and can cause nervous disorders. Usual symptoms of Coxsackie and echovirus are fever, mild rash, and mild upper respiratory tract (URT) illness.

Enterovirus 71

Enterovirus 71 (EV-71) is notable as one of the major causative agents for hand, foot and mouth disease (HFMD), and is sometimes associated with severe central nervous system diseases. EV71 was first isolated and characterized from cases of neurological disease in California in 1969. To date, little is known about the molecular mechanisms of host response to EV71 infection, but increases in the level of mRNAs encoding chemokines, proteins involved in protein degradation, complement proteins, and proapoptotis proteins have been implicated.

Poliovirus

There are three serotypes of poliovirus, *PV1*, *PV2*, and *PV3*; each with a slightly different capsid protein. Capsid proteins define cellular receptor specificity and virus antigenicity. *PV1* is the most common form encountered in nature; however, all three forms are extremely infectious. Poliovirus can affect the spinal cord and cause poliomyelitis.

Polioviruses were formerly classified as a species belonging to the genus Enterovirus in the family Picornaviridae. The Poliovirus species has been eliminated from the genus Enterovirus. The following serotypes, Human poliovirus 1, Human poliovirus 2, and Human poliovirus 3, were assigned to the species Human enterovirus C, in the genus Enterovirus in the family Picornaviridae. The type species of the genus Enterovirus was changed from Poliovirus to Human enterovirus C. This has been ratified in April 2008. The 39th Executive Committee (EC39) of the International Committee on Taxonomy of Viruses (ICTV) met in Canada during June 2007 with new taxonomic proposals.

Two of the proposals with three changes were:

- Code 2005.261V.04: To remove the following species Poliovirus from the existing genus Enterovirus in the family Picornaviridae.
- Code 2005.262V.04: To assign the viruses; PV-1, PV-2, PV-3 to the existing species Human enterovirus C in the genus Enterovirus in the family Picornaviridae.
- Code 2005.263V.04: To change the type species Poliovirus from the existing genus Enterovirus in the family Picornaviridae to the type species Human enterovirus C.

Proposals approved at the (EC39) meeting of 2007, were sent to members of ICTV via email for ratification and have become official taxonomy. There have been a total of 215 taxonomic proposals, which have been approved and ratified since the 8th ICTV Report of 2005.

The ratification process was performed by email. The proposals were sent electronically via email on March 18, 2008 to ICTV members with a request to vote on whether to ratify the taxonomic proposals, with a 1-month deadline. The following are two of the taxonomic proposals with three changes that were ratified by ICTV members in April 2008:

Picornaviruses

- 2005.261V.04: To remove the following species from the existing genus Enterovirus in the family Picornaviridae: Poliovirus. (Note: Poliovirus hereby loses its status as a virus species).
- 2005.262V.04: To assign the following viruses to the species Human enterovirus C in the existing genus Enterovirus in the family Picornaviridae: Human poliovirus 1, Human poliovirus 2, Human poliovirus 3. (This is not strictly necessary as a taxonomic proposal because it concerns entities below the species level, but it is left in to clarify this reorganization of the Picornaviridae).
- 2005.263V.04: To change the type species of the genus Enterovirus in the family Picornaviridae, from Poliovirus to Human enterovirus C.

The ICTVdb, International Committee on Taxonomy of Viruses data base, based on the ICTV Master Species List, 8th Report, June 2005 is obsolete.

Rhinovirus

There are three species of Rhinoviruses: Human Rhinovirus A, Human Rhinovirus B, and Human Rhinovirus C which contain over 100 serotypes. Rhinoviruses are the most suspected causative agents of the common cold. This makes it difficult to develop a single vaccine against so many serotypes.

Diseases caused by enterovirus infection

- Poliomyelitis is the most notable disease caused by enterovirus infection.
- Nonspecific febrile illness is the most common presentation of enterovirus infection. Other than fever, symptoms include muscle pain, sore throat, gastrointestinal distress, and headache. Abdominal discomfort may also be reported in some patients.
- Enteroviruses are by far the most common causes of aseptic meningitis in children. In the United States, enteroviruses are responsible for 30,000 to 50,000 meningitis hospitalizations per year as a result of 30 million to 50 million infections.
- Pleurodynia is characerized by severe paroxysmal pain in the chest and abdomen, along with fever, and sometimes nausea, headache, and emesis.
- Pericarditis and/or myocarditis are typically caused by enteroviruses; symptoms consist of fever with dyspnea and chest pain. Arrhythmias, heart failure, and myocardial infarction have also been reported.
- Acute hemorrhagic conjunctivitis can be caused by enteroviruses.
- Herpangina is caused by Coxsackie A virus, and causes a vesicular rash in the oral cavity and on the pharynx, along with high fever, sore throat, malaise, and often dysphagia, loss of appetite, back pain, and headache. It is also self-limiting, with symptoms typically ending in 3–4 days.
- Hand, foot and mouth disease is a childhood illness most commonly caused by infection by Coxsackie A virus or EV71.
- Encephalitis is rare manifestation of enterovirus infection; when it occurs, the most frequent enterovirus found to be causing it is echovirus 9.
- Bornholm disease is enteroviral in origin.
- A 2007 study suggested that acute respiratory or gastrointestinal infections associated with enterovirus may be a factor in chronic fatigue syndrome.

Treatment

Treatment for enteroviral infection is mainly supportive. In cases of pleurodynia, treatment consists of analgesics to relieve the severe pain that occurs in patients with the disease; in some severe cases, opiates may be needed. Treatment for aseptic meningitis caused by enteroviruses is also mainly symptomatic. In patients with enteroviral carditis, treatment consists of the prevention and treatment of complications, such as arrhythmias, pericardial effusion, and cardiac failure. Other treatments that have been investigated for enteroviral carditis include intravenous immunoglobulin.

The human enteroviruses are ubiquitous viruses that are transmitted from person to person via direct contact with virus shed from the gastrointestinal or upper respiratory tract. The enteroviruses belong to the Picornaviridae family of viruses and are traditionally divided into 5 subgenera based on differences in host range and pathogenic potential. Each subgenus contains a number of unique serotypes, which are distinguished based on neutralization by specific antisera. The subgenera include polioviruses, coxsackievirus (groups A and B), and echoviruses.

A total of 72 serotypes were originally identified by conventional methods; 64 serotypes remain after recognition of redundant serotypes. Three serotypes comprise the polioviruses, 23 serotypes comprise coxsackievirus group A, 6 serotypes comprise coxsackievirus group B, and 29 serotypes comprise the echoviruses. A new classification scheme has been adopted that divides all nonpolio enterovirus into 4 groups designated A through D based on the homology within RNA region coding for the VP1 capsid protein. More recently, many new serotypes that are not included in the original classification have been characterized by molecular methods, bringing the number of known serotypes to more than 90.

Virology

- The enteroviruses are icosahedral nonenveloped viruses that are approximately 30 nm in diameter.
- They have a capsid composed of 60 subunits each formed from 4 proteins (VP1 to VP4).
- They are stable at a pH from 3-10, distinguishing them from other picornaviruses (including rhinoviruses), which are unstable below pH 6.
- A linear, single-strand RNA genome of about 7.5 kb is enclosed by the capsid; the translation product is a single polyprotein that is cleaved after translation by viral-coded proteases into the structural proteins (VP1 to VP4), RNA polymerase, proteases, and other nonstructural proteins.
- Enteroviruses resist lipid solvents, ether, chloroform, and alcohol. They are inactivated at temperatures above 50°C but remain infectious at refrigerator temperature.
- Molar MgCl2 reduces thermolability at higher temperatures.
- The viruses are inactivated by ionizing radiation, formaldehyde, and phenol.

Enteroviruses cause a wide range of infections. Poliovirus, the prototypical enterovirus, can cause a subclinical or mild illness, aseptic meningitis, or paralytic poliomyelitis, a disease that has been eradicated in the United States and other developed countries. The nonpolio viruses (group A and B coxsackieviruses, echoviruses, enteroviruses) continue to be responsible for a wide spectrum of diseases in persons of all ages, although infection and illness occur most commonly in infants.

Coxsackievirus infection is the most common cause of viral heart disease. Group A coxsackieviruses may cause flaccid paralysis, while group B coxsackieviruses cause spastic paralysis. Other diseases associated with group A coxsackievirus infections include hand-foot-and-mouth disease (HFMD) and hemorrhagic conjunctivitis, while group B coxsackievirus is associated with herpangina, pleurodynia, myocarditis, pericarditis, and meningoencephalitis. Aseptic meningitis and the common cold are associated with both groups.

Diseases caused by echoviral infections range from the common cold and fever to aseptic meningitis and acute hemorrhagic conjunctivitis (AHC).

Pathophysiology

Enteroviruses are transmitted predominantly via the fecal-oral route. However, there are some exceptions, including coxsackievirus A21, which is spread mainly by respiratory secretions, and enterovirus 70, which is shed in tears and spread via fingers and fomites.

Upon entry into the oropharynx, the virus replicates in submucosal tissues of the distal pharynx and alimentary tract. Viral particles are shed in the feces and in upper respiratory tract secretions for days prior to symptom onset. The average incubation period is 3-10 days, during which the virus migrates to regional lymphoid tissue and replicates. Minor viremia results, which is associated with the onset of symptoms and viral spread to the reticuloendothelial system (spleen, liver, bone marrow).

Dissemination to target organs follows, and viral replication in target organs produces the major viremia with possible secondary seeding of the CNS. Potential target organs include the skin and CNS. Infectious virus is shed from the upper respiratory tract for 1-3 weeks and from the feces for 3-8 weeks. Enteroviruses undergoes a high rate of mutation during replication in the gastrointestinal tract, where single-site mutations can occur in the 5' noncoding region of the attenuated polioviruses; this can lead to prolonged excretion and neurovirulence.

The neuropathy of paralytic diseases caused by enteroviruses is due to direct cellular destruction. Neuronal lesions occur mainly in anterior horn cells of the spinal cord. The 3 serotypes of poliovirus all bind to the cell surface receptor CD155.

Immunity and immune response

- Immunity to enterovirus is serotype-specific. Intact humoral immunity is required for the control and eradication of enteroviral disease.
- T lymphocytes do not contribute to viral clearance and, in coxsackievirus B3 myocarditis, may contribute to myocardial inflammation.
- Humoral immunity (antibody-mediated) mechanisms operate both in the alimentary tract (to prevent mucosal infection) and in the blood (to prevent dissemination to target organs).
- Secretory immunoglobulin A (IgA) appears in nasal and alimentary secretions 2-4 weeks after the administration of live-attenuated oral poliovirus vaccine

(OPV) and persists for at least 15 years. Upon re-exposure to infectious virus, high titers of secretory IgA antibodies prevent or substantially reduce poliovirus shedding; higher secretory IgA titers lead to better immunity.

- Immunoglobulin M (IgM) antibodies appear as early as 1-3 days after enteroviral challenge and disappear after 2-3 months.
- Immunoglobulin G (IgG) antibody, which is generally detected 7-10 days after infection, is mostly of the IgG_1 and IgG_3 subtypes. Serum neutralizing IgG antibodies persist for life after natural enteroviral infections.
- Macrophage function is also a critical component of the immune response in enteroviral infections; ablation of macrophage function in experimental animals markedly enhances the severity of coxsackievirus B infections.

Epidemiology

United States

- Nonpolio enteroviruses are responsible for 10-20 million symptomatic infections per year and are more prevalent among children of lower socioeconomic class, probably because of crowding, poor hygiene, and opportunities for fecal contamination.
- AHC was first recognized in the United States in 1981 during an epidemic in Florida; few cases have been reported since. The prevalence is higher in southern areas than in northern areas.
- Between 2002 and 2004, echoviruses 9 and 30 were the most commonly reported enterovirus serotypes in the United States. In contrast, other enterovirus serotypes (eg, echovirus 1, coxsackievirus B6, and enteroviruses 68 and 69) are rarely reported and appear to have little epidemic potential. However, difficulty in isolation of enterovirus 68 may bias the data, leading to an underestimation of its prevalence.
- Coxsackievirus A is likely underrepresented because only some serotypes are readily isolated in cell culture.
- National or regional outbreaks of aseptic meningitis are occasionally reported, such as the echovirus 30 outbreaks in the United States between 1989 and 1992 and in 2003 and echovirus 13 and echovirus 18 outbreaks in 2001. Aseptic meningitis is no longer a nationally notifiable disease in the United States.

International

- Enteroviruses are distributed worldwide and are influenced by season and climate. Infections occur in summer and early fall in temperate areas, while tropical and semitropical areas bear the brunt all year.
- AHC occurs as epidemics in tropical countries during the hot and rainy season. It was first recognized in 1969 in Ghana (Apollo disease) and Indonesia. AHC is also epidemic in India and the Far East.

- The worldwide prevalence of poliomyelitis has decreased significantly because of improved economic conditions and availability of vaccines. The last case of wild polio in the Americas occurred in Peru in 1991. In 1994, the World Health Organization declared polio eradicated from the Western Hemisphere. In 2000, 7 cases of poliomyelitis due to a mutated polio strain related to oral polio vaccine were reported from Haiti and the Dominican Republic. Polio remains a significant disease in the developing world, and, in 2003, 6 endemic countries were identified: Afghanistan, Egypt, India, Niger, Nigeria, and Pakistan.
- In 2008, 1,652 confirmed cases of paralytic polio were reported worldwide. Polio is endemic in 4 countries: Afghanistan, India, Nigeria, and Pakistan. In addition, 14 other previously polio-free countries (Angola, Burkina Faso, Benin, Central Africa Republic, Chad, Côte d'Ivoire, The Democratic Republic of Congo, Ghana, Ethiopia, Nepal, Niger, Sudan, Tango) have reported cases in 2008-2009 (114 cases through August 2009) as a result of importations. As of September 2009, 969 cases of polio (including wild polio strains and oral vaccine–derived) had been reported in endemic and nonendemic countries.
- In a Korean study of children during an outbreak of aseptic meningitis, echovirus 6 or 30 infection was the most common manifestation.

Mortality/Morbidity

- More than 90% of infections caused by the nonpolio enteroviruses are asymptomatic or result in only an undifferentiated febrile illness.
- Myopericarditis carries a mortality rate of 0%-4%. Myocarditis carries a higher mortality rate than pericarditis. Additionally, murine model studies have suggested that a deficiency of complement receptors 1 and 2 leads to increased morbidity in coxsackie B3 infections, including myocarditis, dilated cardiomyopathy, and fibrosis.
- Prior to the vaccine era, the mortality rate in polio epidemics was 5%-7%.
- The overall risk of OPV-related disease is estimated to be 1 case per 2.6 million doses of OPV. The inactivated poliovirus vaccine (IPV) was incorporated into the routine polio vaccination in Europe and Canada in the 1980s. IPV has been used in the United States since 2000; OPV is no longer used in the United States.
- Despite the risk of OPV-related paralysis, it is still the preferred vaccine for global polio eradication in developing nations (see Deterrence/Prevention).

Sex

- The male-to-female ratio of myopericarditis is 2:1. The risk of cardiac involvement is higher during pregnancy and immediately postpartum.
- The prevalence of polio infection is equal in boys and girls, although paralysis is more common in boys. Among adults, women are at increased risk of infection and the postpolio syndrome.

- Aseptic meningitis is approximately twice as common in males as in females.

Age

- Enteroviral infections are most common in young children. Herpangina primarily affects children aged 3 months to 16 years. Poliomyelitis is observed in children younger than 15 years. Aseptic meningitis due to enteroviral infection is more common in infants than in adults. Most cases of pleurodynia occur in children and adults younger than 30 years.
- Myopericarditis is most prevalent in young adults, especially those who are physically active. AHC is most prevalent in adults aged 20-50 years.
- Neonates are at high risk for severe sepsis due to enterovirus infections.

