Geno-Sen's

Influenza Group typing (Rotor Gene)

Real Time PCR Kit

Quantitative

for use with the
Rotor Gene™ 2000/3000/6000
(Corbett Research Australia)

IVD

PACK INSERT
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(An ISO 13485:2003, 9001:2000 Certified Company)

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Influenza Virus typing Geno-Sen's Real Time PCR Kit for use with the *Rotor Gene™ 2000/3000/6000*[*] (Corbett Research).

1. Contents of the Kit:

Color Code	Contents	REF 9111111 100 rxns	REF 9111112 50 rxns	REF 9111113 25 rxns
R1 Blue	H1 Super mix.	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R2 Blue	N1 Super mix.	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R3 Blue	Influenza A Super mix.	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R4 Blue	Influenza B Super mix.	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R5 Blue	H3 Super mix.	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R6 Blue	N2 Super mix.	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R8 Yellow	Influenza Mg Sol	1 Vial	1 Vial	1 Vial
H1-PC Red	*H1* Positive Control	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
N1-PC Red	*N1* Positive Control	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
H3-PC Red	H3 Positive Control	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
N2-PC Red	*N2* Positive Control	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
INFI-A - PC Red	*Influenza A* Positive Control	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
INFI-B-PC Red	Influenza B Positive Control	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 (R7) Green	Influenza IC RG	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

[*] The *Rotor Gene*™ 2000/3000/6000 is a registered trademark of Corbett Research, Australia.

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.) Influenza Virus Group Information

Application

Influenza A & Influenza B Information

Application

Types of Influenza Viruses

There are three types of influenza viruses: A, B and C. Influenza A and B viruses cause seasonal epidemics of disease almost every winter in the United States. Influenza type C infections cause a mild respiratory illness and are not thought to cause epidemics.

Influenza A viruses are divided into subtypes based on two proteins on the surface of the virus: the hemagglutinin (H) and the neuraminidase (N). There are 16 different hemagglutinin subtypes and 9 different neuraminidase subtypes, Influenza A viruses can be further broken down into different strains. The current subtypes of influenza A viruses found in people are A (H1N1) and A (H3N2).

Influenza B viruses are not divided into subtypes. Influenza B viruses also can be further broken down into different strains.

Influenza viruses can change in two different ways.

One is called "antigenic drift." These are small changes in the virus that happen continually over time. Antigenic drift produces new virus strains that may not be recognized by the body's immune system. This process works as follows: a person infected with a particular flu virus strain develops antibody against that virus. As newer virus strains appear, the antibodies against the older strains no longer recognize the "newer" virus, and reinfection can occur. This is one of the main reasons why people can get the flu more than one time. In most years, one or two of the three virus strains in the influenza vaccine are updated to keep up with the changes in the circulating flu viruses. So, people who want to be protected from flu need to get a flu shot every year.

The other type of change is called "antigenic shift." Antigenic shift is an abrupt, major change in the influenza A viruses, resulting in new hemagglutinin and/or new hemagglutinin and neuraminidase proteins in influenza viruses that infect humans. Shift results in a new influenza A subtype. When shift happens, most people have little or no protection against the new virus. While influenza viruses are changing by antigenic drift all the time, antigenic shift happens only occasionally. Type A viruses undergo both kinds of changes; influenza type B viruses change only by the more gradual process of antigenic drift.

Influenza (the flu) is a contagious respiratory illness caused by influenza viruses. It can cause mild to severe illness, and at times can lead to death. The best way to prevent the flu is by getting a flu **vaccination** each year. Every year in the United States, on average 5% to 20% of the population gets the flu; more than 200,000 people are hospitalized from flu complications, and; about 36,000 people die from flu-related causes. Some people, such as older people, young children, and people with certain health conditions, are at high risk for serious flu complications.

Influenza Symptoms

Influenza (also known as the flu) is a contagious respiratory illness caused by flu viruses. It can cause mild to severe illness, and at times can lead to death. The flu is different from a cold. The flu usually comes on suddenly and may include these symptoms:

- Fever (usually high)
- Headache
- Extreme tiredness
- Dry cough
- Sore throat
- Runny or stuffy nose
- Muscle aches
- Stomach symptoms, such as nausea, vomiting, and diarrhea, also can occur but are more common in children than adults

These symptoms are usually referred to as "flu-like symptoms."

Anyone Can Get the Flu, But the Disease Is More Severe for Some People

Most people who get influenza will recover in a few days to less than 2 weeks, but some people will develop life-threatening complications (such as pneumonia) as a result of the flu. Millions of people in the United States — about 5% to 20% of U.S. residents — will get influenza each year. An average of about 36,000 people per year in the United States die from influenza-related causes, and more than 200,000 have to be admitted to the hospital as a result of influenza-related causes. Anyone can get the flu (even healthy people), and serious problems from influenza can happen at any age. People age 65 years and older, people of any age with chronic medical conditions (such as asthma, diabetes, or heart disease), pregnant women, and young children are more likely to get complications from influenza. Pneumonia, bronchitis, and sinus and ear infections are three examples of complications from flu. The flu can make chronic health problems worse. For example, people with asthma may experience asthma attacks while they have the flu, and people with chronic congestive heart failure may have worsening of this condition that is triggered by the flu.

How To Know if You Have the Flu

Your respiratory illness might be the flu if you have sudden onset of body aches, high fever, and respiratory symptoms, and your illness occurs during the usual flu season in the Northern Hemisphere. However, during this time, other respiratory illnesses can cause similar symptoms to the flu. In addition, influenza can also occur outside of the typical flu season. It is impossible to tell for sure if you have the flu based on symptoms alone. Doctors can perform tests to see if you have the flu if you are in the first few days of your illness.

Flu is a serious disease and it's important to take action to protect yourself. If you do get sick, however, there are steps you can take to treat the flu.

Influenza A (H1N1), A (H3N2), and influenza B strains are included in each year's influenza vaccine. Getting a flu vaccine can protect against influenza A and B viruses. The flu vaccine does not protect against influenza C viruses.

Influenza A virus subtype H1N1, also known as **A (H1N1)**, is a subtype of influenza virus A and the most common cause of influenza (flu) in humans. Some strains of H1N1 are endemic in humans, including the strain(s) responsible for the 1918 flu pandemic which killed 50–100 million people worldwide. Less virulent H1N1 strains still exist in the wild today, worldwide, causing a small fraction of all influenza-like illness and a large fraction of all seasonal influenza. H1N1 strains caused roughly half of all flu infections in 2006. Other strains of H1N1 are endemic in pigs and in birds.

In March and April 2009, hundreds of laboratory-confirmed infections and a number of deaths were caused by an outbreak of a new strain of H1N1.

Spanish flu

1918 flu pandemic

The Spanish flu, also known as *La Gripe Española*, or *La Pesadilla*, was an unusually severe and deadly strain of avian influenza, a viral infectious disease, that killed some 50 million to 100 million people worldwide over about a year in 1918 and 1919. It is thought to be one of the most deadly pandemics in human history. It was caused by the H1N1 type of influenza virus.

The Spanish flu caused an unusual number of deaths because it may have caused a cytokine storm in the body. (The recent epidemic of bird flu, also an Influenza A virus, had a similar effect.) The Spanish flu virus infected lung cells, leading to over stimulation of the immune system via release of cytokines into the lung tissue. This leads to extensive leukocyte migration towards the lungs, causing destruction of lung tissue and secretion of liquid into the organ. This makes it difficult for the patient to breathe. In contrast to other pandemics, which mostly kill the old and the very young, the 1918 pandemic killed unusual numbers of young adults, which may have been due to their healthy immune systems being able to mount a very strong and damaging response to the infection.

The term "Spanish" flu was coined because Spain was at the time the only European country where the press were printing reports of the outbreak, which had killed thousands in the armies fighting the First World War. Other countries suppressed the news in order to protect morale.

Russian flu

See Influenza A virus subtype H2N2#Russian flu for the 1889–1890 Russian flu

The more recent Russian flu was a 1977–1978 flu epidemic caused by strain *Influenza A/USSR/90/77 (H1N1)*. It infected mostly children and young adults under 23 because a similar strain was prevalent in 1947–57, causing most adults to have substantial immunity. Some have called it a flu pandemic but because it only affected the young it is not considered a true pandemic. The virus was included in the 1978–1979 influenza vaccine.

2009 swine flu outbreak

The swine influenza virus isolated from patients in the United States was found to be made up of genetic elements from four different flu viruses – North American Mexican influenza, North American avian influenza, human influenza, and swine influenza virus typically found in Asia and Europe – "an unusually mongrelised mix of genetic sequences." This new strain appears to be a result of reassortment of human influenza and swine influenza viruses, in all four different strains of subtype H1N1. However, as the virus has not yet been isolated in animals to date and also for historical naming reasons, the World Organisation for Animal Health (OIE) suggests it be called "North-American influenza". On April 30, 2009 the World Health Organization began referring to the outbreak as "Influenza A (H1N1)" instead of "swine flu", presumably to countermand the consequences of the misleading less scientifically labeled name as referenced by WHO spokesperson Dick Thompson.

Several complete genome sequences for U.S. flu cases were rapidly made available through the Global Initiative on Sharing Avian Influenza Data (GISAID). Preliminary genetic characterization found that the hemagglutinin (HA) gene was similar to that of swine flu viruses present in U.S. pigs since 1999, but the neuraminidase (NA) and matrix protein (M) genes resembled versions present in European swine flu isolates. The six genes from American swine flu are themselves mixtures of swine flu, bird flu, and human flu viruses. While viruses with this genetic makeup had not previously been found to be circulating in humans or pigs, there is no formal national surveillance system to determine what viruses are circulating in pigs in the U.S.

Influenza A virus subtype H3N2 (also **H3N2**) is a subtype of viruses that cause influenza (flu). H3N2 viruses can infect birds and mammals. In birds, humans, and pigs, the virus has mutated into many strains. H3N2 is increasingly abundant in seasonal influenza, which kills an estimated 36,000 people in the United States each year.

H3N2 is a subtype of the viral genus Influenzavirus A, which is an important cause of human influenza. Its name derives from the forms of the two kinds of proteins on the surface of its coat, hemagglutinin (H) and neuraminidase (N). By reassortment, H3N2 exchanges genes for internal proteins with other influenza subtypes.