History

Polio

- Disease due to wild-type poliovirus infection no longer occurs in the Western Hemisphere, and a World Health Organization (WHO) international eradication program is making significant progress in the rest of the world.
- Patients with abortive polio present with symptoms similar to those of other viral infections, including fever, headache, sore throat, loss of appetite, vomiting, and abdominal pain. Neurologic symptoms are typically not reported.
- The symptoms of nonparalytic polio are similar to those of abortive polio but are more intense. Patients report stiffness of the posterior muscles of the neck, trunk, and limbs.
- Paralytic polio presents similarly to nonparalytic polio. It is an acute febrile illness characterized by aseptic meningitis and weakness or paralysis of one or more extremities, along with weakness of one or more muscle groups. Exercise increases the severity of paralytic polio, especially during the first 3 days of the major illness. Intramuscular injections or skeletal muscle injury predisposes to localization of polio to that extremity (termed provocation poliomyelitis).
    - Spinal: Patients have a prolonged prodrome, with features of aseptic meningitis followed in 1-2 days by weakness and, eventually, paralysis.
    - Bulbar: Cranial nerves are involved, most commonly IX, X, and XII. Tonsillectomy increases the risk of bulbar polio. Patients are unable to swallow smoothly. They accumulate pharyngeal secretions, have a nasal twang to the voice, and develop paralysis of vocal cords, causing hoarseness, aphonia, and, eventually, asphyxia.

- Polioencephalitis: This form is principally reported in children. Unlike in other forms of polio, seizures are common and paralysis may be spastic.

Nonpolio viruses

More than 90% of infections caused by the nonpolio enterovirus are asymptomatic or result only in an undifferentiated febrile illness.

- Pleurodynia
  - Group B coxsackieviruses, particularly B3 and B5, are the most important causes of epidemic pleurodynia. Multiple family members may be affected.
  - Pleurodynia manifestations include a sudden onset of fever accompanied by muscular pain in the chest and abdomen. The pain is spasmodic in nature, with spasms lasting 15-30 minutes and worsening during inspiration or coughing. This paroxysmal pain is characteristically associated with fever, peaking within 1 hour after onset of each paroxysm and subsiding with the subsequent paroxysm. Headache, nausea, and vomiting are also frequently reported.
- Myopericarditis
  - Enteroviruses appear to be the most common viral cause of myopericarditis and account for at least half of all cases of acute myopericarditis.
  - Neonatal infections typically develop within the first week of life, and involvement is predominantly myocardial. In contrast, older children and adults usually present with symptoms of pericarditis.
  - The typical presentation in adolescents and adults is shortness of breath, chest pain, and fever 1-2 weeks following an upper respiratory tract infection. Chest pain may be dull or sharp; it is worsened by inspiration and may improve with sitting and leaning forward. It can be differentiated from angina by lack of response to nitroglycerin.
  - Enteroviral myocarditis can present as acute myocardial infarction associated with arrhythmias and heart failure. Some patients with myocardial infarction who have normal findings on coronary angiographic studies have been shown to have myocarditis by radiolabeled antimyosin antibody cardiac scanning.
- Acute hemorrhagic conjunctivitis
  - This highly contagious ocular infection can cause large-scale epidemics. AHC was first described in 1969. Enterovirus 70 is the most common etiology in epidemics. Coxsackievirus A24 causes a similar

disease. AHC was initially recognized in Ghana and Indonesia and is now epidemic in India and the Far East.

- The first reported outbreak of AHC in United States was Key West, Florida, in 1981; subsequently, 2,500 cases were reported in Miami. Since then, with the exception of few imported cases, AHC activity has not been reported in the United States.
- The mode of transmission is from finger or fomite to eye. AHC is highly contagious, and crowding and unsanitary conditions favor spread. Reuse of water for bathing and sharing of towels have been implicated as factors contributing to the spread of infection.
- Onset is abrupt, and the most common symptoms include ocular pain and burning, swelling of the eyelids, and the sensation of a foreign body in the eye. Patients may also experience photophobia and watery discharge. The other eye becomes involved within hours of the first eye.
- Nonspecific symptoms such as fever, malaise, and headache may be present. The symptoms typically improve by the second or third day of infection, and recovery is complete within 7-10 days.

- Nonspecific febrile illness
  - This is the most common presentation of enterovirus infection.
  - More than 90% present with a nonspecific febrile illness that manifests as sudden fever (temperature, 101-104°F). The fever may last for as long as a week and may show a biphasic pattern.
  - Patients may also report myalgia, headache, sore throat, nausea, vomiting, mild abdominal discomfort, and diarrhea.
  - Human enterovirus 68 infection in children may produce a respiratory outbreak characterized by pneumonia and wheezing.
- Aseptic meningitis
  - Enteroviral infections (group B coxsackievirus and echovirus) account for 90% of cases of aseptic meningitis in patients younger than one year and 50% of cases in older children and adults.
  - The clinical presentations of aseptic meningitis vary greatly. Prodromal symptoms include fever, chills, headache, photophobia, and nuchal rigidity. Rash and upper respiratory tract symptoms may also occur. In infants, fever and irritability are the most common symptoms.
  - Fever and meningeal signs subside within 2-7 days.
  - Enterovirus 71, which causes HFMD, has also been associated with a particularly more aggressive and, in some instances, fatal CNS infection in children. It manifests as flaccid motor paralysis and brain

stem encephalitis. Large outbreaks were reported in the late 1990s in Eastern Europe, Russia, Thailand, and Taiwan.

- Most of the enterovirus-positive 758 children in a Korean outbreak experienced fever, headache, vomiting, and neck stiffness, although some also showed cold symptoms, sore throat, altered mental status, and seizures. More than 80% of these had either echovirus types 6 or 30. The majority recovered uneventfully.

- Herpangina
  - Coxsackie A virus is the main etiologic agent of herpangina, described as a vesicular enanthem of the tonsillar fauces and soft palate that principally affects children aged 3-10 years. Other serotypes have been isolated including enterovirus 71 (EV71), which has cause recent outbreaks and epidemics in South-East Asia
  - Symptoms include sudden onset of fever, sore throat, and difficulty swallowing, followed a day later by a painful vesicular eruption of the oral mucosa. The posterior pharynx and tonsils may also be involved. Most disease occurs in the summer.
  - Patients may report anorexia, malaise, irritability, headache, backache, and diarrhea. Symptoms resolve in 3-4 days.
- Hand-foot-and-mouth disease
  - This is mainly a disease of children; most patients are younger than 10 years. Epidemics of HFMD occur approximately every 3 years.
  - Coxsackievirus A16 is the most common etiologic agent, although enterovirus 71 and numerous other coxsackievirus serotypes may also cause the disease.
  - Following an incubation period of 3-6 days, patients experience prodromal symptoms such as fever, cough, sore throat, malaise, and anorexia. The prodrome lasts from 12-36 hours; afterward, vesicular eruptions of the hands, feet, and oral cavity develop. This may cause decreased oral intake in young children. The lesions self-resolve within 5-7 days.
  - Infection with enterovirus 71 may be accompanied by severe neurologic disease including encephalitis, meningitis, and poliolike paralysis.
- Encephalitis
  - Frank encephalitis is an unusual manifestation of enterovirus infection.
  - Echovirus 9 is the most common etiologic agent.

- Clinical manifestations have ranged from lethargy, drowsiness, and personality change to seizures, paresis, and coma. Children with focal encephalitis present with partial motor seizures, hemichorea, and acute cerebellar ataxia; this may mimic herpes simplex encephalitis.

- Nonpoliovirus paralytic disease
  - Enterovirus 71 and coxsackievirus A7 have been associated with large outbreaks of poliomyelitislike disease in Russia, Eastern Europe, Thailand, and Taiwan. Some cases have manifested as brainstem encephalitis or noncardiogenic pulmonary edema, with some having a fatal course.
  - Paralytic disease caused by nonpolioviruses other than enterovirus 71 is usually less severe and is associated with paralysis. It manifests as muscle weakness and complete unilateral oculomotor palsy.
  - Guillain-Barré syndrome and transverse myelitis has been reported in a small number of patients infected with coxsackievirus serotypes A2, A5, A9, and B4 and with echovirus serotypes 5, 6, and 22.
- Neonatal infections: Refer to the article Enteroviral Infections in eMedicine's Pediatrics volume.

Physical

Physical examination findings in enteroviral disease vary greatly depending on the type of illness and etiologic agent.

- Nonspecific febrile illness: Physical findings are those of general viral illness; mild pharyngeal erythema or conjunctivitis may be present.
- Pleurodynia: Paroxysmal chest pain is characteristic, has no prodrome, and begins with an abrupt onset of spasmodic pain, typically over the lower part of the rib cage or the upper abdominal region. Fever often occurs within one hour of the onset of pain and subsides as the pain recedes. During paroxysms, respirations are rapid and shallow. The pain is reproducible, and patients appear healthy between paroxysms of pain. Auscultation may reveal a pleural friction rub.
- Myopericarditis: The most common symptoms are dyspnea, chest pain, fever, and malaise. Pain in the precordial area maybe sharp or dull and is often exacerbated by the recumbency position. A pericardial friction rub is transient, if present. Signs of congestive heart failure are present in 20% of cases.
- AHC: The hallmark physical findings include ocular erythema and subconjunctival hemorrhage, which seems to be more profuse in young patients. Palpebral edema, chemosis, and ocular discharge may also be noted. Preauricular lymphadenopathy is an associated finding in AHC.
- Aseptic meningitis: Meningeal signs (nuchal rigidity, bulging fontanelles in infants) may be present, along with a positive Kernig and/or Brudzinski sign.

Some patients develop a rash. Adults may experience a more prolonged period of headache and fever than children. Approximately 5%-10% of infants with aseptic meningitis experience complications such as febrile seizures, complex seizures, lethargy, coma, and movement disorders early in the course.

- Encephalitis: Manifestations range from lethargy, drowsiness, and personality change to seizures, paresis, coma, motor seizures, hemichorea, and acute cerebellar ataxia.
- Herpangina: Examination of the oral mucosa reveals punctate macular lesions that evolve into vesicles and eventually ulcerate. The most common site of involvement is the anterior tonsillar pillar and soft palate (mimics pharyngitis or tonsillitis). The lesions are tender and subside within one week.
- HFMD: Vesicular lesions develop on the hands and feet and in the oral cavity. Hands are involved more commonly than feet. The skin lesions consist of mixed papules. Clear vesicles appear gray and are surrounded by erythematous rings. Lesions are tender and resemble those of herpes simplex or varicella zoster infection. They resolve in approximately one week.
- Poliomyelitis
    - Nonparalytic polio: Signs of meningeal irritation are present, and patients may have positive Kernig and Brudzinski signs. In infants, the head drop sign can be elicited.
    - Paralytic polio: In early-stage disease, reflexes are normally active. A change in the character of reflexes precedes paralysis by 12-24 hours. Superficial reflexes are the first to decrease, followed in 8-24 hours by loss of deep tendon reflexes. The resultant paralysis is flaccid and characteristically asymmetric in distribution. Proximal limb muscles are involved more than distal muscles. The lower extremities are affected more commonly than the upper extremities.
- Orchitis: In some remote cases, the presentation of coxsackievirus B infection clinically resembles mumps orchitis.

Causes

- The most common mode of transmission of enteroviruses is via the fecal-oral route. Poor sanitation, low socioeconomic status, and crowded living conditions all facilitate the spread of infection. Direct contact with feces occurs with activities such as diaper changing. Indirect transmission due to poor sanitary conditions may occur via numerous routes, including via contaminated water, food, fingers, fomites, or contaminated ophthalmological instruments (eg, AHC).
- Respiratory-oral spread may also be the mode of transmission for coxsackievirus A21 and other coxsackievirus serotypes.
- Transmission of enteroviruses has been described among travelers swimming in sewage-contaminated seawater.

Differential Diagnosis Required with

- Adenoviruses
- Botulism
- Ehrlichiosis
- Hand-Foot-and-Mouth Disease
- Herpangina
- Herpes Simplex
- Lyme Disease
- Myocardial Infarction
- Pharyngitis, Bacterial
- Pharyngitis, Viral
- Pleurodynia
- Rocky Mountain Spotted Fever
- Varicella-Zoster Virus

Laboratory Studies

- Diagnosis of enterovirus infections is often clinical. Laboratory diagnosis can be achieved with serological tests, viral isolation by cell culture, and polymerase chain reaction (PCR)/. Real Time PCR is the fastest method to detect the presence or absence of Enteroviruses.

    - Serology: The microneutralization test is the most widely used method for detecting antibodies to enteroviruses. Serological examination reveals a 4-fold increase in antibodies to enteroviruses between the acute and convalescent phases of illness. This diagnostic modality is infrequently used since it is serotype-specific, relatively insensitive, poorly standardized, labor intensive, and too slow for clinical purposes.

    - Viral isolation: The virus can be isolated from CSF, blood, or feces, depending on the site affected, and the yield is increased if multiple sites are sampled. Enterovirus produces a characteristic cytopathic effect in cultured cells. Poliovirus is easily cultured from stool and nasopharyngeal secretions, but isolation from the CSF is more difficult. The cytopathic effect is confirmed by indirect immunofluorescence using a broadly specific monoclonal antibody. The sensitivity of viral culture ranges from 60%-75%.

- **Real Time PCR: This rapid test is highly sensitive and specific for detecting enteroviral RNA in CSF specimens, with a sensitivity of 100% and specificity of 100%. PCR provides rapid results and is the best diagnostic test for use in CSF but is limited by availability in some areas and cost in underdeveloped regions.**
- Cardiac enzyme levels may be elevated in persons with myopericarditis, indicating myocardial damage.
- CSF analysis: The CSF profile in patients with aseptic meningitis usually reveals a mildly elevated white blood cell count, and the differential invariably shifts to a predominance of lymphocytes during the initial 1-2 days of illness. Glucose levels are normal or mildly decreased, while the protein level is normal or slightly increased.

Imaging Studies

- Chest radiography: In patients with myopericarditis, chest radiography may reveal cardiomegaly secondary to pericardial effusion or cardiac dilation. In pleurodynia, chest radiographic findings are normal.
- Echocardiography: Transient wall motion abnormalities may be detectable in mild cases. Severe cases may demonstrate acute ventricular dilation and reduced ejection fraction.

Other Tests

- ECG: Nonspecific ST-T changes may be observed in persons with myopericarditis. Severe disease may cause Q waves, ventricular tachyarrhythmias, and heart block. ECG findings may demonstrate evolution through several stages of myopericarditis, as follows:
  - Stage I - Diffuse ST elevation with PR depression
  - Stage II - Normalization of ST and PR segments
  - Stage III - Deep symmetric inversion of T waves
  - Stage IV - May revert to normal or permanent T-wave inversions
- Electroencephalography: This test may be useful for evaluating the extent and severity of illness in patients with encephalitis.
- Ophthalmic slit-lamp examination: In persons with AHC, corneal erosions may be visualized using a fluorescein stain. Enterovirus 70 and coxsackievirus A24 can often be recovered from conjunctival swabs during the first 3 days of infection.
- Histopathologic findings in most enterovirus infections are usually nonspecific, consisting primarily of lymphocytic infiltrates and cellular destruction.

- Histologic findings in patients with polio have been well studied. Evidence of infection is pronounced in the spinal cord, medulla, pons, and mid brain. Neuronal destruction is observed, along with an inflammatory infiltrate composed of lymphocytes, macrophages, and polymorphonuclear leukocytes.

Medical Care

- Polio management is supportive in nature.
    - Abortive polio: Treatment with bed rest and minimal exertion may be done at home. Supportive treatment with analgesics and sedatives may be used.
    - Nonparalytic polio: Management is similar to that of abortive polio. Combine analgesic therapy with hot packs for pain relief.
    - Paralytic polio: In contrast to abortive and nonparalytic polio, which can be managed at home, patients with paralytic polio require hospitalization.
    - Bed rest is required during the early stages of the disease because exertion may worsen the paralysis.
    - Applying hot packs to affected muscles may alleviate pain.
    - Align the body in a neutral position to minimize deformity. Patients should start physical therapy soon after the resolution of pain. Physical therapy should include both active and passive exercises.
    - Mechanical ventilation may be required if respiratory muscles are affected.
    - Postural drainage and suction should be implemented in mild bulbar polio.
    - Patients with weakness or paralysis of the bladder may be treated with cholinergic agents, the sound of running water, or catheterization.
- Pleurodynia: Treatment is symptomatic, using analgesics and heat application for pain relief. Severe pain may require opiate analgesics.
- Aseptic meningitis: Treatment is symptomatic, with analgesics for headache relief. Headache is often severe and prolonged in adults; potent analgesics should be administered, when necessary.
- Myopericarditis
    - Treatment is mainly supportive in nature and involves management of pericardial pain, pericardial effusion, arrhythmias, and heart failure.
    - Bed rest is important since exercise can increase the degree of myocardial necrosis.