Seasonal H3N2 flu

Seasonal influenza kills an estimated 36,000 people in the United States each year. Flu vaccines are based on predicting which mutants of H1N1, H3N2, H1N2, and influenza B will proliferate in the next season. Separate vaccines are developed for the northern and southern hemispheres in preparation for their annual epidemics. In the tropics, influenza shows no clear seasonality. In the past ten years, H3N2 has tended to dominate in prevalence over H1N1, H1N2, and influenza B. Measured resistance to the standard antiviral drugs amantadine and rimantadine in H3N2 has increased from 1% in 1994 to 12% in 2003 to 91% in 2005.

Seasonal H3N2 flu is a human flu from H3N2 that is slightly different from one of last year's flu season H3N2 variants. Seasonal influenza viruses flow out of overlapping epidemics in East and Southeast Asia, then trickle around the globe before dying off. Identifying the source of the viruses allows global health officials to better predict which viruses are most likely to cause the most disease over the next year. An analysis of 13,000 samples of influenza A/H3N2 virus that were collected across six continents from 2002 to 2007 by the WHO's Global Influenza Surveillance Network showed that newly emerging strains of H3N2 appeared in East and Southeast Asian countries about 6 to 9 months earlier than anywhere else. The strains generally reached Australia and New Zealand next, followed by North America and Europe. The new variants typically reached South America after an additional 6 to 9 months, the group reported.[2]

Swine flu

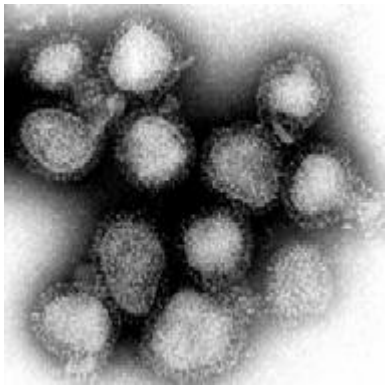
Pigs can harbor influenza viruses adapted to humans and others that are adapted to birds, allowing the viruses to exchange genes and create a pandemic strain.

"In swine, 3 influenza A virus subtypes (H1N1, H3N2, and H1N2) are circulating throughout the world. In the United States, the classic H1N1 subtype was exclusively prevalent among swine populations before 1998; however, since late August 1998, H3N2 subtypes have been isolated from pigs. Most H3N2 virus isolates are triple reassortants, containing genes from human (HA, NA, and PB1), swine (NS, NP, and M), and avian (PB2 and PA) lineages. [...] Present vaccination strategies for SIV control and prevention in swine farms typically include the use of 1 of several bivalent SIV vaccines commercially available in the United States. Of the 97 recent H3N2 isolates examined, only 41 isolates had strong serologic cross-reactions with antiserum to 3 commercial SIV vaccines. Since the protective ability of influenza

vaccines depends primarily on the closeness of the match between the vaccine virus and the epidemic virus, the presence of nonreactive H3N2 SIV variants suggests that current commercial vaccines might not effectively protect pigs from infection with a majority of H3N2 viruses."

Avian influenza virus H3N2 is endemic in pigs in China and has been detected in pigs in Vietnam, increasing fears of the emergence of new variant strains. Health experts say pigs can carry human influenza viruses, which can combine (i.e. exchange homologous genome sub-units by genetic reassortment) with H5N1, passing genes and mutating into a form which can pass easily among humans. H3N2 evolved from H2N2 by antigenic shift and caused the Hong Kong Flu pandemic of 1968 and 1969 that killed up to 750,000 humans. The dominant strain of annual flu in humans in January 2006 is H3N2. Measured resistance to the standard antiviral drugs amantadine and rimantadine in H3N2 in humans has increased to 91% in 2005. In August 2004, researchers in China found H5N1 in pigs.

Hong Kong Flu (1968–1969)



The influenza viruses that caused Hong Kong flu. (magnified approximately 100,000 times)

The **Hong Kong Flu** was a category 2 flu pandemic caused by a strain of H3N2 descended from H2N2 by antigenic shift, in which genes from multiple subtypes reassorted to form a new virus. This pandemic of 1968 and 1969 killed an estimated one million people worldwide. The pandemic infected an estimated 500,000 Hong Kong residents, 15% of the population, with a low death rate. In the United States, approximately 33,800 people died.

Both the H2N2 and H3N2 pandemic flu strains contained genes from avian influenza viruses. The new subtypes arose in pigs coinfected with avian and human viruses and were soon transferred to humans. Swine were considered the original "intermediate host" for influenza, because they supported reassortment of divergent subtypes. However, other hosts appear capable of similar coinfection (e.g., many poultry species), and direct transmission of avian viruses to humans is possible. H1N1 may have been transmitted directly from birds to humans (Belshe 2005).

The Hong Kong flu strain shared internal genes and the neuraminidase with the 1957 Asian Flu (H2N2). Accumulated antibodies to the neuraminidase or internal proteins may have resulted in much fewer casualties than most pandemics.

However, cross-immunity within and between subtypes of influenza is poorly understood.

The Hong Kong flu was the first known outbreak of the H3N2 strain, though there is serologic evidence of H3N? infections in the late 19th century. The first record of the outbreak in Hong Kong appeared on 13 July 1968 in an area with a density of about 500 people per acre in an urban setting. The outbreak reached maximum intensity in 2 weeks, lasting 6 weeks in total. The virus was isolated in Queen Mary Hospital. Flu symptoms lasted 4 to 5 days.

By July 1968, extensive outbreaks were reported in Vietnam and Singapore. By September 1968, it would reach India, Philippines, northern Australia and Europe. That same month, the virus entered California from returning Vietnam War troops. It would reach Japan, Africa and South America by 1969.

"Three strains of Hong Kong influenza virus isolated from humans were compared with a strain isolated from a calf for their ability to cause disease in calves. One of the human strains. A/Aichi/2/68, was detected for five days in a calf, but all three failed to cause signs of disease. Strain A/cal/Duschanbe/55/71 could be detected for seven days and caused an influenza-like illness in calves."

Fujian flu (2003–2004)

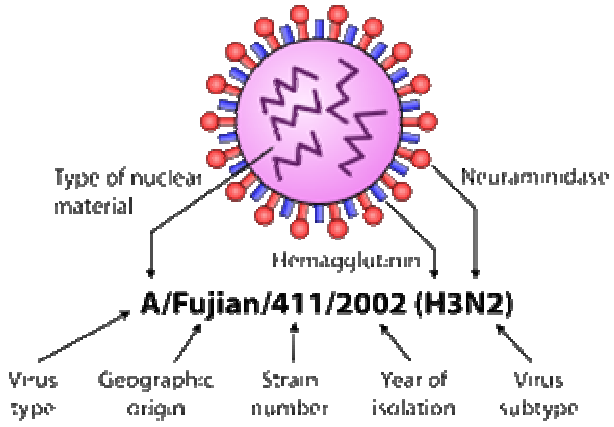


Diagram of influenza virus nomenclature.

Fujian flu refers to flu caused by either a Fujian human flu strain of the H3N2 subtype of the *Influenza A virus* or a Fujian bird flu strain of the H5N1 subtype of the *Influenza A virus*. These strains are named after Fujian, a coastal province of the People's Republic of China that is across the Taiwan strait from Taiwan.[12]

A/Fujian (H3N2) human flu (from A/Fujian/411/2002(H3N2) -like flu virus strains) caused an unusually severe 2003–2004 flu season. This was due to a reassortment event that caused a minor clade to provide a haemagglutinin gene that later became part of the dominant strain in the 2002–2003 flu season. A/Fujian (H3N2) was made part of the trivalent influenza vaccine for the 2004–2005 flu season and its descendants are still the most common human H3N2 strain.

2004–2005 flu season

The 2004–05 trivalent influenza vaccine for the United States contained A/New Caledonia/20/99-like (H1N1), A/Fujian/411/2002-like (H3N2), and B/Shanghai/361/2002-like viruses.

2005–2006 flu season

The vaccines produced for the 2005–2006 season use:

- an A/New Caledonia/20/1999-like(H1N1);
- an A/California/7/2004-like(H3N2) (or the antigenically equivalent strain A/New York/55/2004);
- a B/Jiangsu/10/2003-like viruses.

2006–2007 flu season

The 2006–2007 influenza vaccine composition recommended by the World Health Organization on 15 February 2006 and the U.S. FDA's Vaccines and Related Biological Products Advisory Committee (VRBPAC) on 17 February 2006 use:

- an A/New Caledonia/20/99 (H1N1)-like virus;
- an A/Wisconsin/67/2005 (H3N2)-like virus (A/Wisconsin/67/2005 and A/Hiroshima/52/2005 strains);
- a B/Malaysia/2506/2004-like virus from B/Malaysia/2506/2004 and B/Ohio/1/2005 strains which are of B/Victoria/2/87 lineage.

2007–2008 flu season

The composition of influenza virus vaccines for use in the 2007–2008 Northern Hemisphere influenza season recommended by the World Health Organization on 14 February 2007 was:

- an A/Solomon Islands/3/2006 (H1N1)-like virus;
- an A/Wisconsin/67/2005 (H3N2)-like virus (A/Wisconsin/67/2005 (H3N2) and A/Hiroshima/52/2005 were used at the time);
- a B/Malaysia/2506/2004-like virus

"A/H3N2 has become the predominant flu subtype in the United States, and the record over the past 25 years shows that seasons dominated by H3N2 tend to be worse than those dominated by type A/H1N1 or type B." Many H3N2 viruses making people ill in this 2007–2008 flu season differ from the strains in the vaccine and may not be well covered by the vaccine strains. "The CDC has analyzed 250 viruses this

season to determine how well they match up with the vaccine, the report says. Of 65 H3N2 isolates, 53 (81%) were characterized as A/Brisbane/10/2007-like, a variant that has evolved [notably] from the H3N2 strain in the vaccine—A/Wisconsin/67/2005."

2009 H1N1 influenza outbreak

2009 H1N1 influenza outbreak

The British Columbia Center for Disease Control reported on 5 May 2009 that on 28 April 2009 it had isolated a new variant H3N2 virus from a person who recently visited Mexico. It has been suggested that some of the differences in the pathology of the influenza in Mexico may be due to the presence of this variant H3N2 and not H1N1.

What is Fujian-like strain A influenza?

All forms of flu are caused by influenza viruses and they fall into three categories: A, B and C. Fujian-like influenza is an A-type virus.

It is the A-types that are normally responsible for flu epidemics. While influenza B and C viruses are less easily spread and tend to cause less severe illness.

There are many different strains of the A-type flu virus and new ones develop every year. Depending on the way that the virus has developed, the strains can be spread more easily (or less easily) and cause more (or less) severe illness.

The Fujian-like strain has developed in a way that makes it spread more easily than other A-type strains that have been circulating in the UK population over the past few years and it causes more severe symptoms. It also seems to spread easily among children and young adults.