  - Intravenous immunoglobulin (IVIG) therapy has shown some benefit in small case-control studies. Nevertheless, most reports lack statistical significance, and randomized trials are needed.
  - Capsid-binding inhibitors belong to a class of drugs that have shown benefit in some immunosuppressed patients with myocarditis. However, these drugs are not available for use in the United States.
  - Corticosteroids yield little or no benefit, and immunosuppressive therapy is contraindicated during the acute phase of viral myocarditis because they have been shown to cause clinical deterioration.

- Acute hemorrhagic conjunctivitis
  - Treatment is primarily symptomatic in nature.
  - Antimicrobial agents are not indicated unless bacterial superinfection occurs. Corticosteroids are contraindicated.
  - Cold compresses may be used, along with antihistamine/decongestant eye drops.
- Herpangina and hand-foot-and-mouth disease
  - Symptomatic treatment for sore throat is the mainstay of treatment, including analgesics, topical anesthetics, mouth wash, and saline rinses.
  - Viscous lidocaine (2% solution) may be helpful.

Medication Summary

Management is supportive and addresses symptoms. No antiviral medications are currently approved for the treatment of enterovirus infections.

- Hygienic measures such as hand washing and adequate disposal of infected secretions help prevent the spread of enteroviral infections.
- Poliovirus vaccines have been instrumental in the effort to eradicate polio; the vaccine is available in 2 forms.
  - The OPV is a live attenuated vaccine that contains all 3 serotypes. It was developed by Sabin. OPV administration decreases replication of the virus in the small intestine and increases production of high titers of IgA in the mucosa. The advantages of OPV include easy administration, stimulation of local and generalized immunity, and herd immunity. Adverse effects include vaccine-associated paralytic poliomyelitis and seroconversion rates lower than those achieved with IPV. OPV should not be administered to immunocompromised patients or to household contacts of these patients.

  - OPV is used in developing nations because of it lower cost, ease of administration, and superior secretory immunity in the GI tract in comparison to IPV.
  - IPV was originally developed by Salk in 1955. Current formulations of IPV are more immunogenic than those available before 1987. This vaccine elicits higher IgG antibody titers and has few adverse effects but is inferior to OPV in providing secretory immunity in the GI tract. It is the recommended polio vaccine in United States when the risk of vaccine-associated paralytic polio disease due to OPV exceeds that of wild-type polio disease. Higher costs for production and supply and the delivery route, along with the lack of herd immunity, makes its use less desirable in developing countries.
  - Combined immunization with OPV and IPV provides the highest serum level of antibody response, with equivalent mucosal immunity to that produced by OPV alone.

- The spread of AHC is prevented by hand washing and using separate towels.
- Intensified efforts to eradicate polio have led to the introduction of new monovalent OPV type 1 (mOPV1) and type 3 (mOPV3) vaccines to more rapidly eliminate the final strains of poliovirus in circulation.
- Further efforts to simplify administration of the two monovalent vaccines have resulted in the development of a bivalent oral polio vaccine (bOPV). This vaccine consists of live-attenuated (weakened) poliovirus strains of type 1 and type 3, which simultaneously target the two remaining types of wild poliovirus (type 1 and type 3).
- Recent trials demonstrated the superiority of bOPV over tOPV and noninferiority to the respective mOPVs in achieving seroconversion.
- As of 2009, the use of bOPV or mOPVs as supplementary immunization activity to complement tOPV is recommended.

Complications

- Polio
  - Respiratory failure secondary to paralysis of respiratory muscles or to lesions of the respiratory center is a life-threatening complication of paralytic polio.
  - Pharyngeal paralysis may occur.
  - Myocarditis is rarely diagnosed clinically.
  - Gastrointestinal hemorrhage results from intestinal erosions and may require transfusion. Gastric dilation is abrupt in onset, and immediate gastric aspiration should be performed.
  - Hypertension is a common complication and may progress to hypertensive encephalopathy.

- Postpolio syndrome occurs 3-4 decades after acute paralytic polio. It is characterized by muscle pain, worsening of prior weakness, or new paralysis. This is more common in women than in men.
- Vaccine-associated poliomyelitis occurs in approximately 1 per 2.6 million people overall and in 1 per 750,000 people who receive the OPV.

- Aseptic meningitis: Complications include lethargy, febrile seizures, and coma.
- Nonpoliovirus paralytic disease: This is usually less severe than polio-associated paralysis.
- Myopericarditis
  - Chronic dilated cardiomyopathy may result from past enteroviral infections, and cardiac transplantation may be required in severe cases.
  - In rare cases, chronic constrictive pericarditis develops 5 weeks to 1 year after resolution.
- Acute hemorrhagic conjunctivitis
  - Secondary bacterial conjunctivitis may occur.
  - In severe cases, keratitis may occur and may last several weeks but is rarely permanent.
  - Paralysis (motor and/or sensory) may follow AHC by 2-5 weeks. It is clinically indistinguishable from polio, although it occurs exclusively in patients older than 20 years. Males are affected more frequently than females.
  - Neurological complications of AHC occur in epidemics caused by enterovirus 70 but not by coxsackievirus A24.

Prognosis

- Polio: Paralytic polio leads to permanent weakness in the affected limb. Permanent weakness occurs in approximately two thirds of patients. Postpolio syndrome is slowly progressive. In the epidemic era, poliomyelitis carried an overall mortality rate of 5%-10%.
- Aseptic meningitis: Fever and signs of meningeal irritation usually resolve within 1-2 weeks in infants; some adults are ill for 2-3 weeks. Long-term prognosis is excellent.
- Pleurodynia: Patients with epidemic pleurodynia completely recover.
- Myopericarditis: The prognosis is good, and mortality rates in acute infection are low. Severe cases can result in dilated or restrictive cardiomyopathy,

persistent electrocardiographic abnormalities, or congestive heart failure. Twenty percent of patients may have recurrent myopericarditis and develop chronic dilated cardiomyopathy. Infants are at a higher risk of developing long-term sequelae.

The *Geno-Sen's* Enterovirus *Quantification Kit for EnteroVirus* genomes is designed for *in vitro* quantification of reverse transcribed most common *EnteroViruses.*

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

Geno Sen's standardized ready-to-use Control and Reaction mix allow fast processing of the samples.

Samples which can be used for Extraction: Whole Blood, serum, C.S.F. Stool, cell Culture supernatants 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
- *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 EnteroVirus*** *PCR* Reagents constitute a ready to use system for detection and quantification of ***EnteroVirus*** 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 ***EnteroVirus*** 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 (EV 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, C.S.F, Cell Culture supernatants.	***Geno Sen's*** ® Viral RNA Extraction Mini Kit (Columns based) OR QIAamp Viral RNA Mini extraction Kit (50)	98001 or 98002 52904	Genome Diagnostics Pvt. Ltd. India. QIAGEN

The ***Geno Sen's***® EnteroVirus Real Time PCR 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 ***EnteroVirus*** *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 *(EV 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 Copies/µl. The following formula has to be applied to convert the values determined using the standard curve into Copies/ml of sample material:

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

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. copies/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 **copies**/µl = 40000000 **copies**/ml
S2: 10^4 **copies**/µl = 4000000 **copies**/ml

S3: 10^3 **copies**/µl = 400000 **copies**/ml
S4: 10^2 **copies**/µl = 40000 **copies**/ml
S5: 10^1 **copies**/µl = 4000 **copies**/ml

If the starting volume of the sample while using the Qiagen QIAamp Viral RNA Mini extraction kit is 140µl & the final Eluted Volume is 50µl then to obtain the direct values i.e. Copies/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 Copies/µl = 35750000 Copies/ml
S2: 10^4 Copies/µl = 3575000 Copies/ml
S3: 10^3 Copies/µl = 357500 Copies/ml
S4: 10^2 Copies/µl = 35750 Copies/ml
S5: 10^1 Copies/µl = 3575 Copies/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 *(EV 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

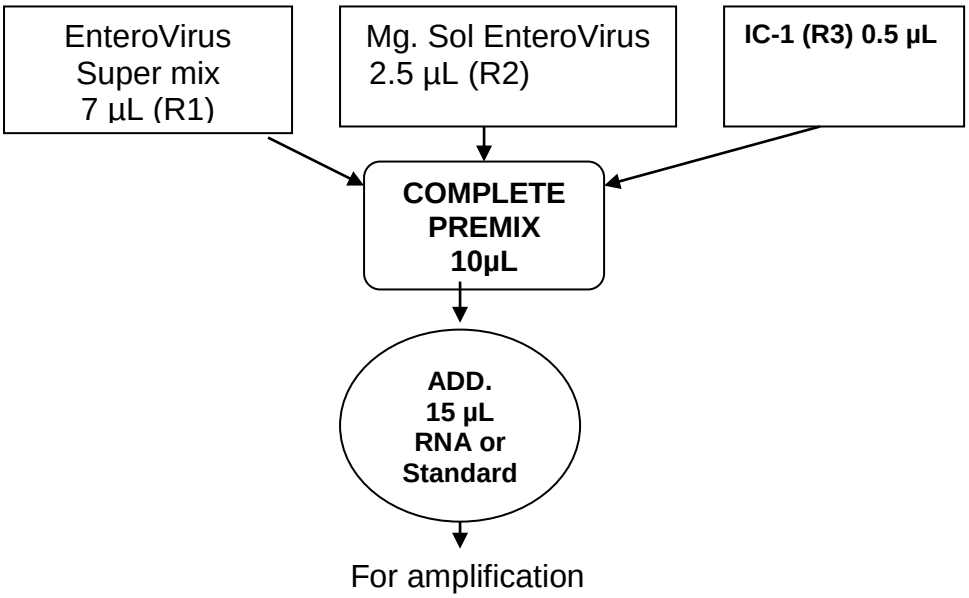


Fig. 4.
Depending upon the number of samples the following pipetting scheme can be followed. e.g. for 10 rxns.

***EnteroVirus* MASTER MIX**	**1 rxns.**	**10 rxns.**
***EnteroVirus* Super Mix (R1)**	**7 µL**	**70 µL**
***EnteroVirus* Mg Sol. (R2)**	**2.5 µL**	**25 µL**
IC-1 (R3) 1 µL	**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 (EV *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 the *RotorGene*™ 2000/3000

The *RotorGene*™ *2000/3000* PCR program for the detection of EnteroVirus 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

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.

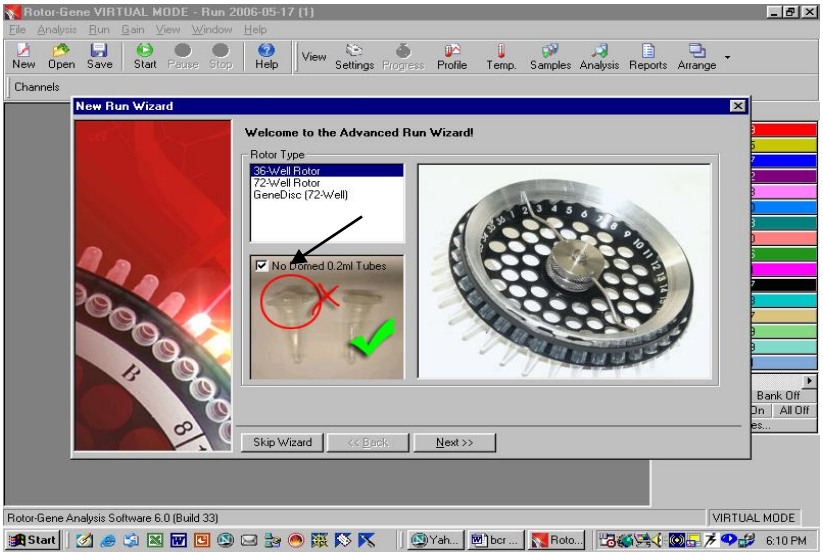


Fig. 6.

Confirmation of Reaction Volume as follows.

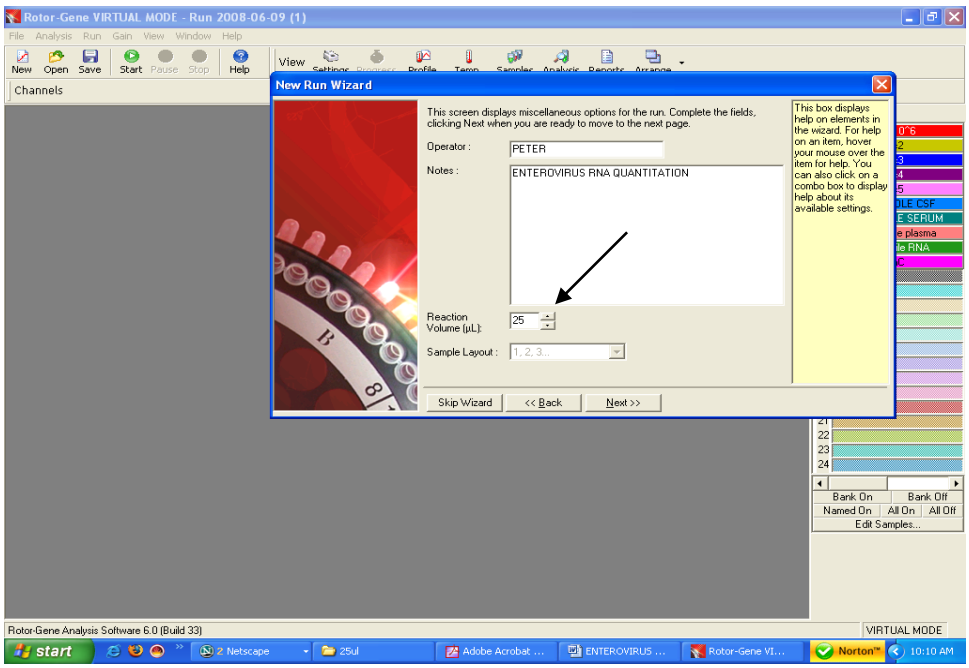


Fig. 7. Setting of general assay parameters.

- 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.

THERMAL PROFILE & CALIBRATION:

Here the thermal profile for the assay will be defined.

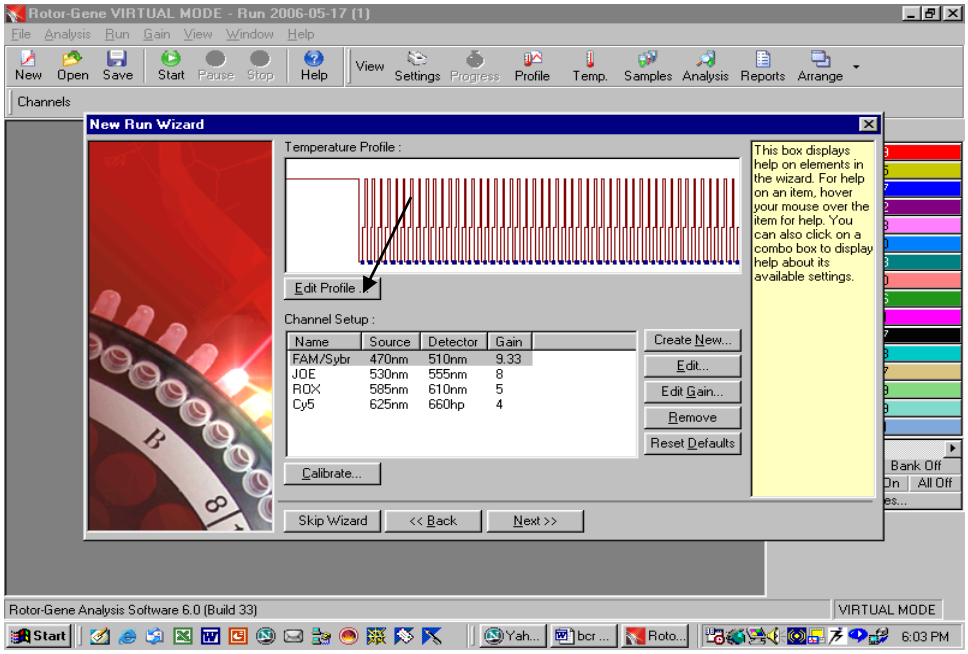


Fig. 8.

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

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

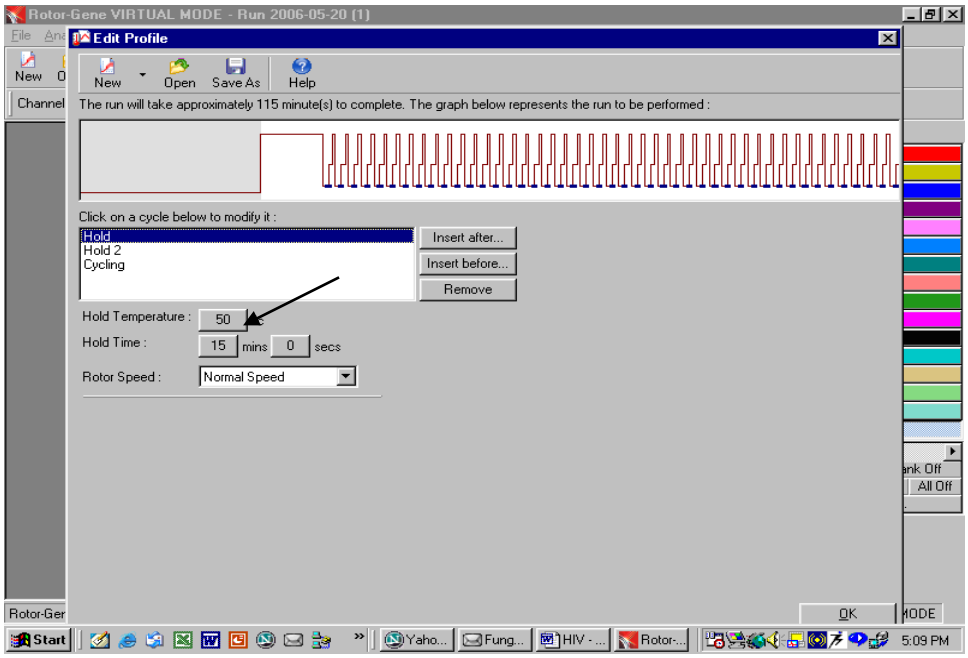


Fig. 9.

CYCLING PROFILE: Second hold 95°C for 10 minutes as below

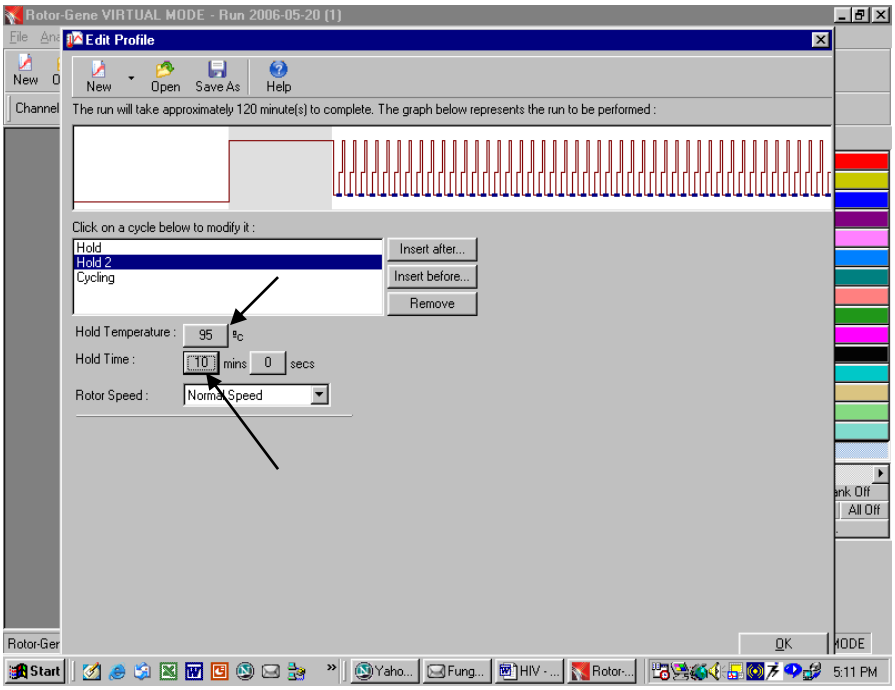


Fig. 10. Initial activation of the Hot Start enzyme.

Setting up of denaturation step in the cycling profile as depicted below i.e.

95°C for 15 seconds.

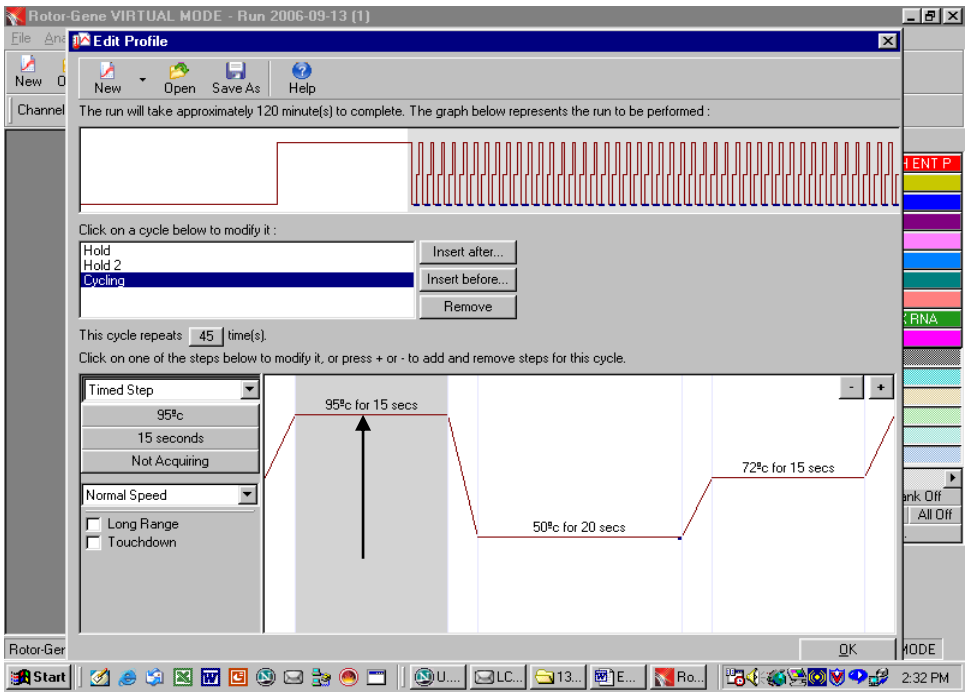


Fig. 11.

Setting up of Anneling step in the cycling profile as depicted below i.e. 50°C for 20 **Seconds and defining the Data acquiring channel i.e FAM & JOE**

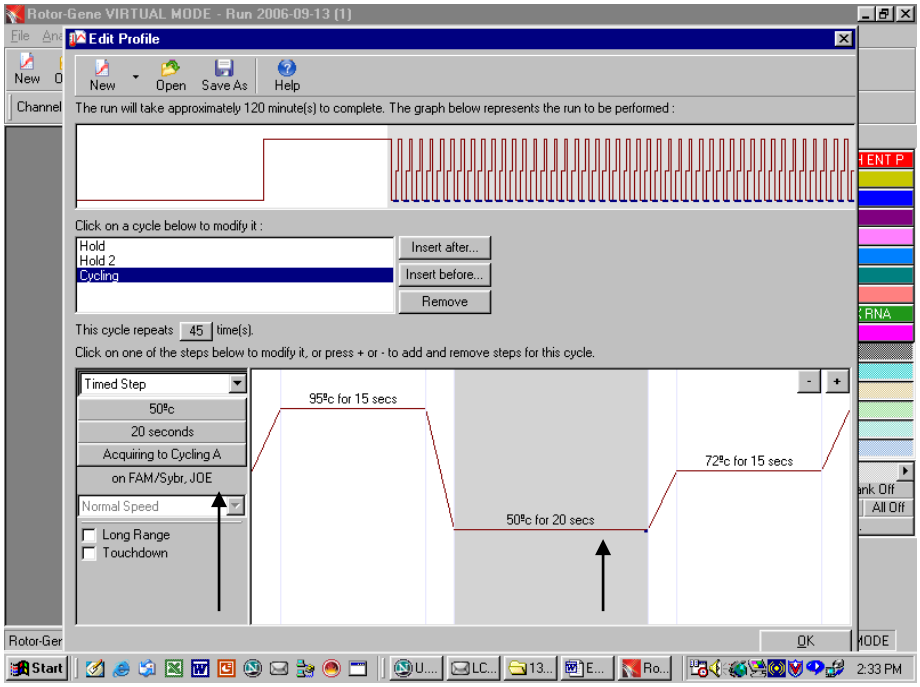


Fig. 12.

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

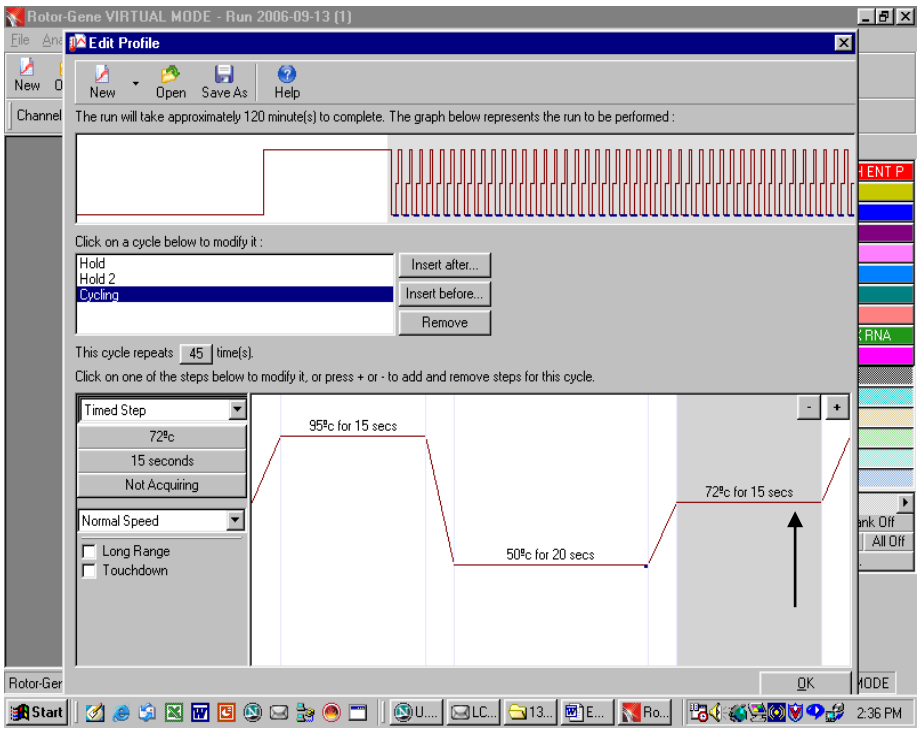


Fig. 13.

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

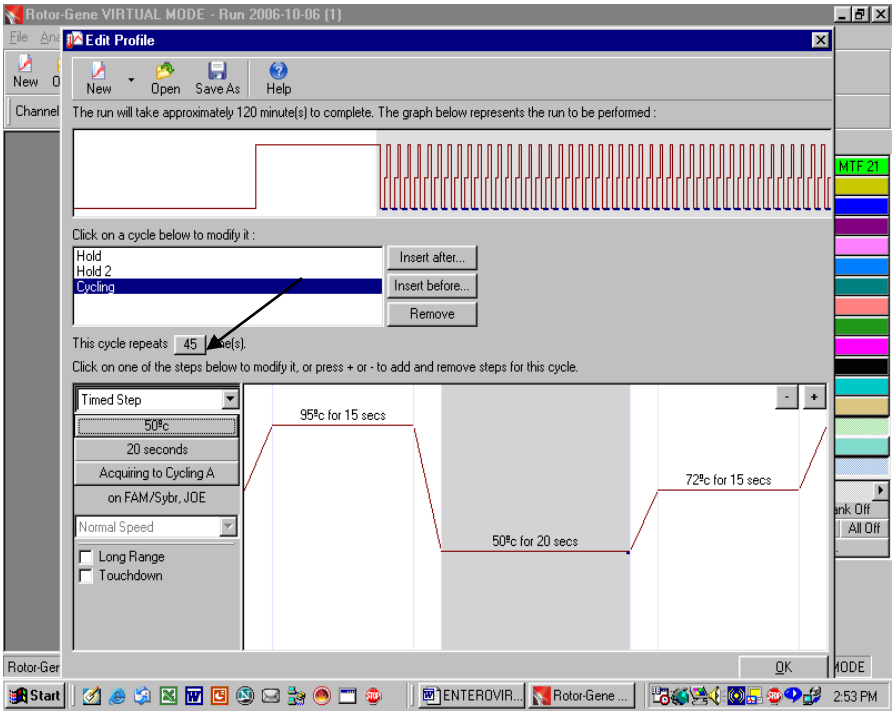


Fig. 14.

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

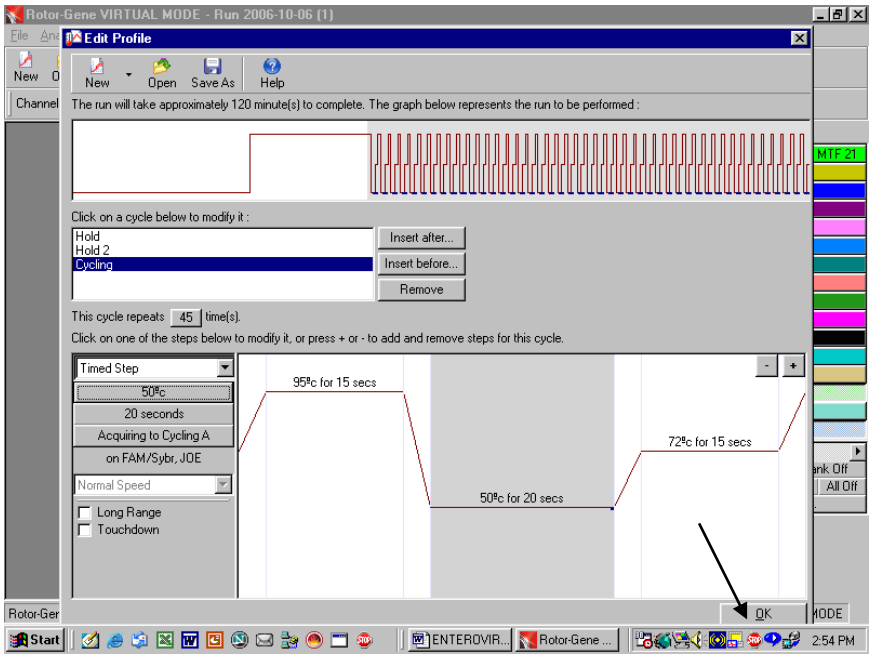


Fig. 15.

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

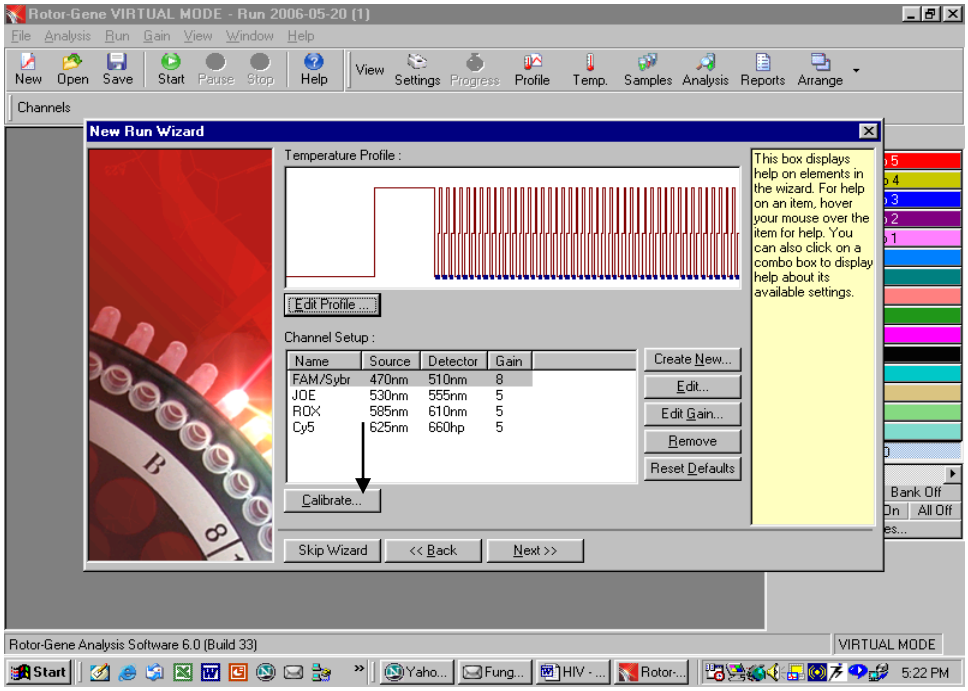


Fig. 16.