The *Geno-Sen's* Influenza Typing assay is developed for laboratory scale or high-throughput qualitative transcript analysis by real time quantitative fluorescence PCR of H1, N1, H3, N2, influenza A, influenza B Gene of Influenza group separately. Geno Sen's standardized ready-to-use Positive Control and Reaction mix allows fast processing of the samples separately for the H1, N1, H3, N2, influenza A, influenza B Gene Amplifications.

Samples which can be used for Extraction: Sputum; throat and nasal swabs in viral transport medium, or lung tissues.

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*** **Influenza Group typing** *PCR* Reagents constitute a ready to use system for detection of **H1, N1, H3, N2, influenza A, influenza B Gene** 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 H1, N1, H3, N2, influenza A, influenza B Gene***s*** which covers gene sequences for all the known subtypes including the recent Mexican H1N1 strain. A part of the gene is amplified to generate amplified product 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 controls (H1-PC, N1-PC, H3-PC, N2- PC, Influenza A-PC & Influenza B-PC) are supplied which allow the determination of the gene. For further information, please refer to section 8.c Positive controls.

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	
Sputum; throat and nasal swabs in viral transport medium, or lung tissues.	QIAamp Viral RNA Mini extraction Kit (50) OR Viral RNA extraction kit Bioneer	52904 K-3033	QIAGEN Bioneer

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.

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 *Influenza Virus Group typing assay 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 RG (R7). To add the IC Gene 0.5 µl/rxn of R7 is added to the premix as shown in the Figure 4 & 5. The results can be visualized in the Joe/Yellow channel.

8.c Positive Controls.

The Positive controls provided in the kit (H1-PC, N1-PC, H3-PC, N2- PC, Influenza A-PC & Influenza B-PC) are treated in the same way as extracted samples and the same volume is used i.e. (15µl) instead of the sample.

8.d Procedure

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 Positive controls & at least one negative control (*Water, PCR grade*) are included per 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

S.No.		H1	N1	H3	N2	Influenza A	Influenza B
	Reagent						
1.	**R1 Blue**	**7µL (R1)**	**----**	--------	----	-----	----
2.	**R2 Blue**		**7µL (R2)**	-------	----	-----	----
3.	**R3 Blue**	-------	----	-----	----	**7µL (R3)**	----
4.	**R4 Blue**	-------	----	-----	----		**7µL (R4)**
5.	**R5 Blue**	----	-----	**7µL (R5)**	-------	----	-----
6.	**R6 Blue**	-------	----	-----	**7µL (R6)**	----	-----
7.	**R8 Yellow**	**2.5 µL**	**2.5 µL**	**2.5 µL**	**2.5 µL**	**2.5 µL**	**2.5 µL**
8.	**IC (R7) Green**	**0.5 µL**	**0.5 µL**	**0.5 µL**	**0.5 µL**	**0.5 µL**	**0.5 µL**
	Sample/ PC/NC	**ADD 15 µL Extracted RNA/ Positive control/Mol. Grade water**	**ADD 15 µL Extracted RNA/ Positive control/Mol. Grade water**	**ADD 15 µL Extracted RNA/ Positive control/Mol. Grade water**	**ADD 15 µL Extracted RNA/ Positive control/Mol. Grade water**	**ADD 15 µL Extracted RNA/ Positive control/Mol. Grade water**	**ADD 15 µL Extracted RNA/ Positive control/Mol. Grade water**
	Total Volume	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL
		To Amplification	To Amplification	To Amplification	To Amplification	To Amplification	To Amplification

For H1 dilution.

H1 **MASTER MIX**	**1 rxns.**	**10 rxns.**
H1 **Super Mix (R1)**	**7 µL**	**70 µL**
Influenza Mg Sol. (R8)	**2.5 µL**	**25 µL**
Influenza IC RG (R7)	**0.5 µL**	**5 µL**
Total	**10µL**	**100µL**

For N1 dilution.

N1 **MASTER MIX**	**1 rxns.**	**10 rxns.**
N1 **Super Mix (R2)**	**7 µL**	**70 µL**
Influenza Mg Sol. (R8)	**2.5 µL**	**25 µL**
Influenza IC RG (R7)	**0.5 µL**	**5 µL**
Total	**10µL**	**100µL**

For Infi A dilution.

Influenza A MASTER MIX	1 rxns.	10 rxns.
Influenza A Super Mix (R3)	7 µL	70 µL
Influenza Mg Sol. (R8)	2.5 µL	25 µL
Influenza IC RG (R7)	0.5 µL	5 µL
Total	10µL	100µL

For Infi B dilution.

Influenza B MASTER MIX	1 rxns.	10 rxns.
Influenza B Super Mix (R4)	7 µL	70 µL
Influenza Mg Sol. (R8)	2.5 µL	25 µL
Influenza IC RG (R7)	0.5 µL	5 µL
Total	10µL	100µL

For H3 dilution.

H3 MASTER MIX	1 rxns.	10 rxns.
H3 Super Mix (R5)	7 µL	70 µL
Influenza Mg Sol. (R8)	2.5 µL	25 µL
Influenza IC RG (R7)	0.5 µL	5 µL
Total	10µL	100µL

For N2 dilution.

N2 MASTER MIX	1 rxns.	10 rxns.
N2 Super Mix (R6)	7 µL	70 µL
Influenza Mg Sol. (R8)	2.5 µL	25 µL
Influenza IC RG (R7)	0.5 µL	5 µL
Total	10µL	100µL

Fig. 5.

Prepare 6 sets of 0.2 ml PCR tubes & Pipette 10 µl of the Master Mix into each respectively 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 Positive controls (H1-PC, N1-PC, H3-PC, N2- PC, Influenza A-PC & Influenza B-PC)

must be used as a Positive Amplification Confirmation controls 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 ***Influenza Group typing*** 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.

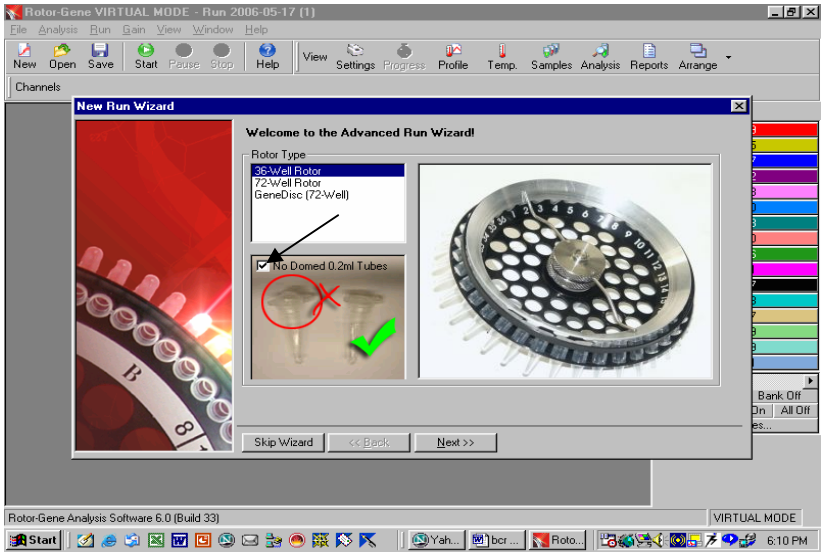


Fig. 6.

Confirmation of Reaction Volume as follows.

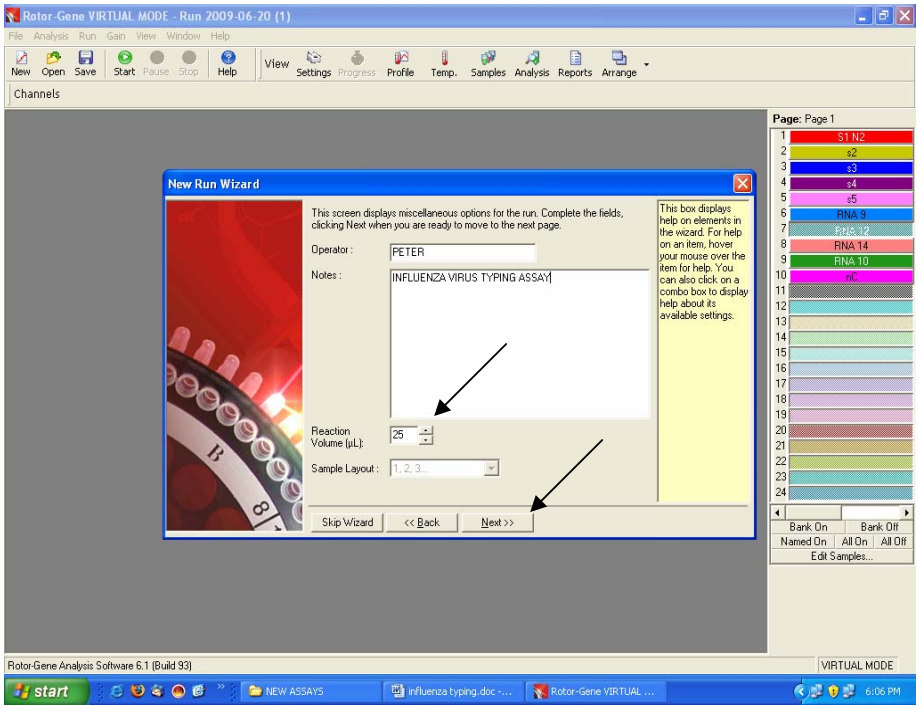


Fig. 7. Setting of general assay parameters.

- 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.

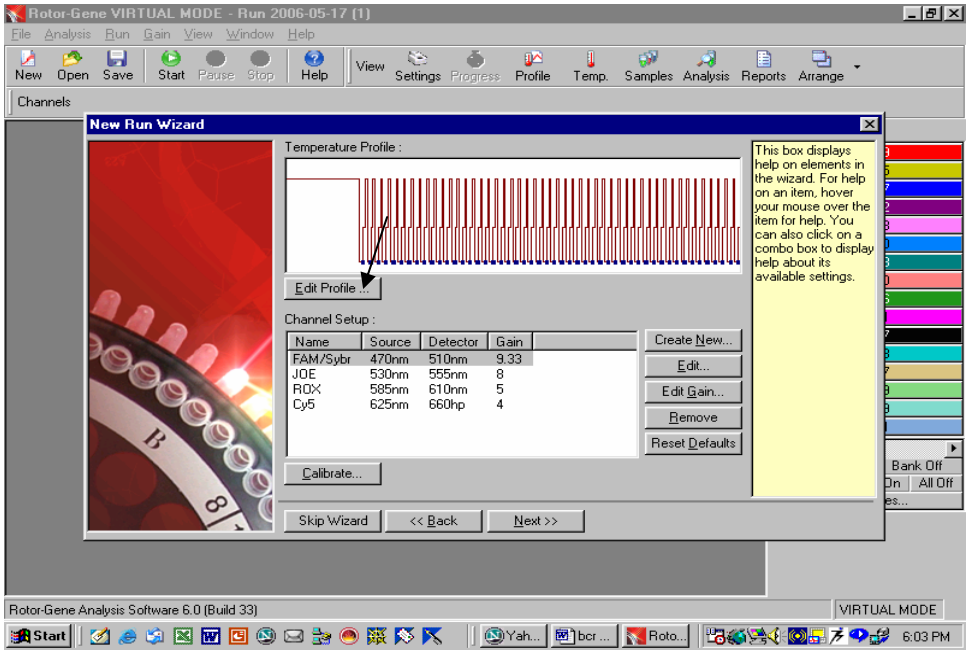


Fig. 8.

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

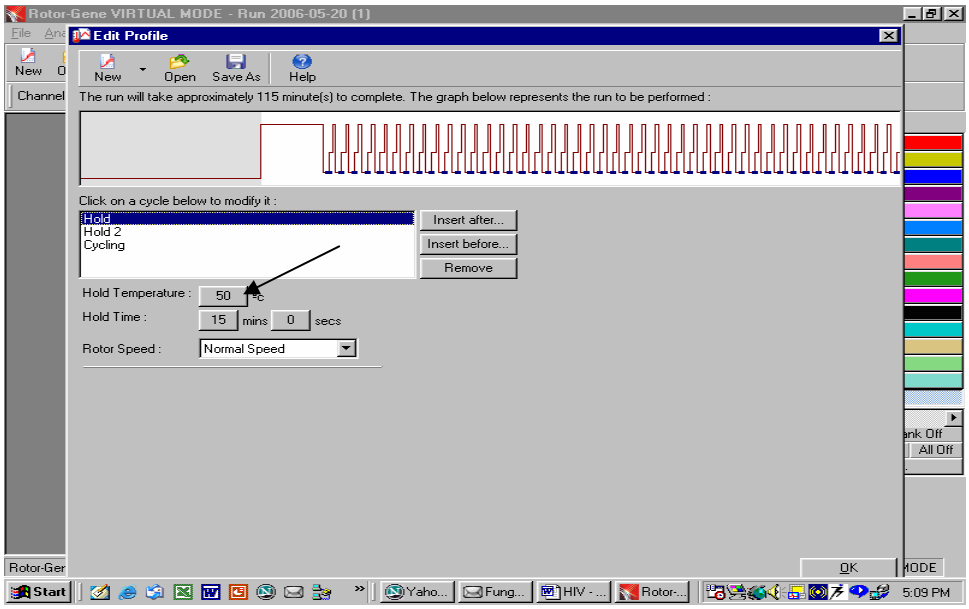


Fig. 9.

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

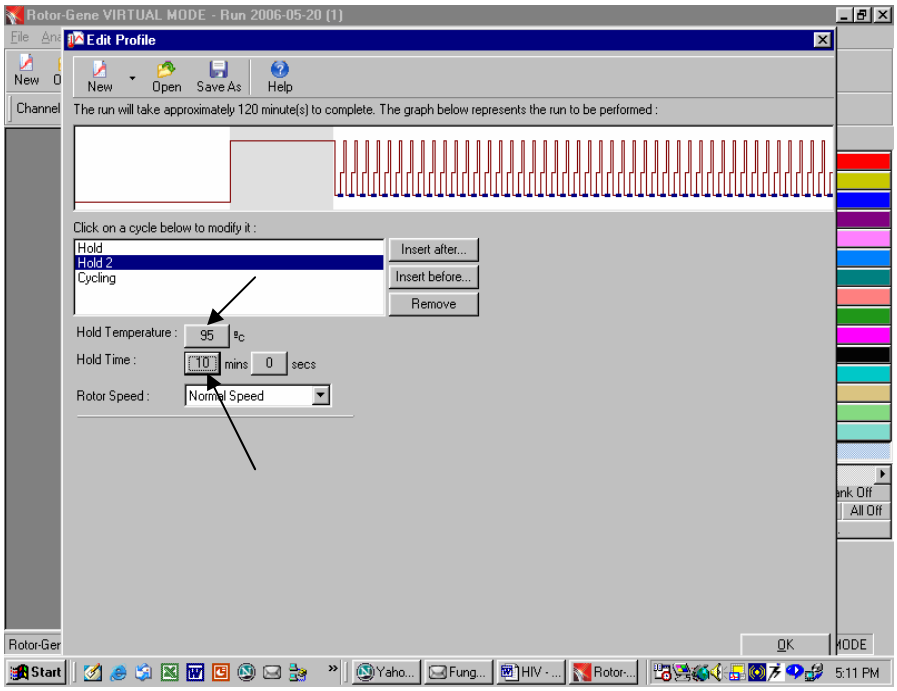


Fig. 10. Initial activation of the Hot Start enzyme.

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

95°C for 15 seconds.

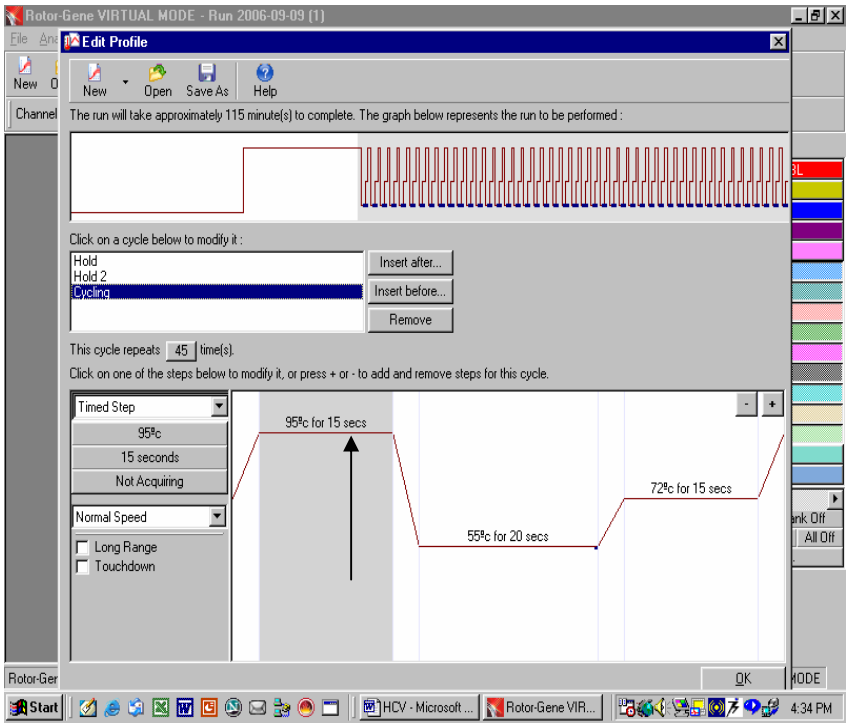


Fig. 11.

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

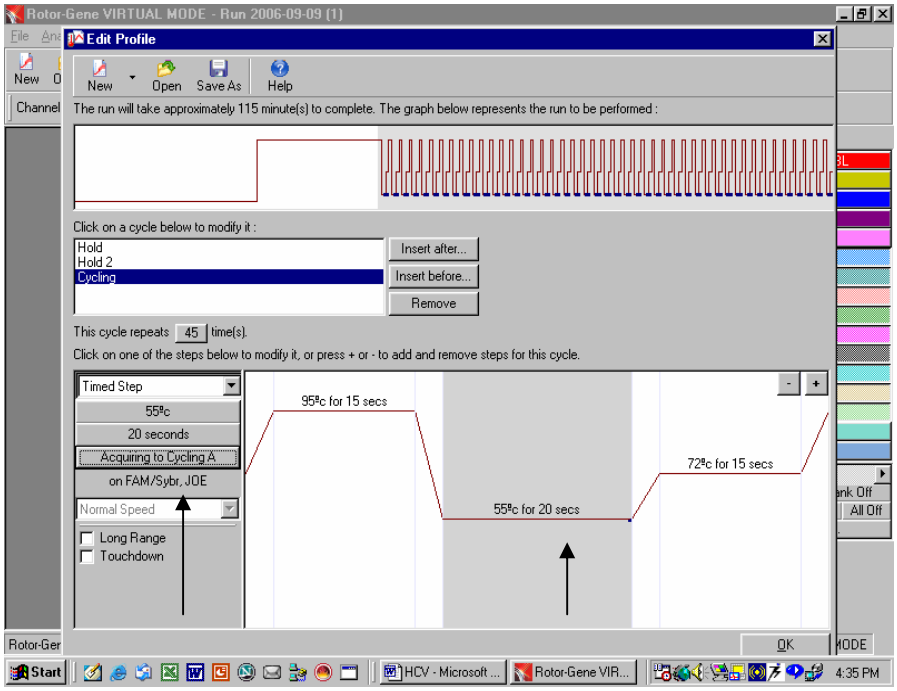


Fig. 12.

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

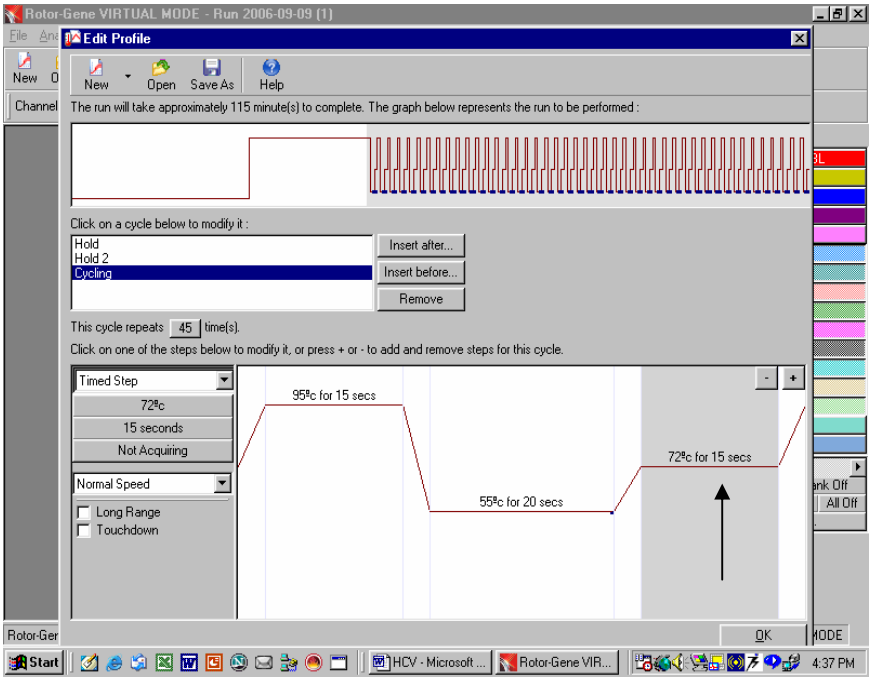


Fig. 13.