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. 17).

Adjustment of the fluorescence channel sensitivity as shown below.

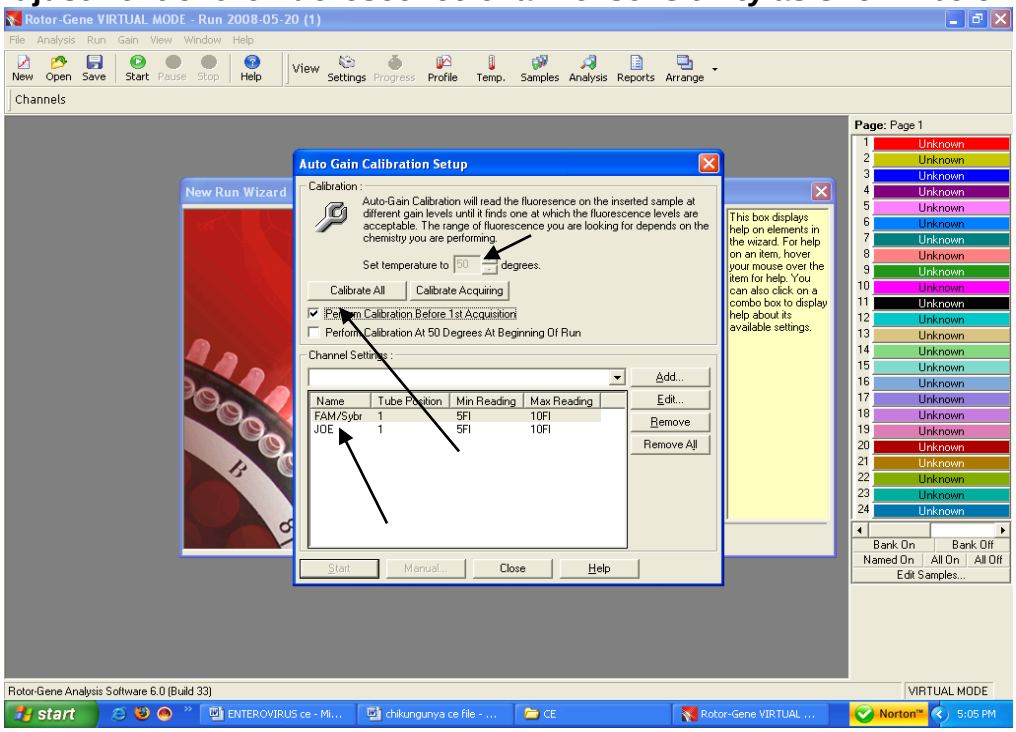


Fig. 17.

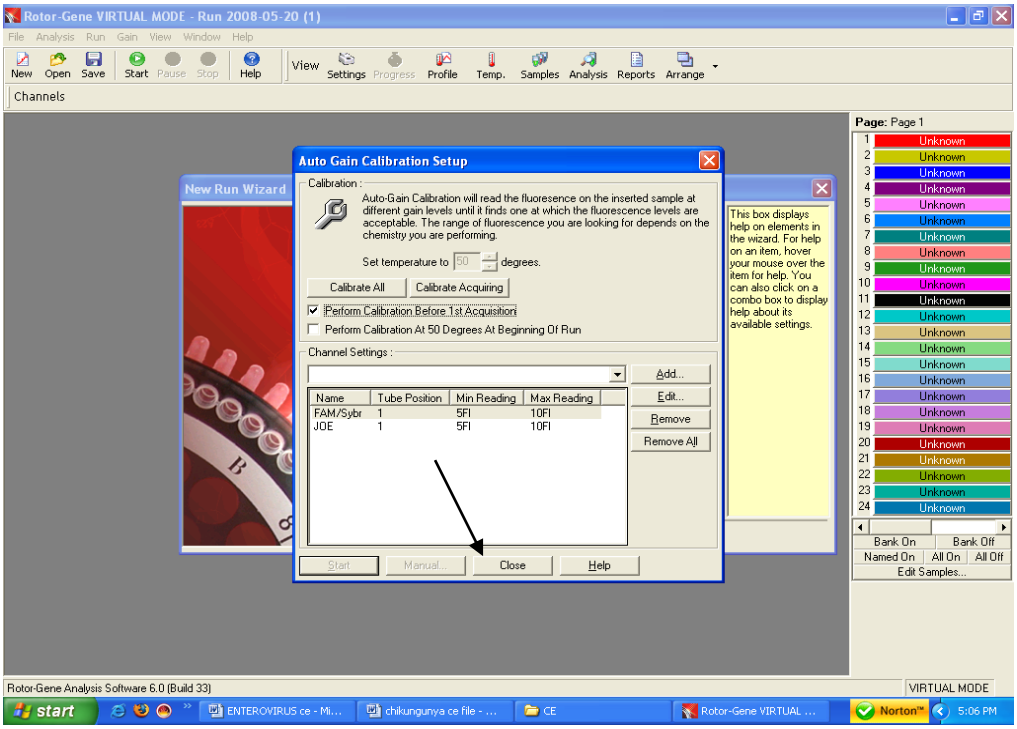


Fig. 18.

Please do not forget to click on the box against " Perform calibration at before 1st acquisition." 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

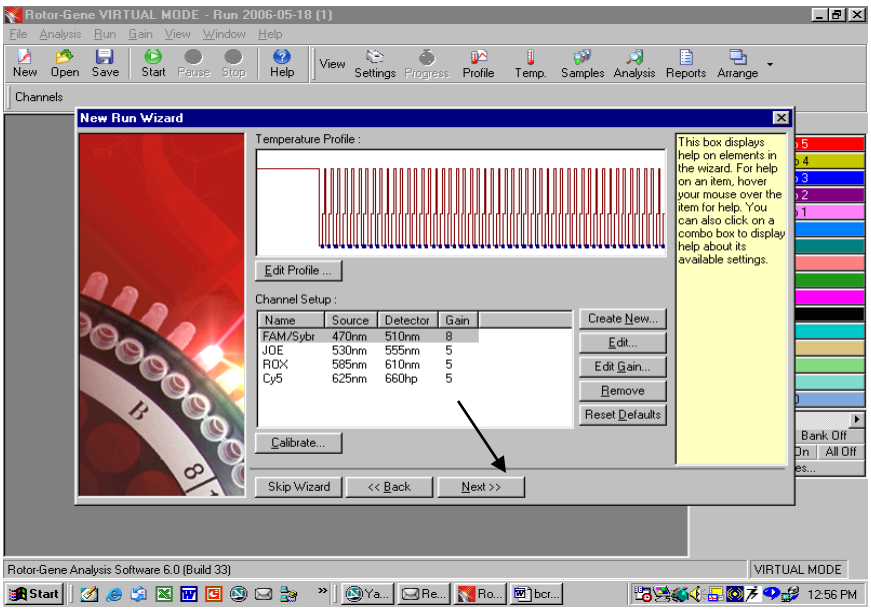


Fig. 19.

Starting of the *Rotor Gene*™ run.

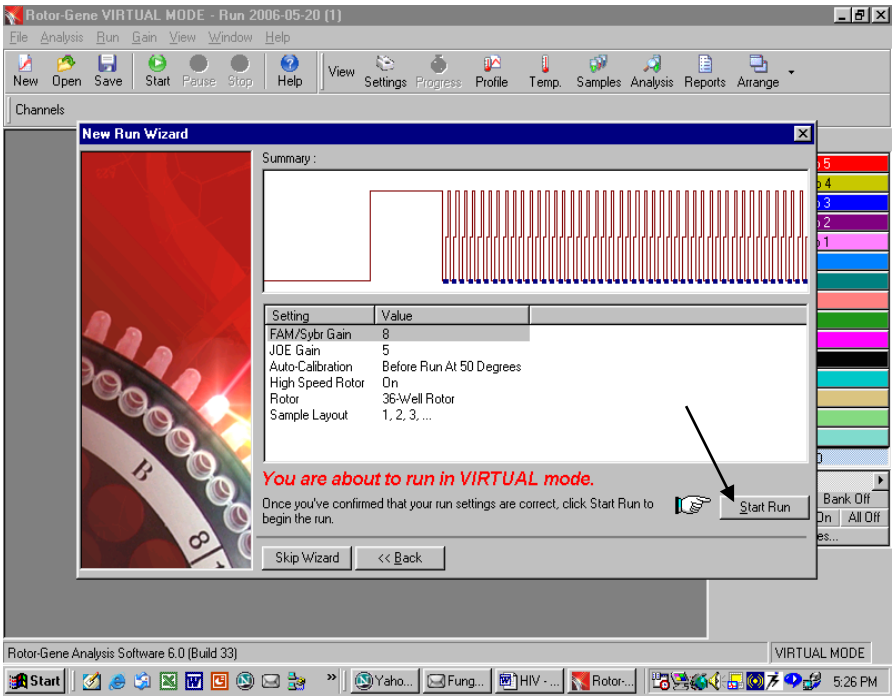


Fig. 20:
Press Start Run Button.

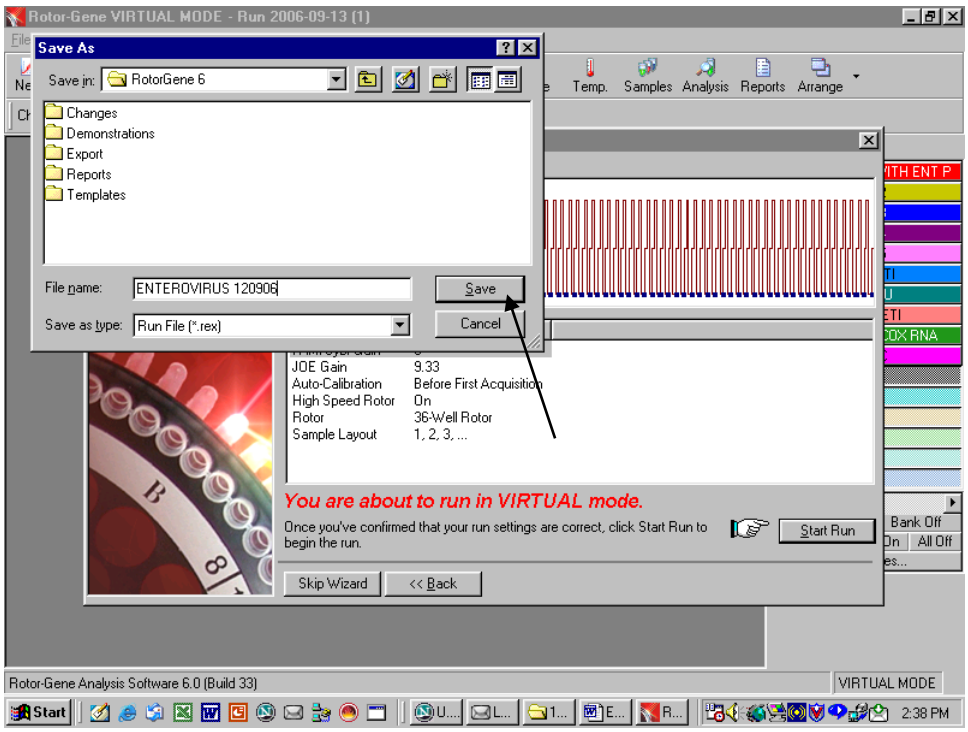


Fig. 21.

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.f. Programming the *RotorGene*™ 6000

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

G. Setting of general assay parameters & reaction volume
H. Thermal Profile & Calibration
I. Cycling profile/ cDNA Synthesis & Initial activation of the Hot Start enzyme
J. Cycling for Amplification of cDNA
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 22-39 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 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.

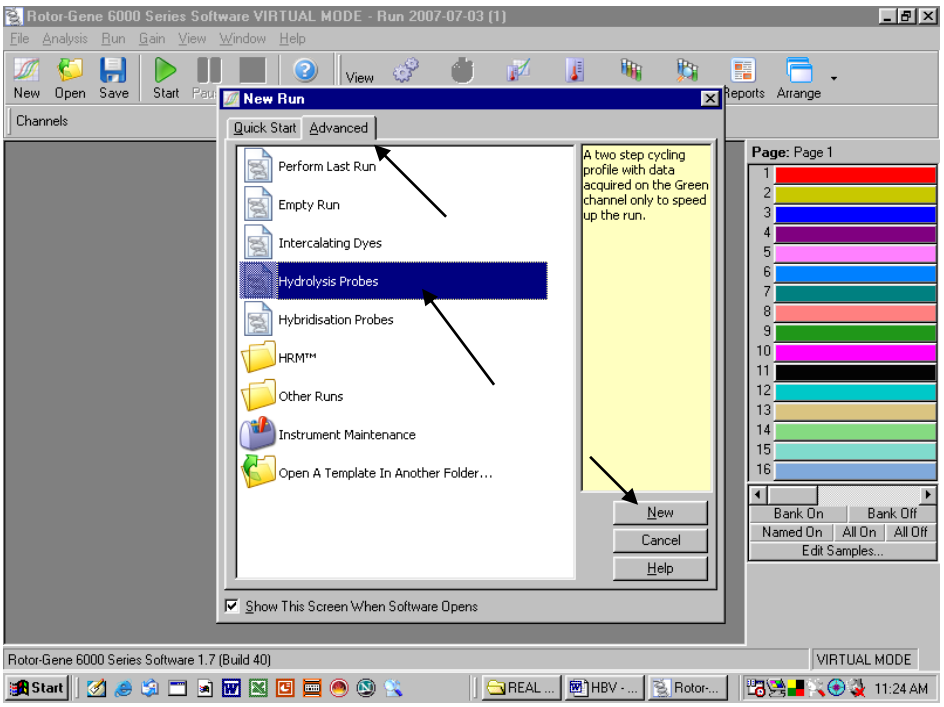


Fig. 22.

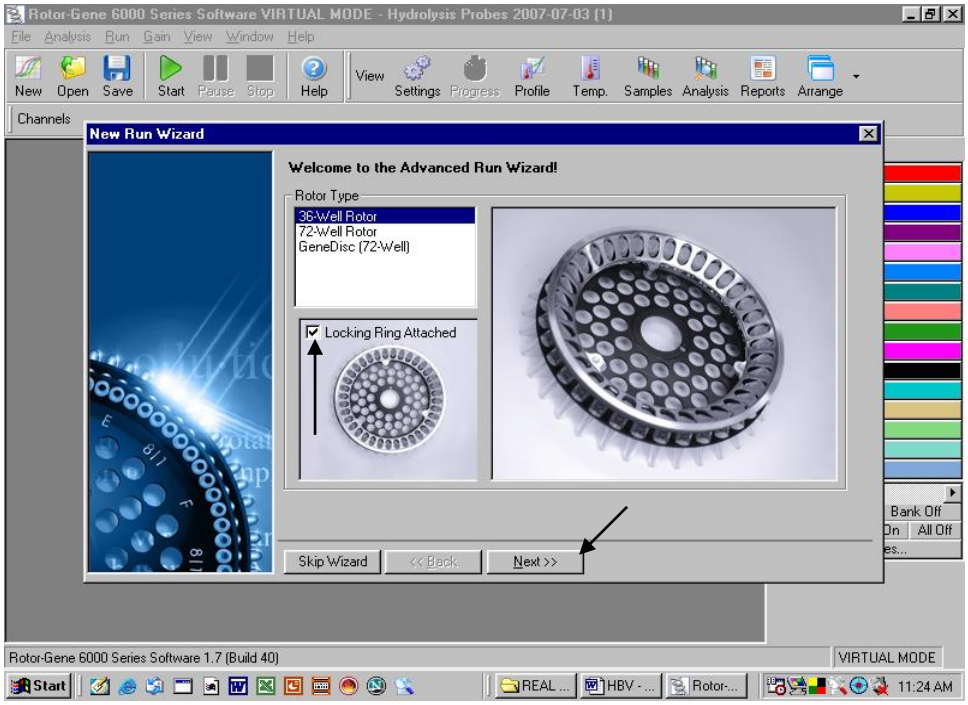


Fig. 23.

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.