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

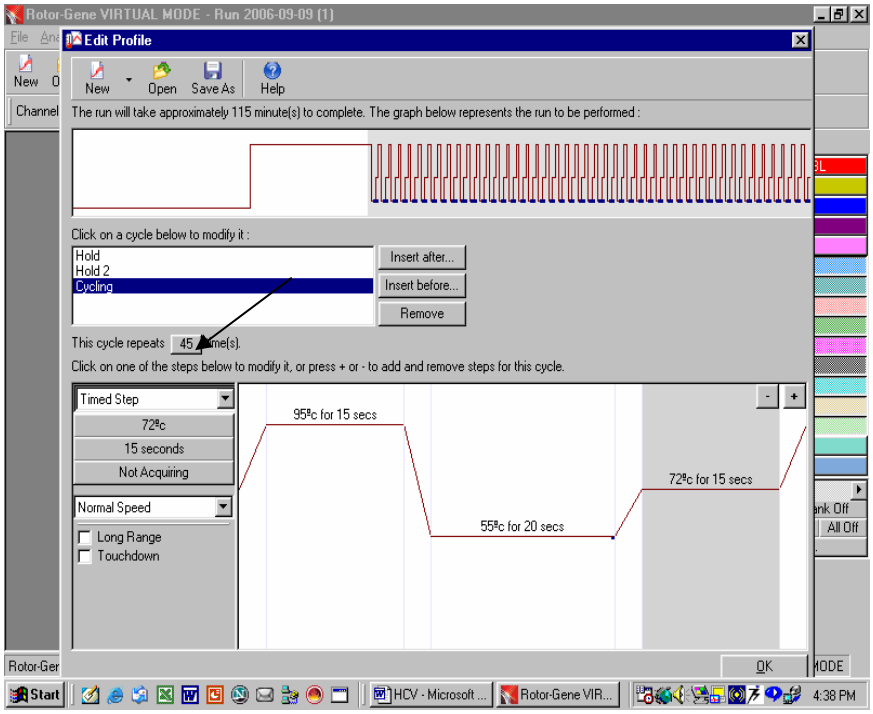


Fig. 14.

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

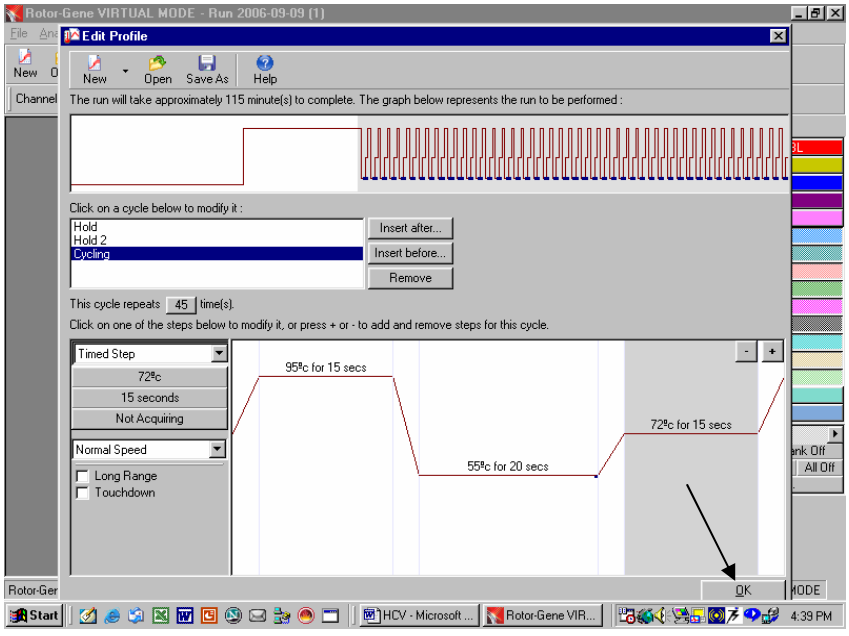


Fig. 15.

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

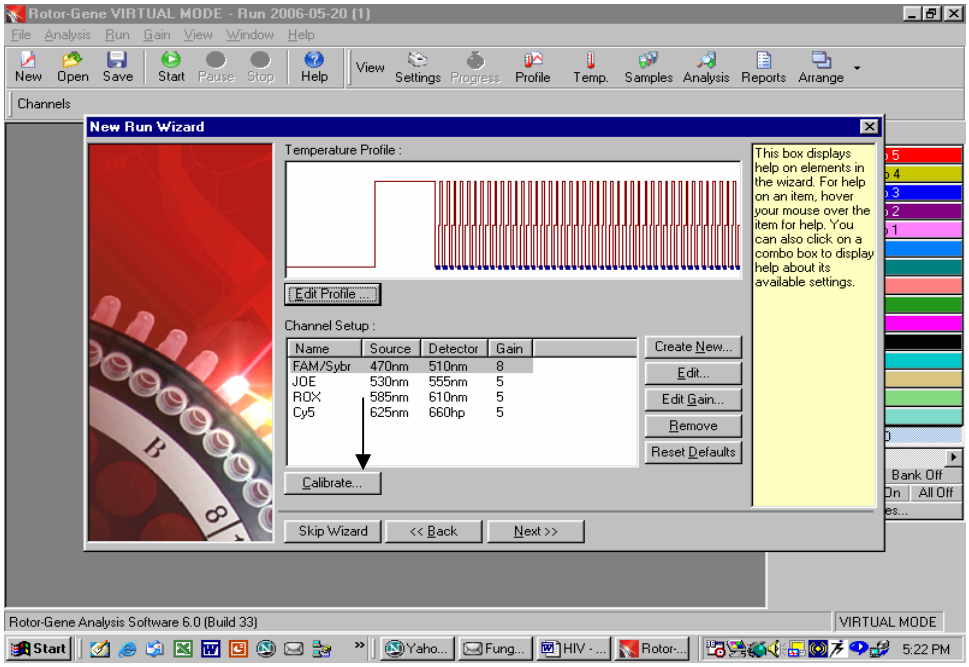


Fig. 16.

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

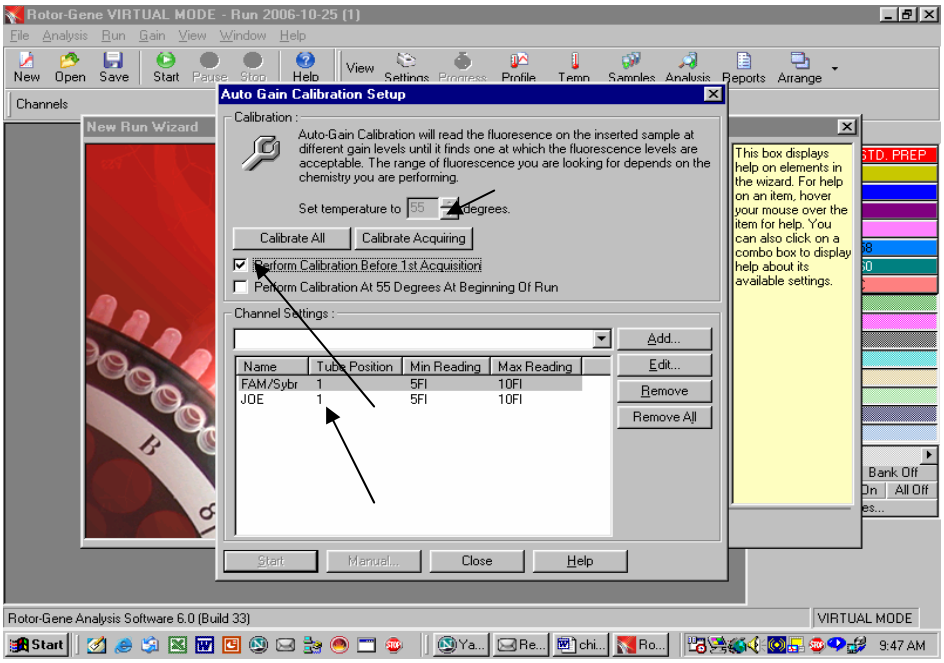


Fig. 17.

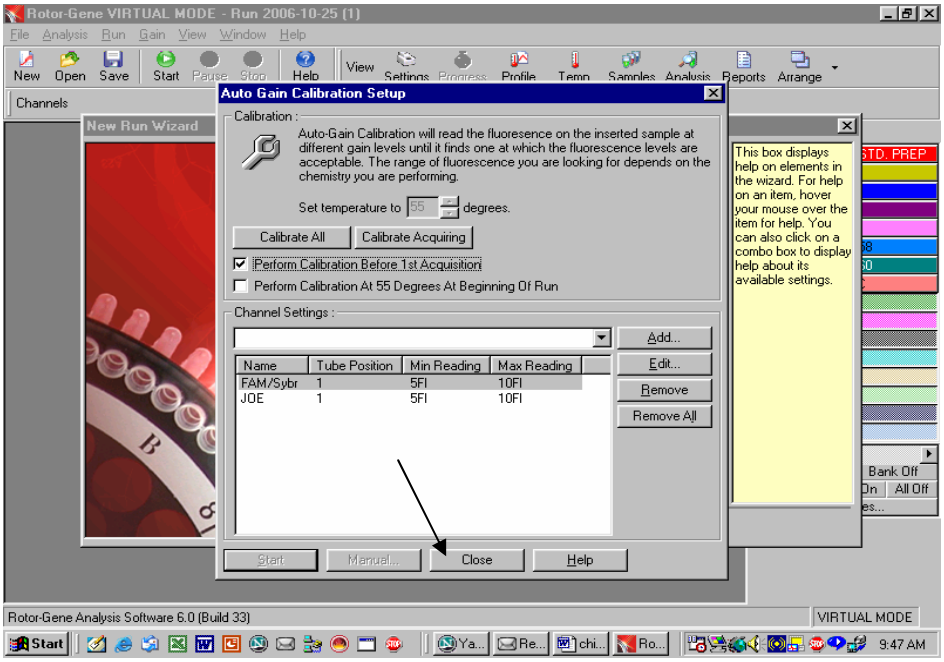


Fig. 18.

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

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

PRESS NEXT

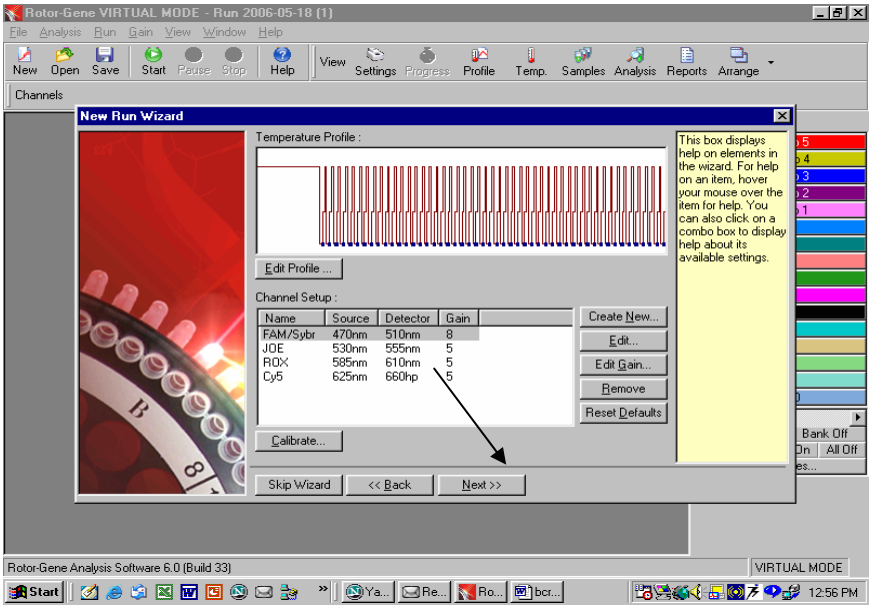


Fig. 19.

Starting of the *Rotor Gene*™ run.

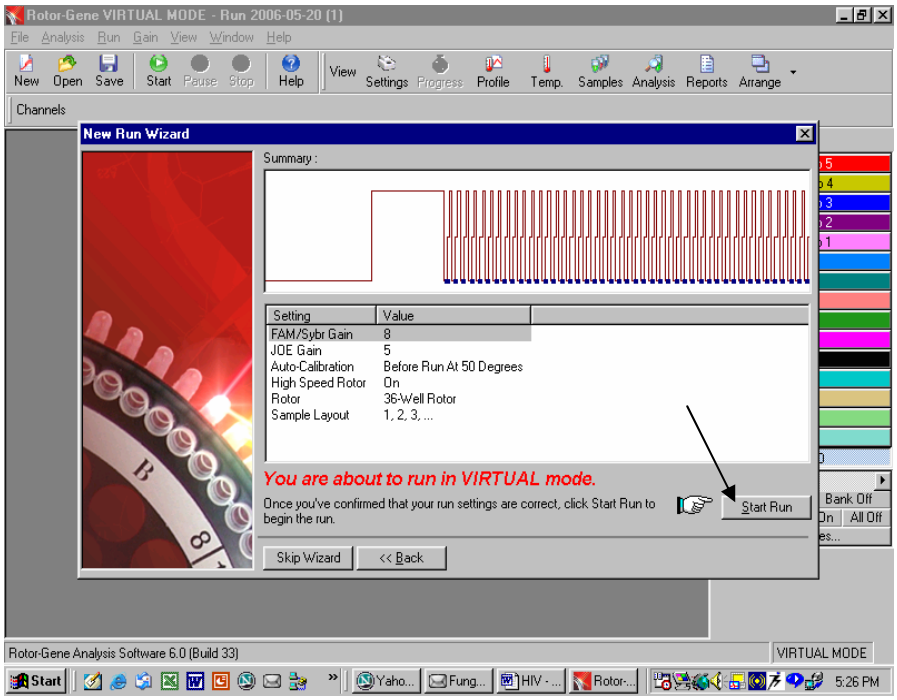


Fig. 20: Press Start Run Button.

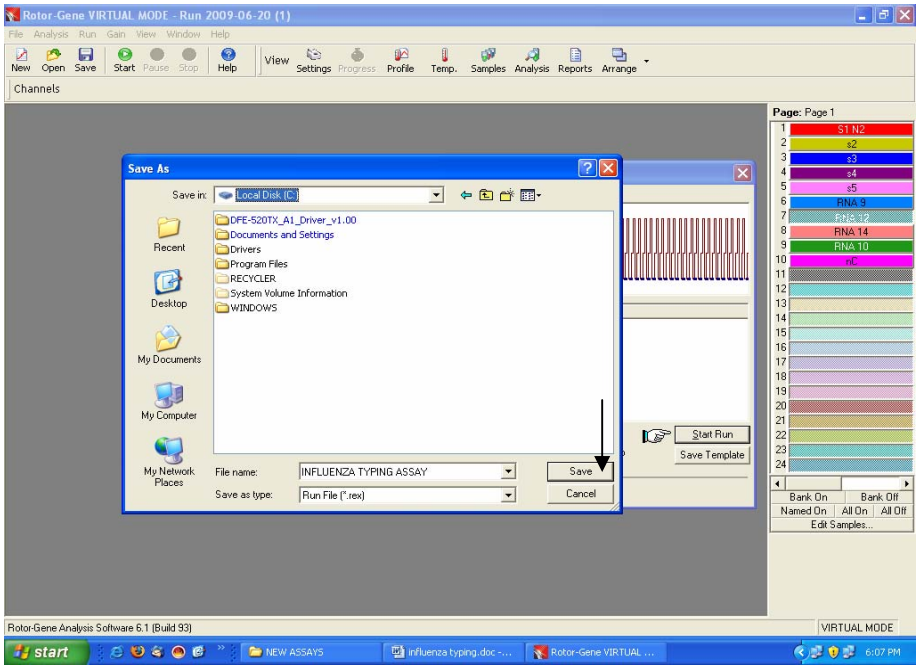


Fig. 21.

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*™ *6000* PCR program for the detection of ***Influenza Group typing*** 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

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.

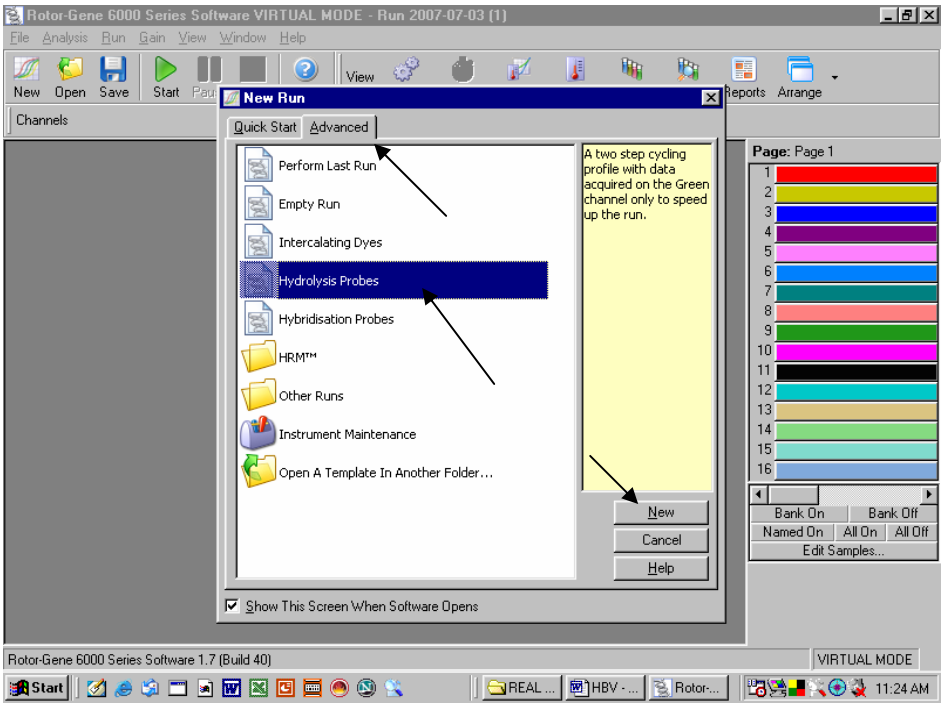


Fig. 22.

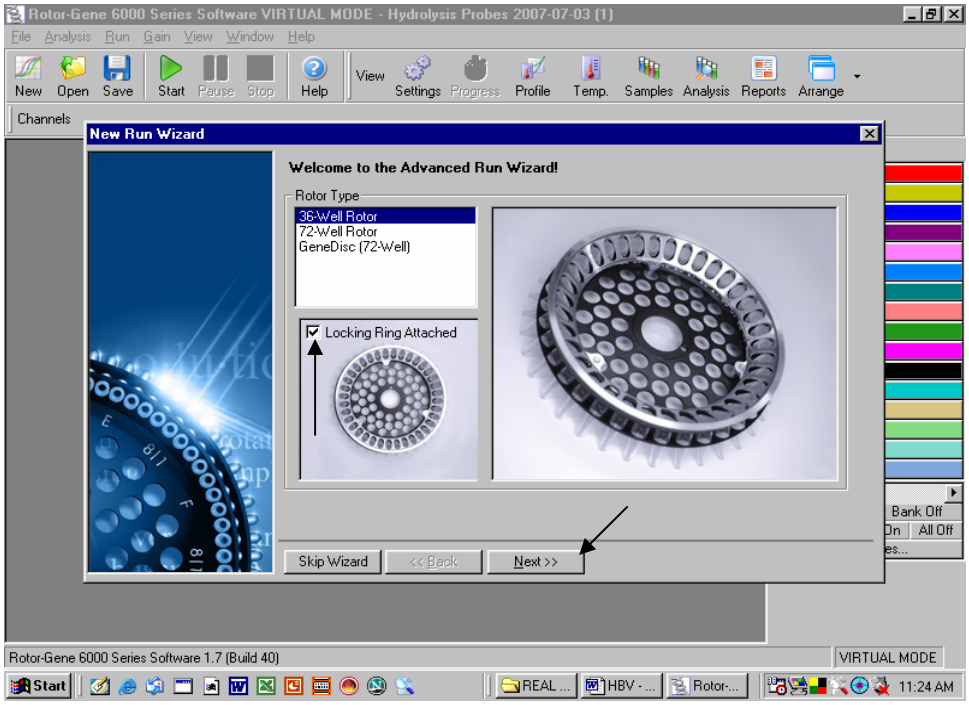


Fig. 23.

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.