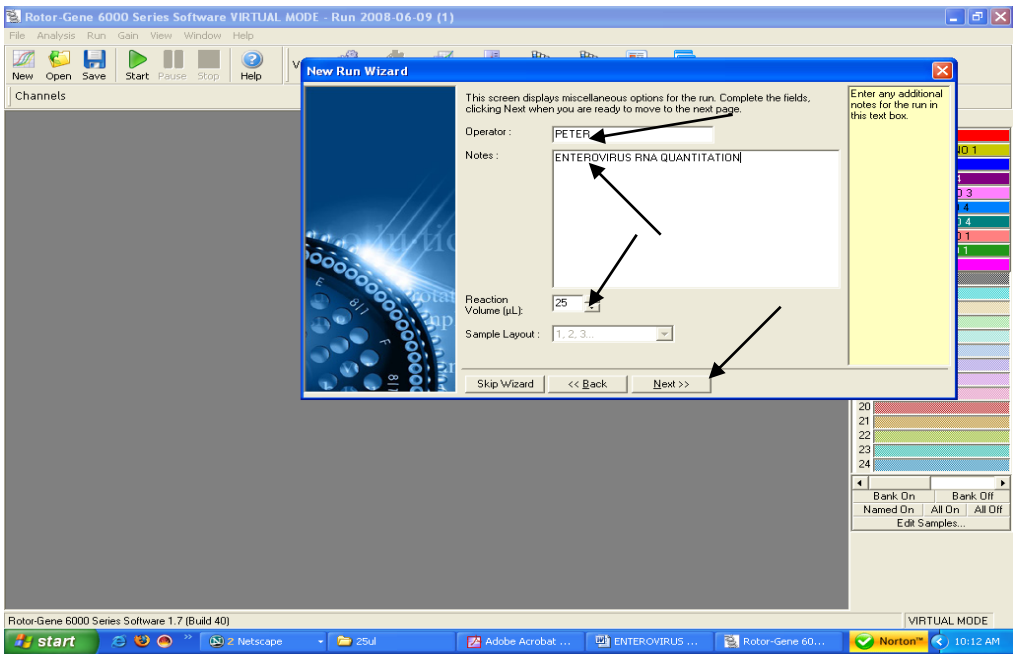


Fig. 24.

- 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.

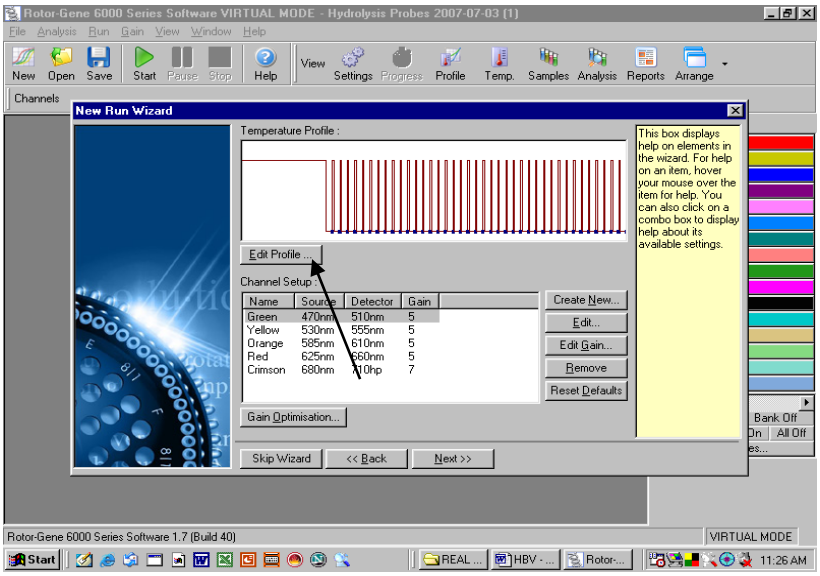


Fig. 25.

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

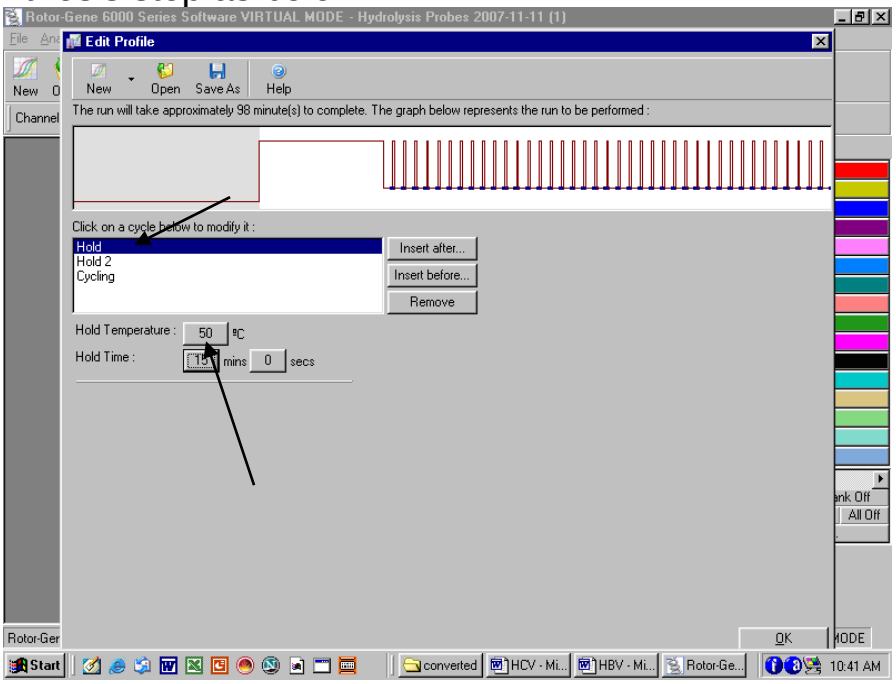


Fig. 26.

Second hold 95°C for 10 minutes as below

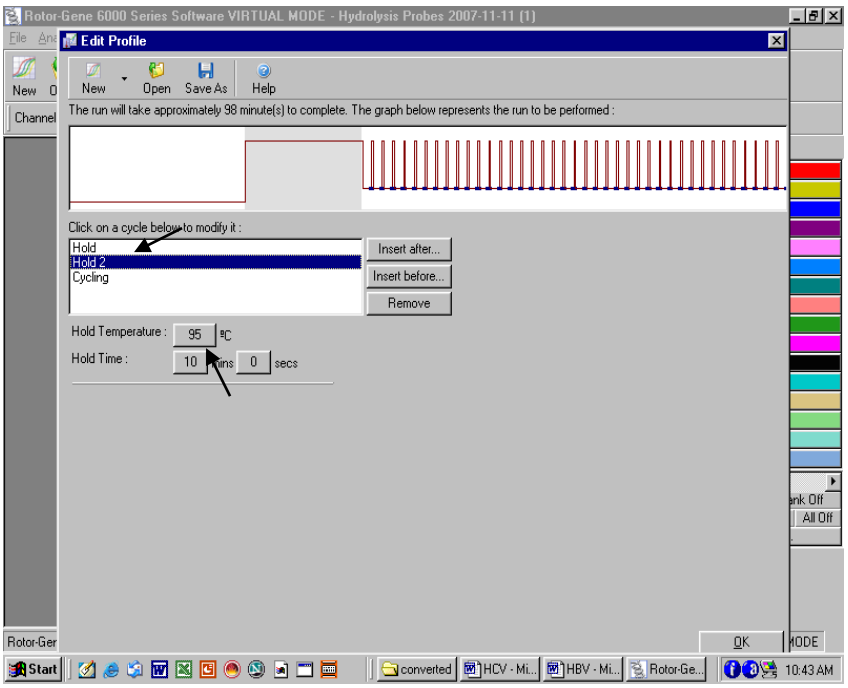


Fig. 27.

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.

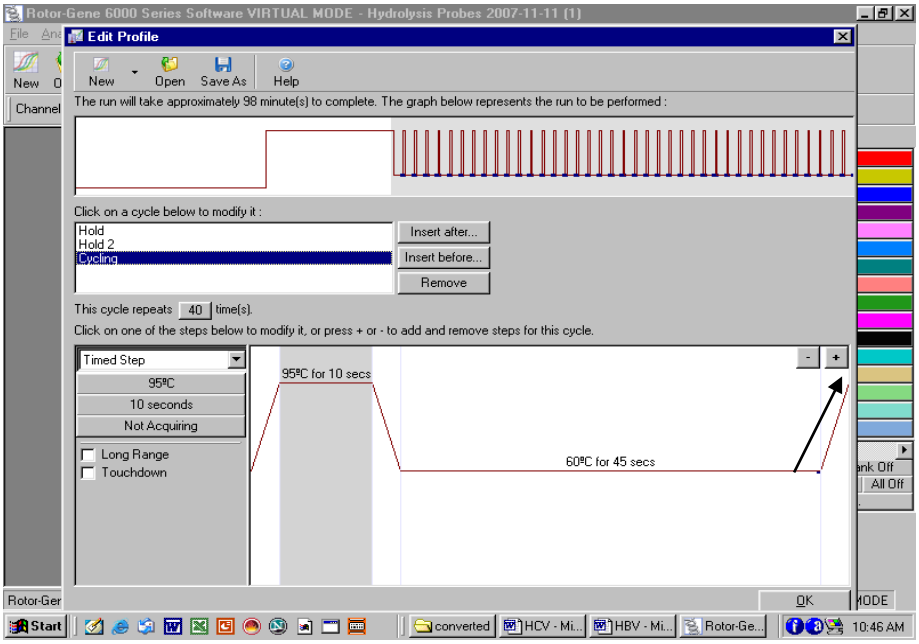


Fig. 28.

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.

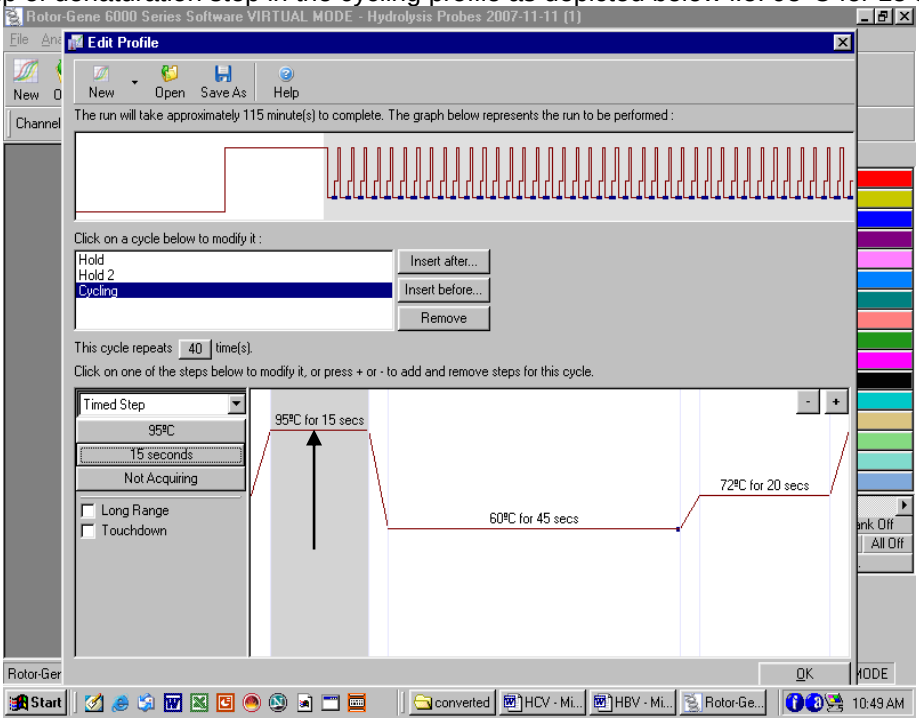


Fig. 29.

Setting up of Anneling step in the cycling profile as depicted below i.e. 50°C for 20 Seconds

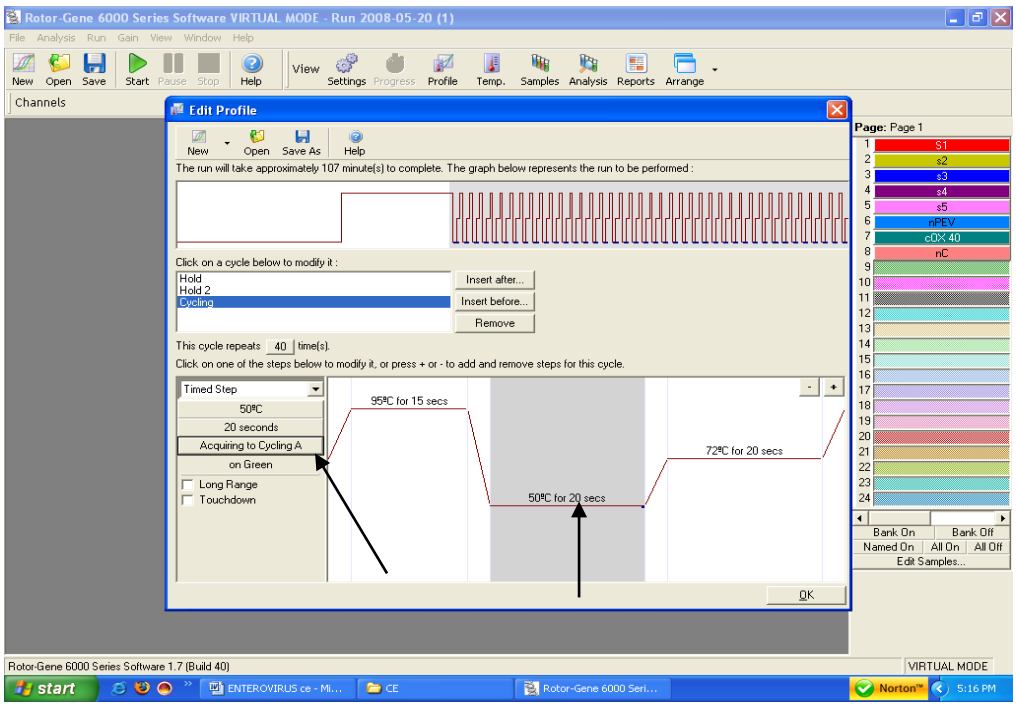


Fig. 30.

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)

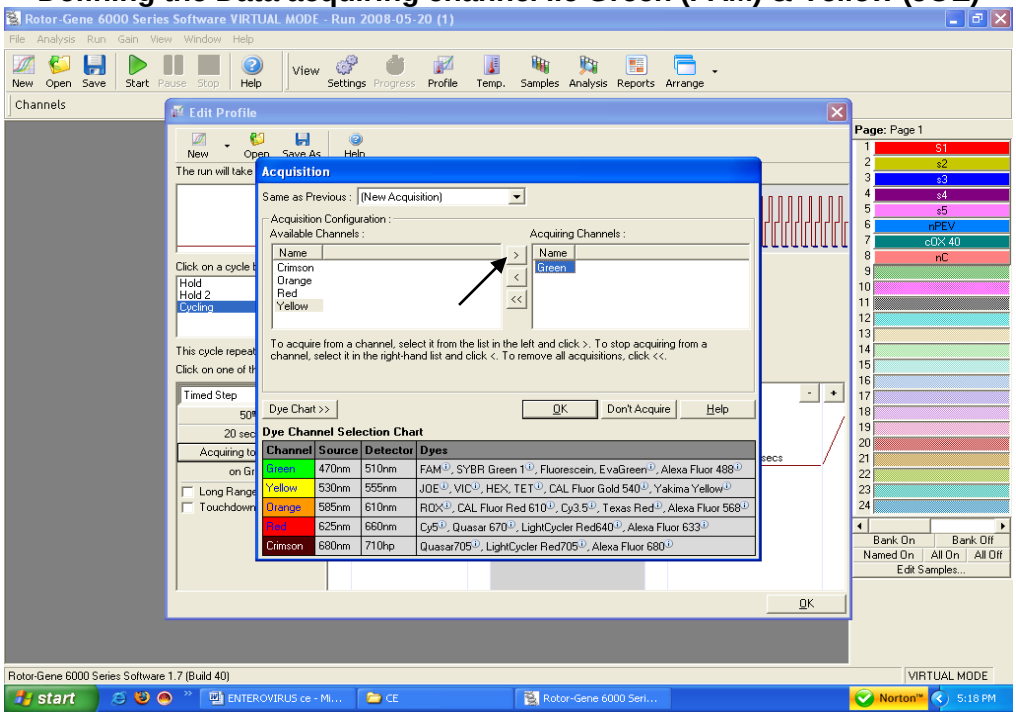


Fig. 31.