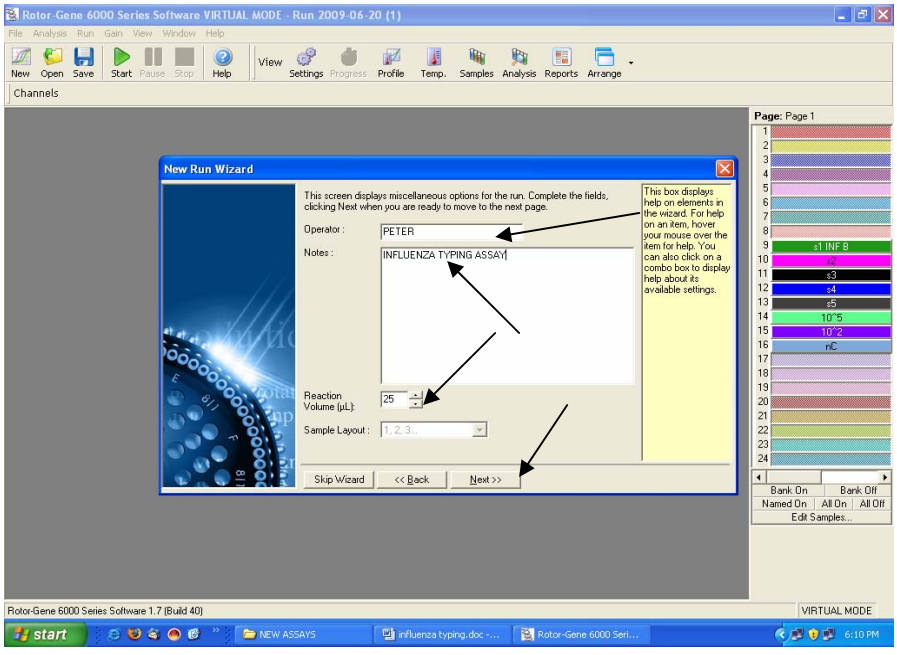


Fig. 24.

- 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.

THERMAL PROFILE & CALIBRATION:

Here the thermal profile for the assay will be defined.

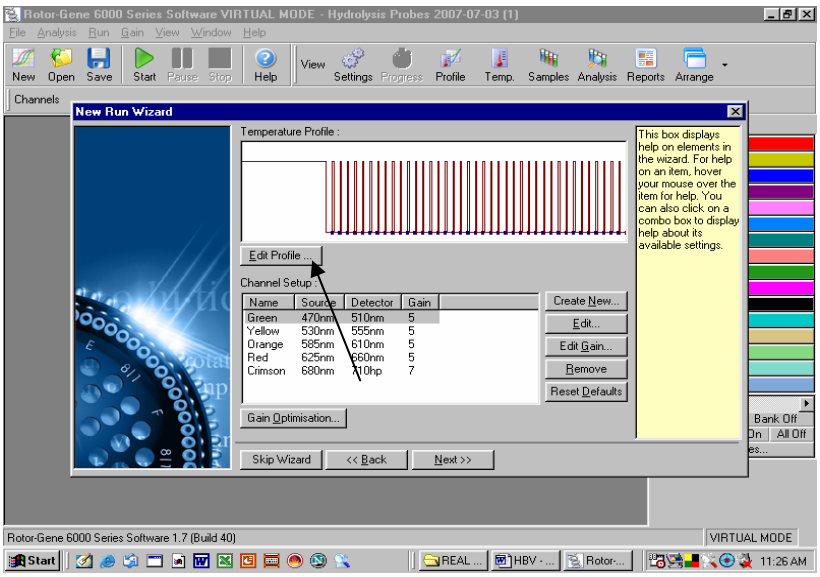


Fig. 25.

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

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

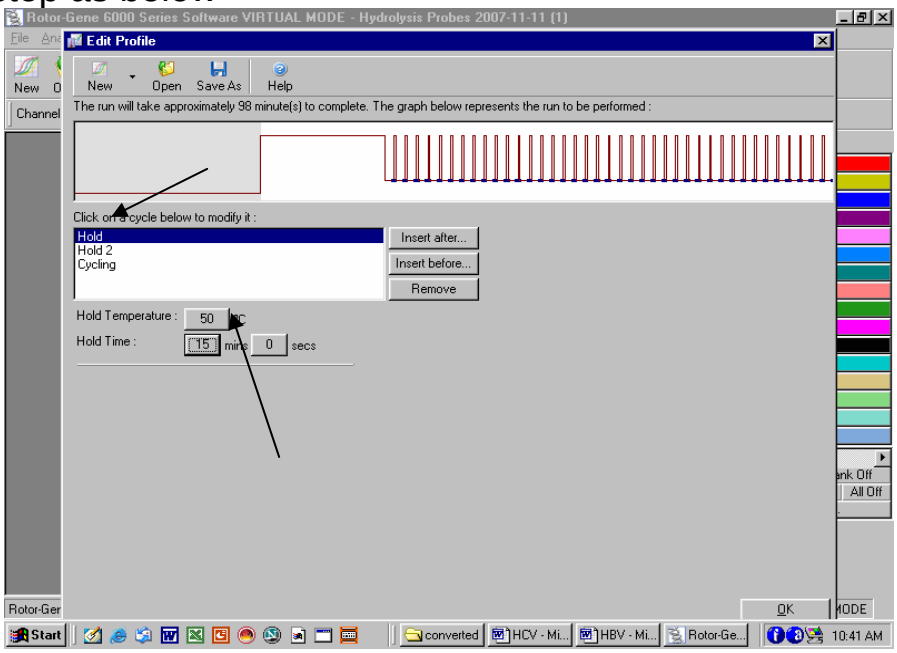


Fig. 26.

Second hold 95°C for 10 minutes as below

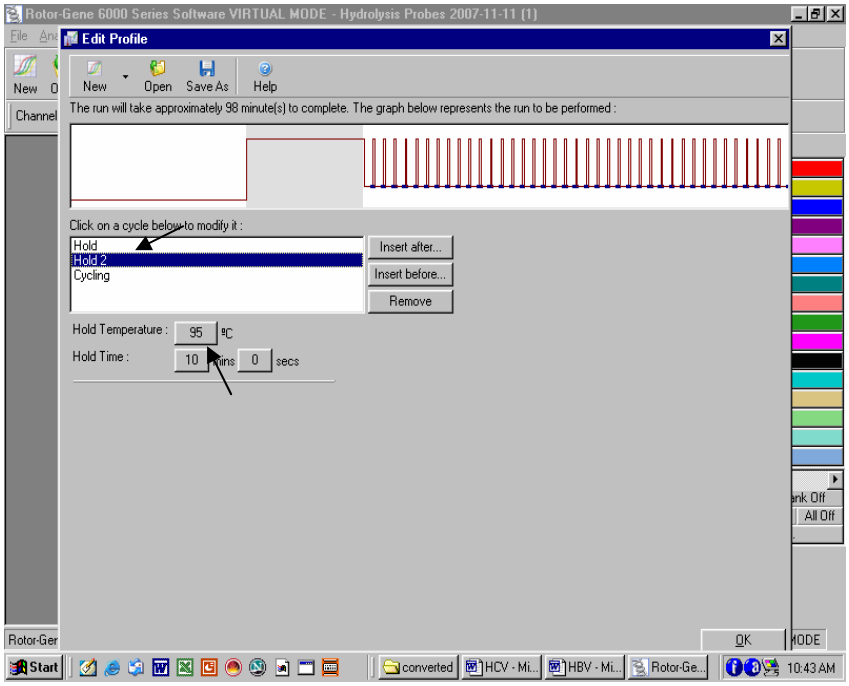


Fig. 27. Initial activation of the Hot Start enzyme. Generally the window will open with the Hold Temp as 95°C and the Hold Time as 10 minutes. In case it is different then set the same to as shown above.

Setting the Cycling and acquisition.

When clicked on Cycling the window will open as below.

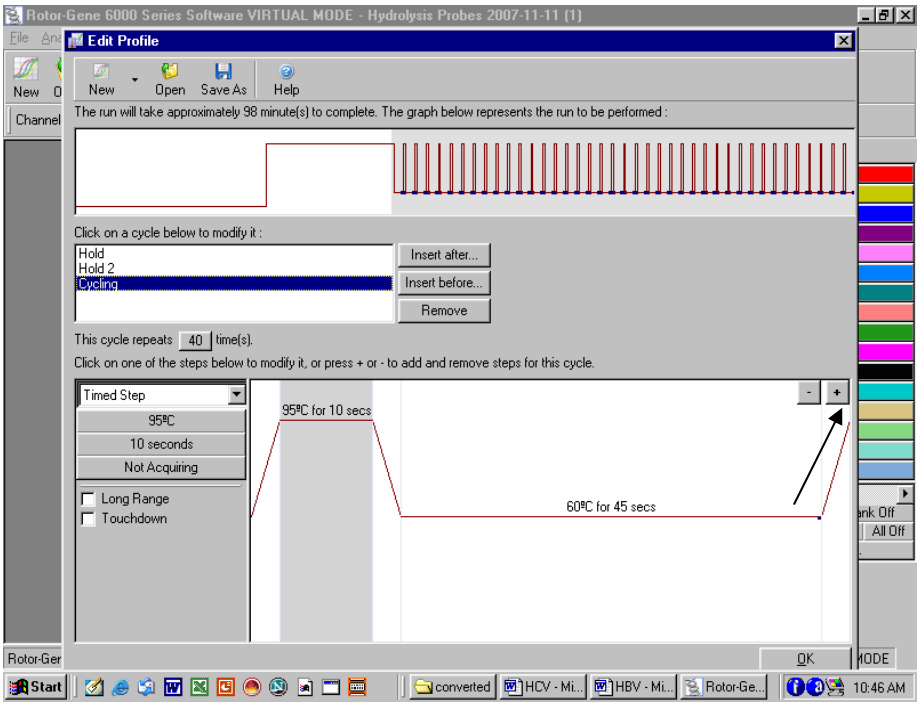


Fig. 28.