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.

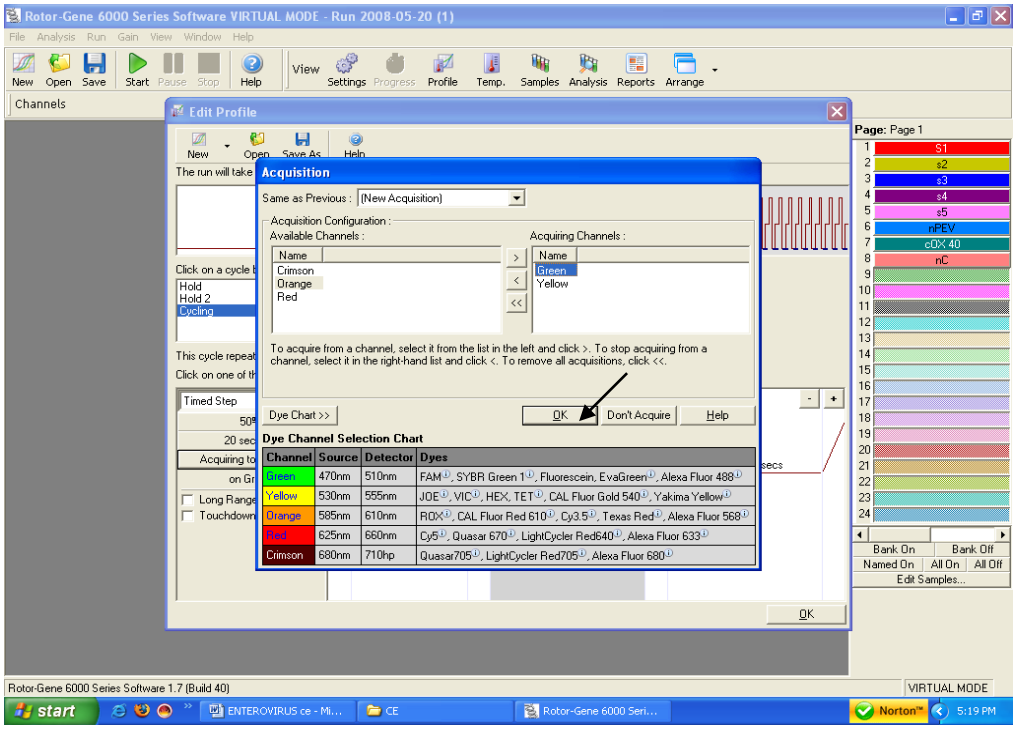


Fig. 32.

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

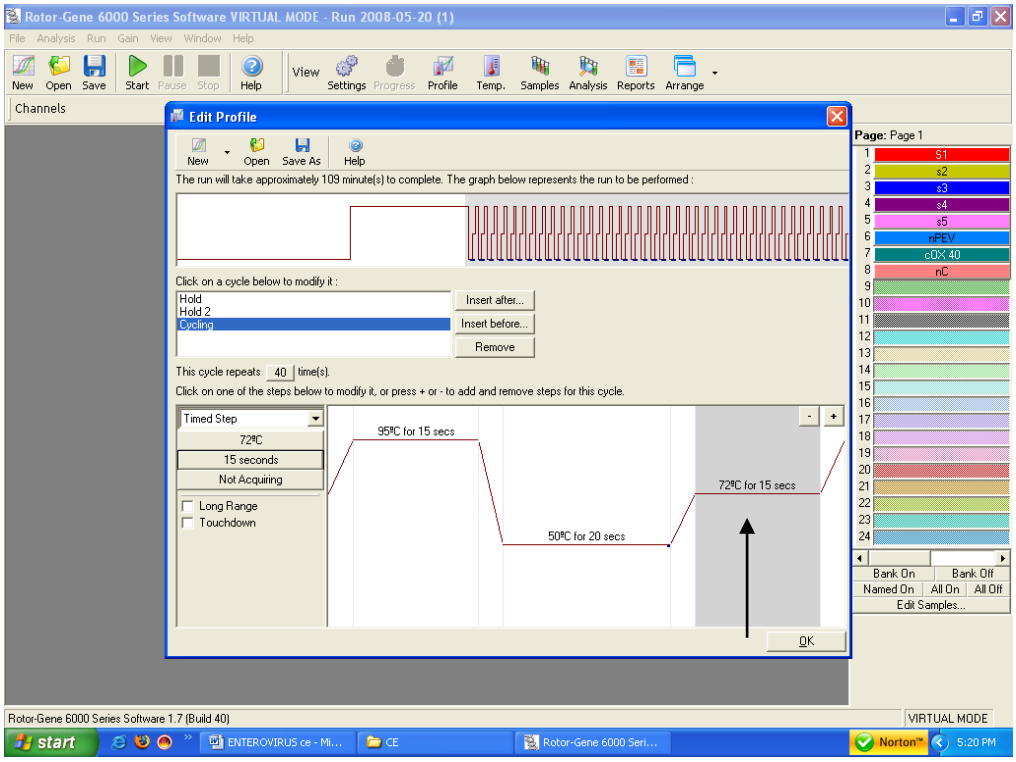


Fig. 33.

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

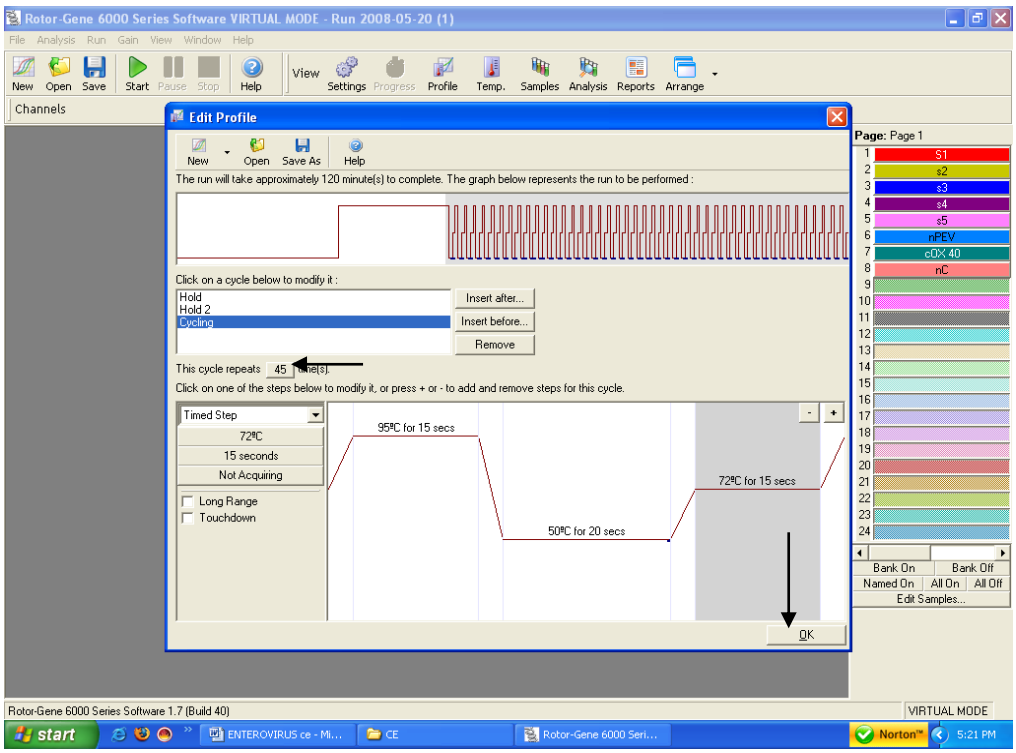


Fig. 34.

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.

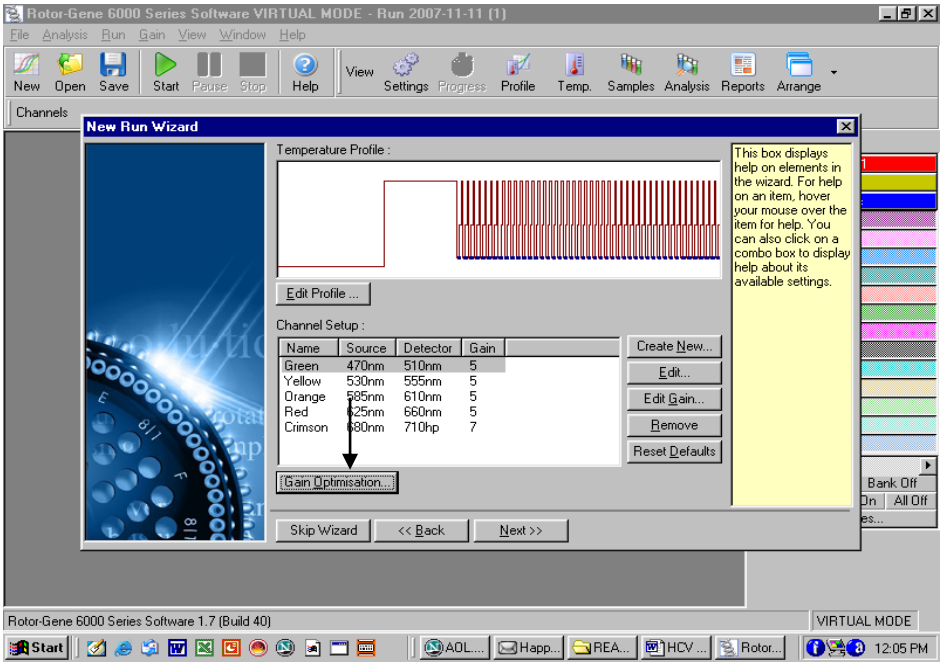


Fig. 35

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 .

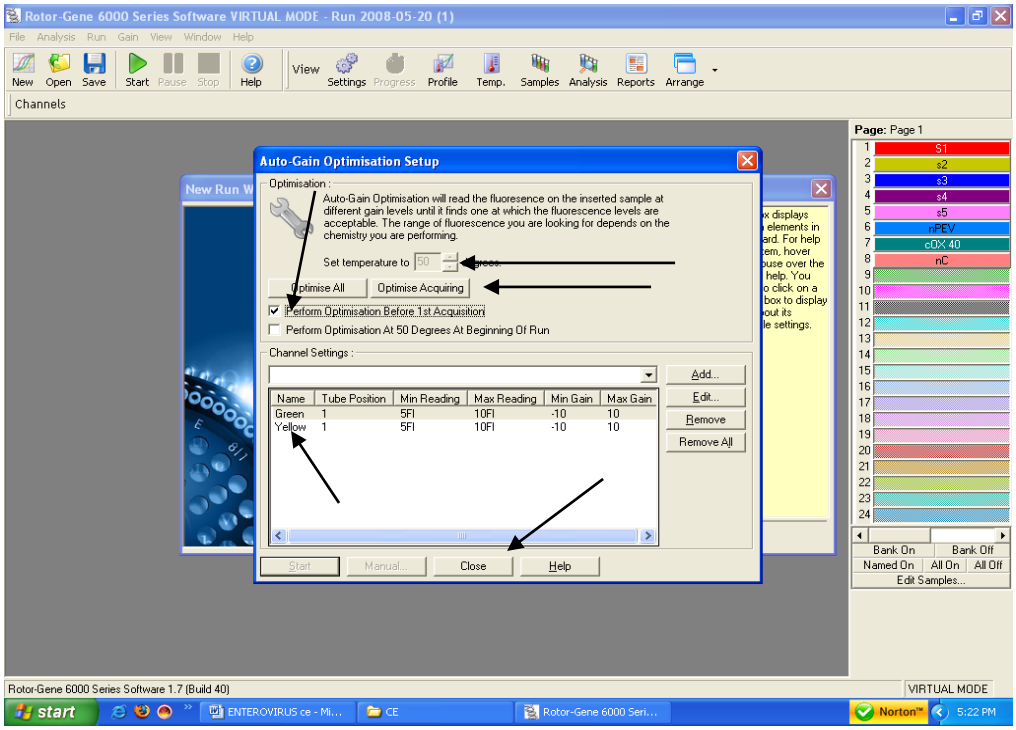


Fig. 36.

The following needs to be done.

- Click on the Optimise Acquiring
- Click on Set temperature To Adjust the Temperature to 50°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.

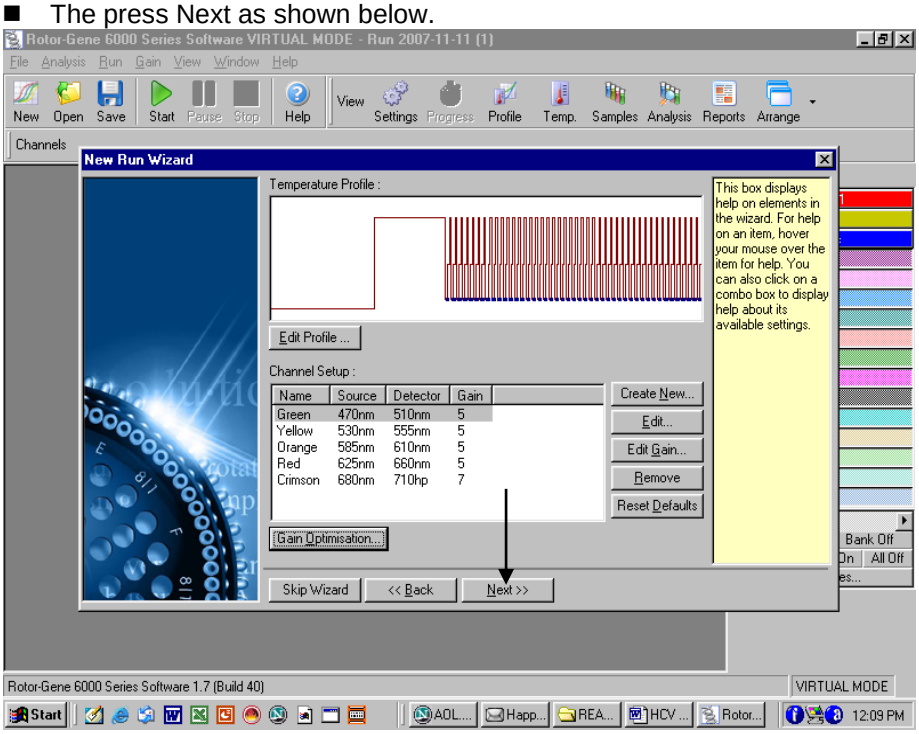


Fig. 37.

L) PRESS Start RUN

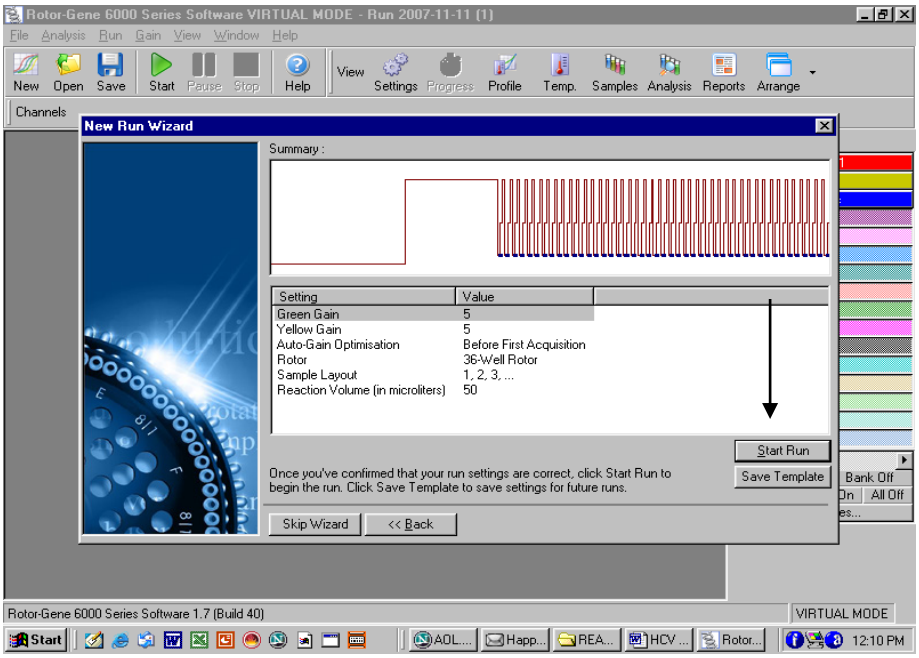


Fig. 38.

Saving the RUN File.

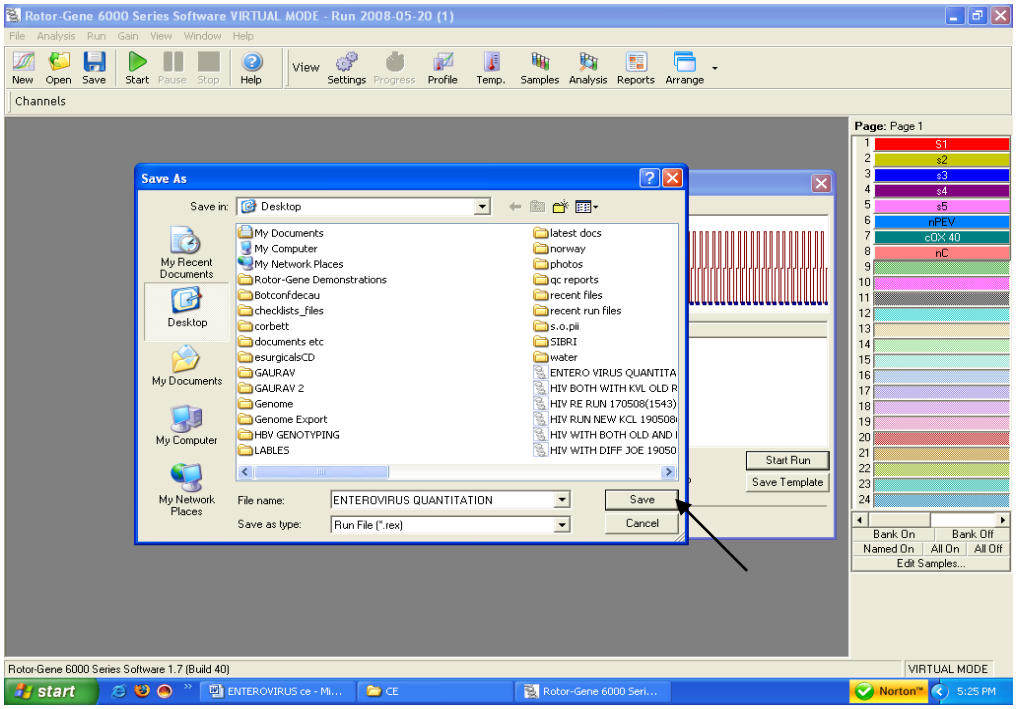


Fig. 39.

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 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 ENTEROVIRUS RNA.

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

No ENTEROVIRUS 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.

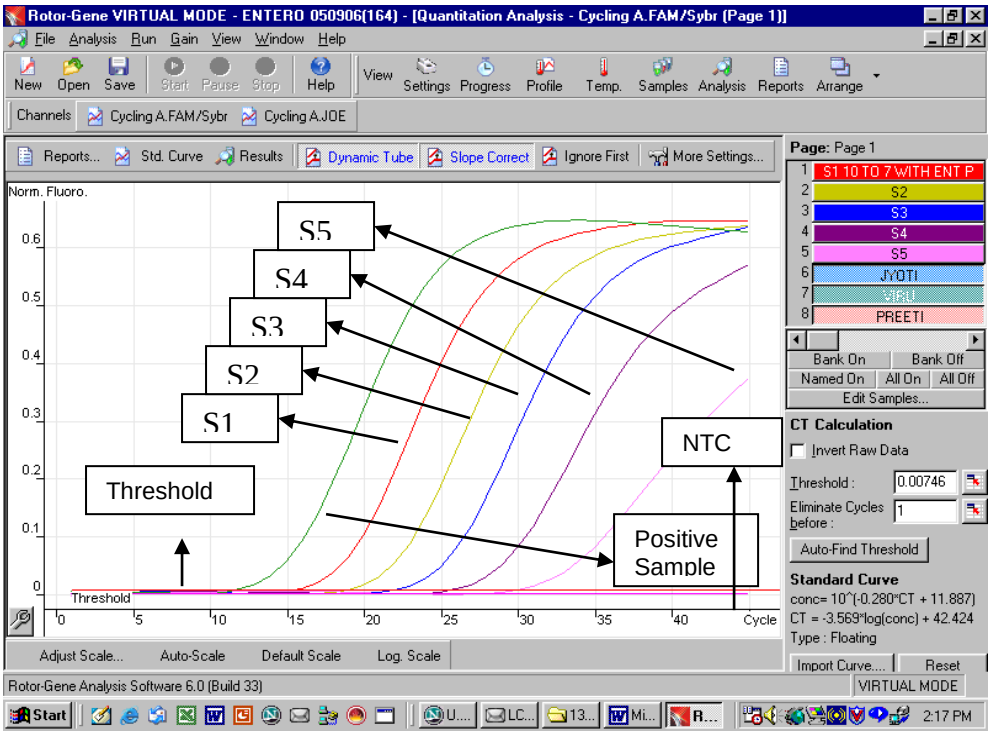
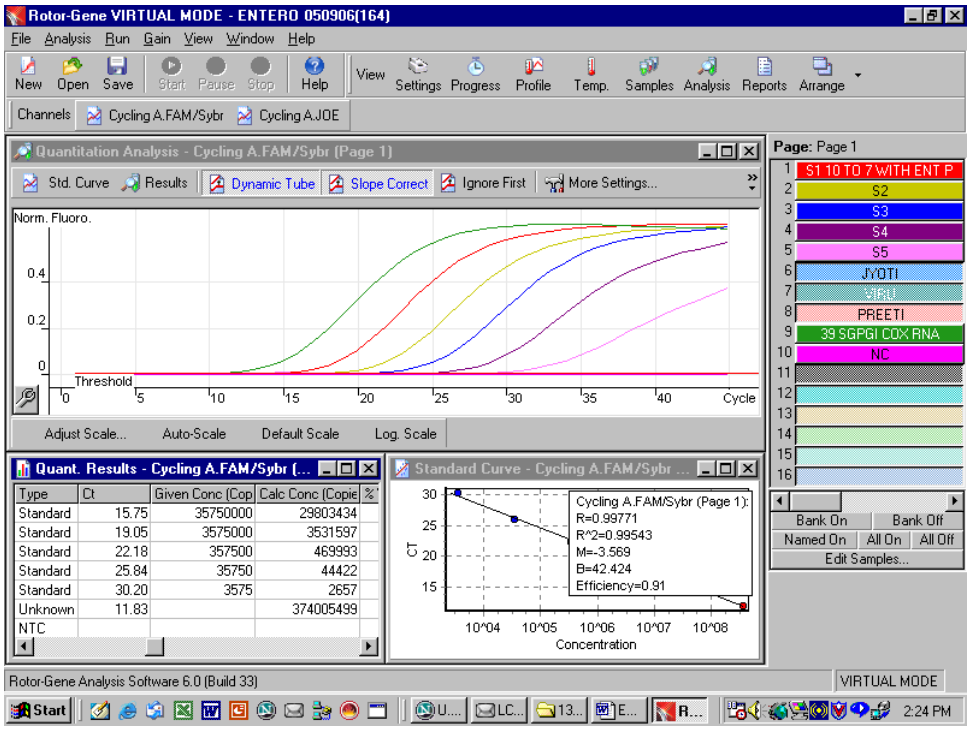


Fig. 40: Detection of the quantitation standards *(EnteroVirus EV S 1-5)* in fluorescence channel Cycling A.FAM. NTC: non-template control.

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



Type	Ct	Given Conc (Cop	Calc Conc (Copie	%
Standard	15.75	35750000	29803434	
Standard	19.05	3575000	3531597	
Standard	22.18	357500	469993	
Standard	25.84	35750	44422	
Standard	30.20	3575	2657	
Unknown	11.83		374005499	
NTC				

Fig. 41.

Example of analysed data for EnteroVirus 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 ENTEROVIRUS 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:

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

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

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

No ENTEROVIRUS 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.

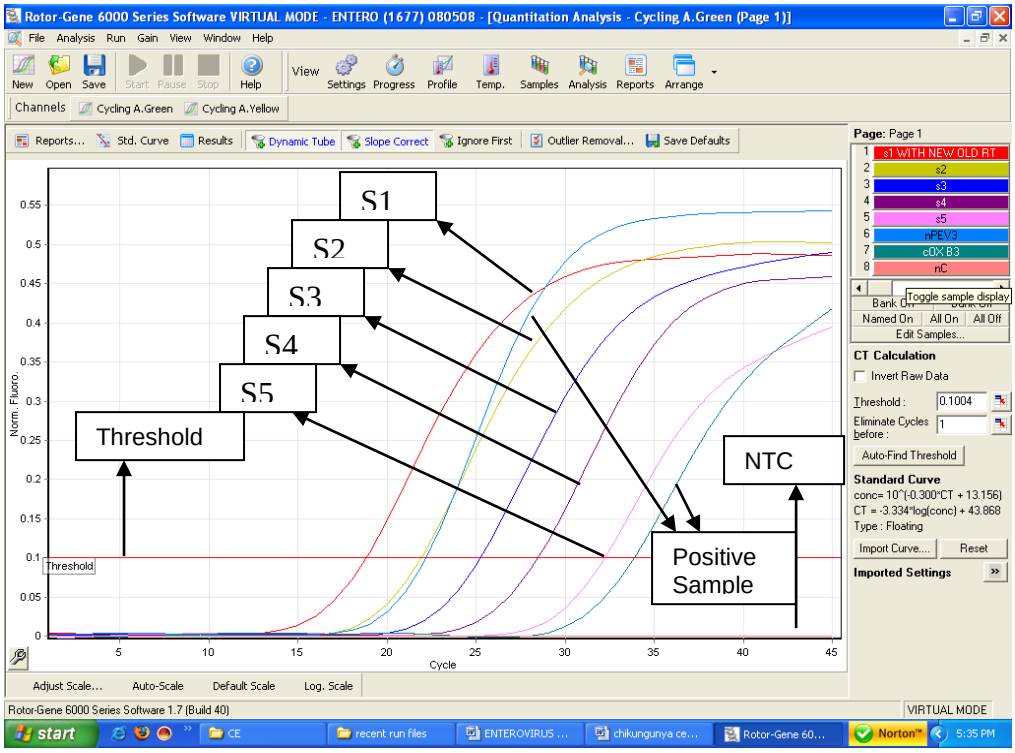


Fig. 42: Detection of the quantitation standards *(EnteroVirus EV S 1-5)* in fluorescence channel Cycling A.Green. NTC: non-template control.

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

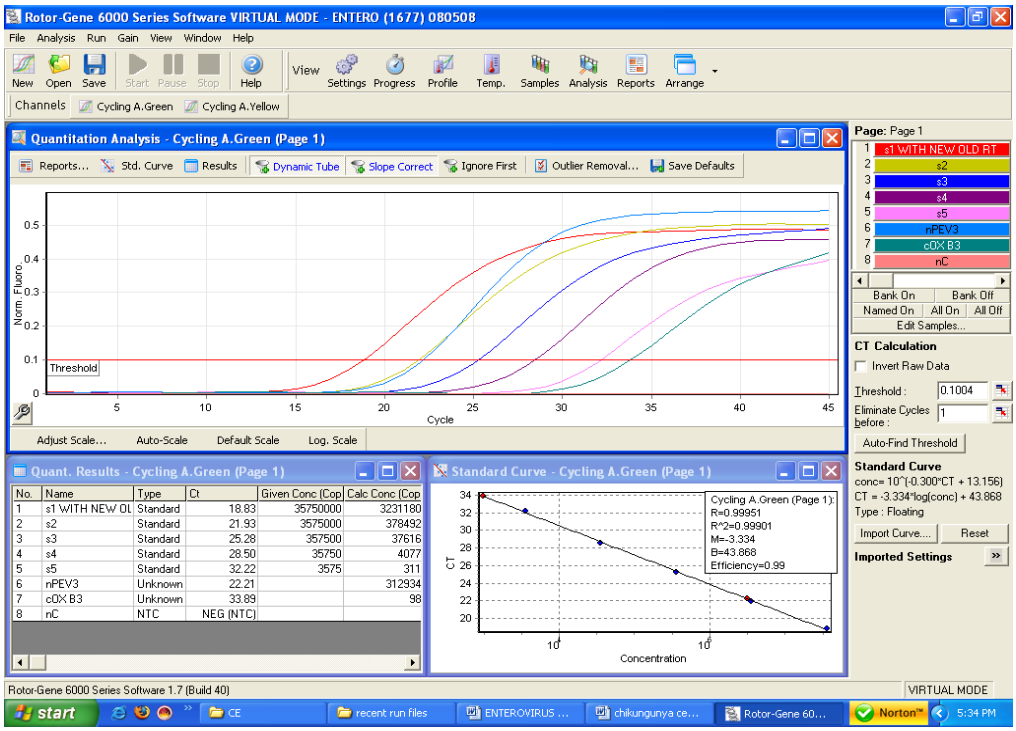


Fig. 43.

Example of analysed data for EnteroVirus 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 ENTEROVIRUS 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

a) For Rotor Gene 2000/3000

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

- Incorrect programming of the *Rotor-Gene™ 2000/3000.*

  ➔ 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 EnteroVirus Super Mix *R1* has been thawed and frozen too often.
- The EnteroVirus 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 EnteroVirus 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.

10. Troubleshooting

b) For Rotor Gene 6000

1. No signal with positive Standards (EnteroVirus EV *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 EnteroVirus Super Mix *R1* has been thawed and frozen too often.
- The EnteroVirus 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 EnteroVirus 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 Analytical Sensitivity and Reproducibility

In order to determine the sensitivity of the ***Geno Sen's®*** EnteroVirus *Real Time PCR RG Kit*, a dilution series has been set up from 10^7 copies/ul down to 10^{-1} Copies/µl of EnteroVirus In-Vitro Transcription and analyzed with the ***Geno Sen's®*** EnteroVirus Real Time PCR RG kit ***using*** *RotorGene 3000/6000 system. 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***® EnteroVirus *Real Time PCR RG Kit* is consistently 40 Copies/ml. This means that there is 95% probability that 40 copies/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***® *EnteroVirus Real Time PCR RG Kit on RotorGene 3000/6000 was determined by Spiking a known negative Serum to a nominal 90 copies/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***® EnteroVirus *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 89 copies/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***® EnteroVirus *Real Time PCR RG Kit on RotorGene 3000/6000 was determined to be 90 copies/ml.*

11.b Specificity

The specificity of the ***Geno Sen's***® EnteroVirus *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 strains of EnteroVirus.*

To further Validate the stringent data In order to check the specificity of the ***Geno Sen's***® EnteroVirus *Real Time PCR RG Kit*, different RNA & DNA listed below were analyzed with ***Geno Sen's***® EnteroVirus *Real Time PCR RG Kit*. None of these led to a positive signal with the ***Geno Sen's***® EnteroVirus *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	Dengue 1-4.	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***® EnteroVirus Real Time PCR RG kit on RotorGene 3000/6000 Machine. 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 Entero Virus RNA Gene.

Sample Type	Serum	Plasma	CSF	Cell Culture
High +ve's	3	0	3	8
Medium +ve's	9	2	8	0
Low +ve's	14	2	12	0
Extremely low +ve's	3	2	3	0
Negative samples.	4	2	6	0
	33	8	32	8

All the above samples were correctly identified by the ***Geno Sen's*®** EnteroVirus Real Time PCR RG kit & all the 12 extremely low samples were accurately detected by the ***Geno Sen's*®** EnteroVirus Real Time PCR RG kit & exhibited copies around 90 copies /ml or less than 90 copies/ml.

Further studies are underway on this aspect of specificity and sensitivity.

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 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 genome24@rediffmail.com

14. Publication and Reference:

I) An epidemic of encephalitis associated with human enterovirus B in Uttar Pradesh, India, 2008.

Kumar A[1], Shukla D, Kumar R, Idris MZ, Misra UK, Dhole TN.

ii) High frequency of enterovirus serotype circulation in a densely populated area of India

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III) Molecular and biological characterization of circulating human enterovirus

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For the Degree of DOCTOR OF PHILOSOPHY
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DEPARTMENT OF BOTANY
UNIVERSITY OF LUCKNOW LUCKNOW-226007 INDIA 2013

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.**

15.

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