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

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

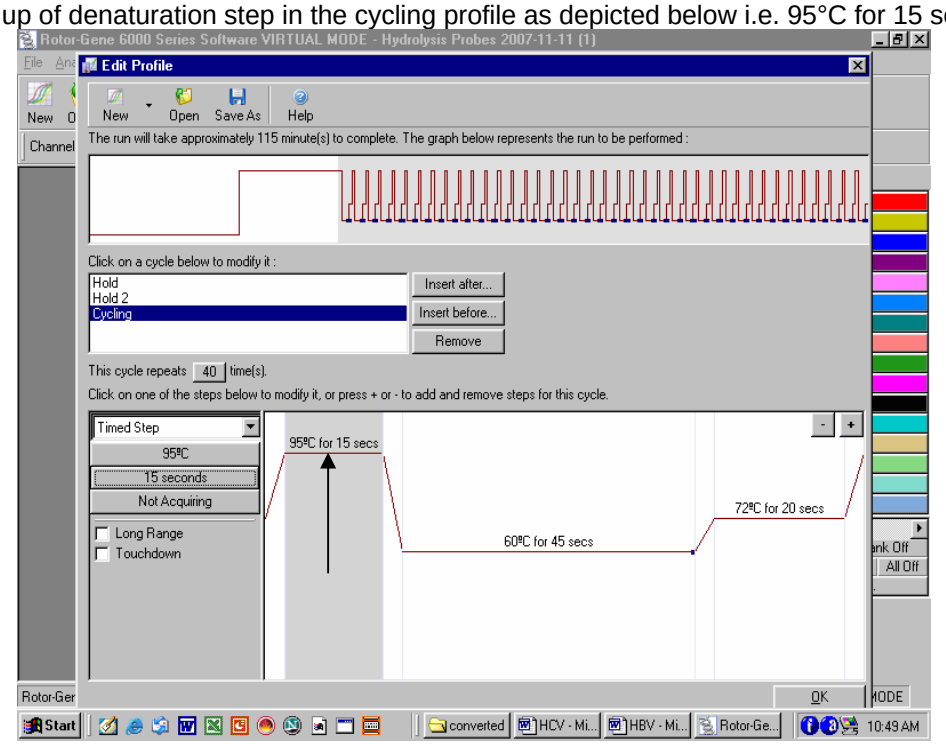


Fig. 29.

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

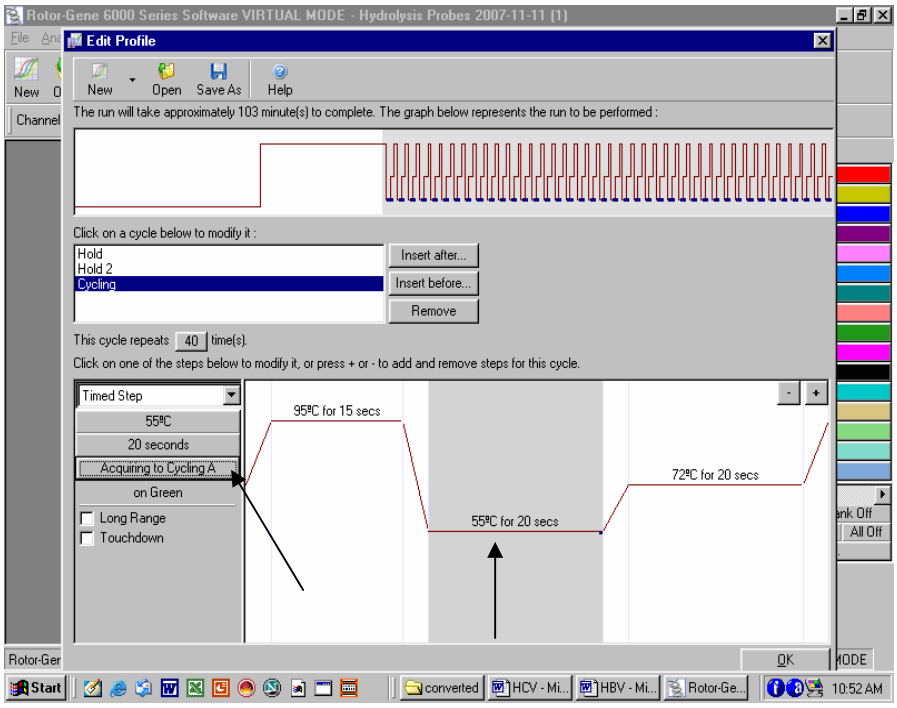


Fig. 30.

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

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

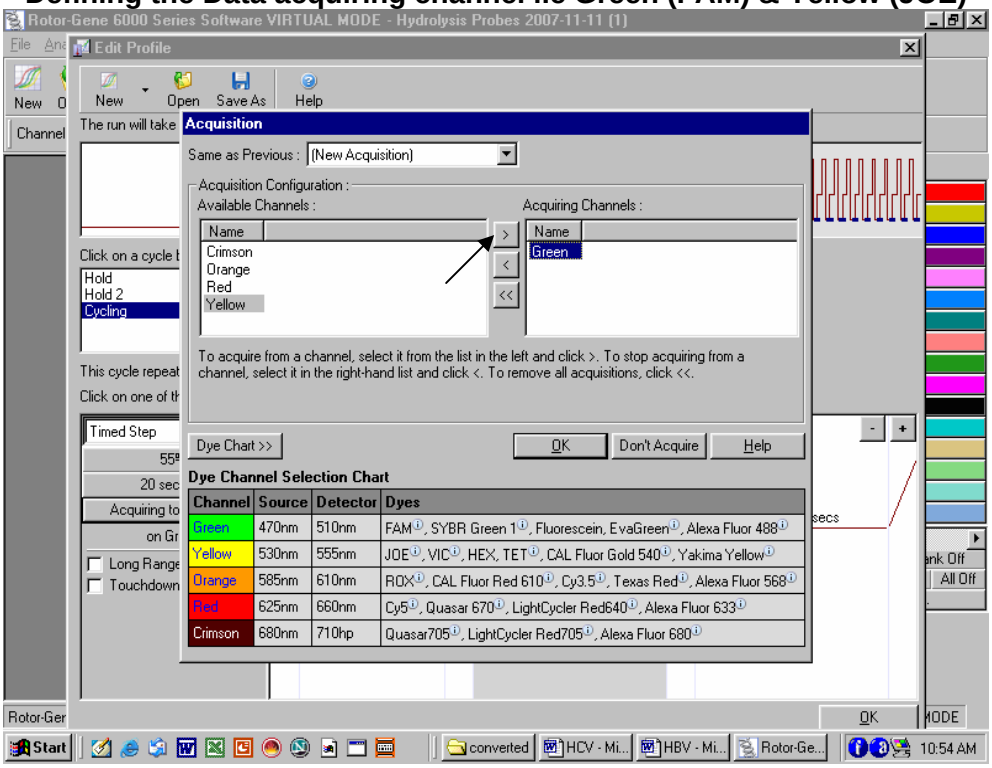


Fig. 31.

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

Confirmation of Channels as shown below.

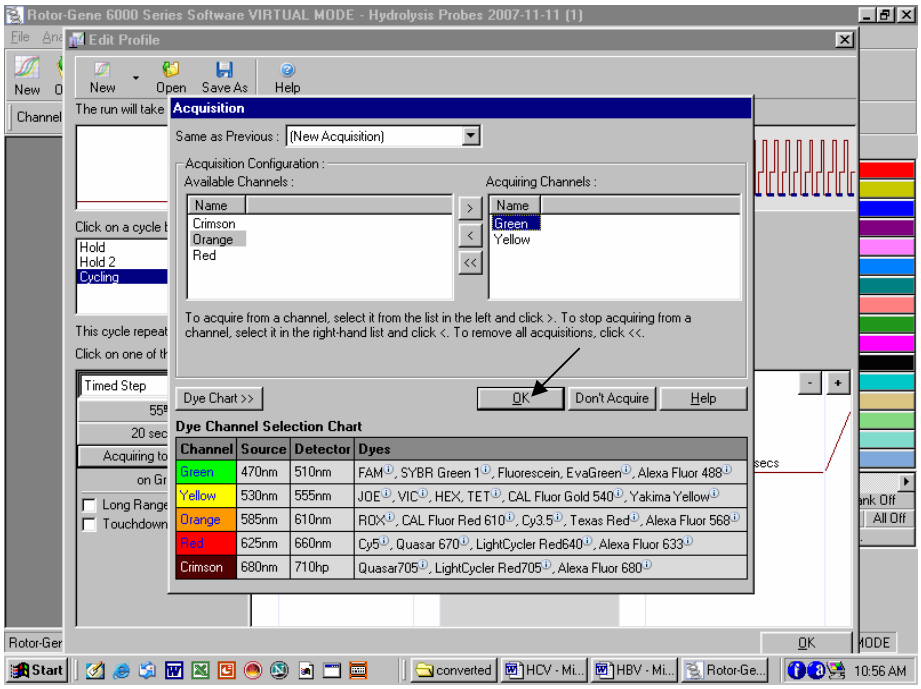


Fig. 32.

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

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

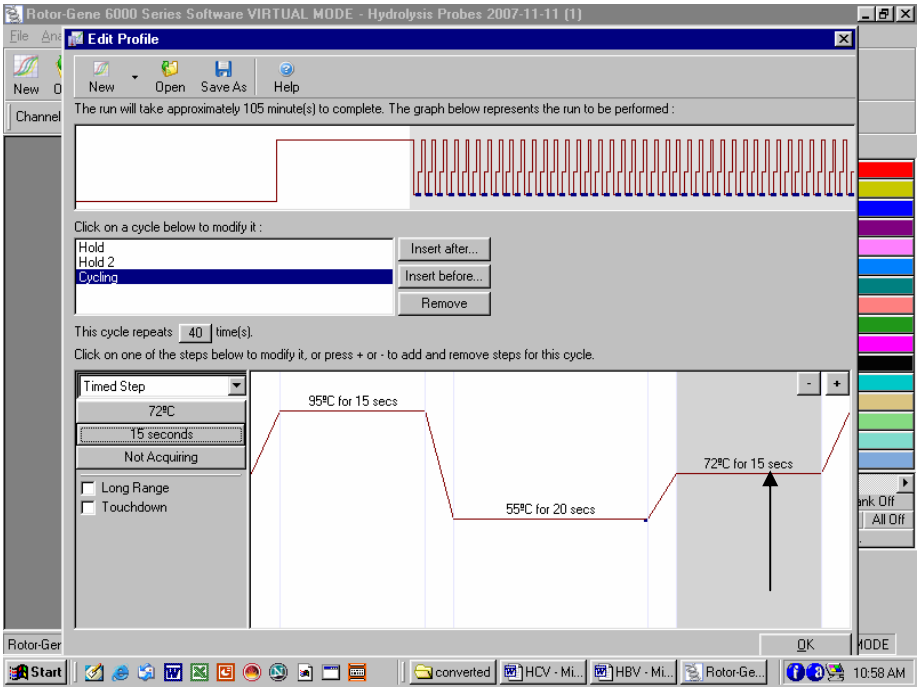


Fig. 33.

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

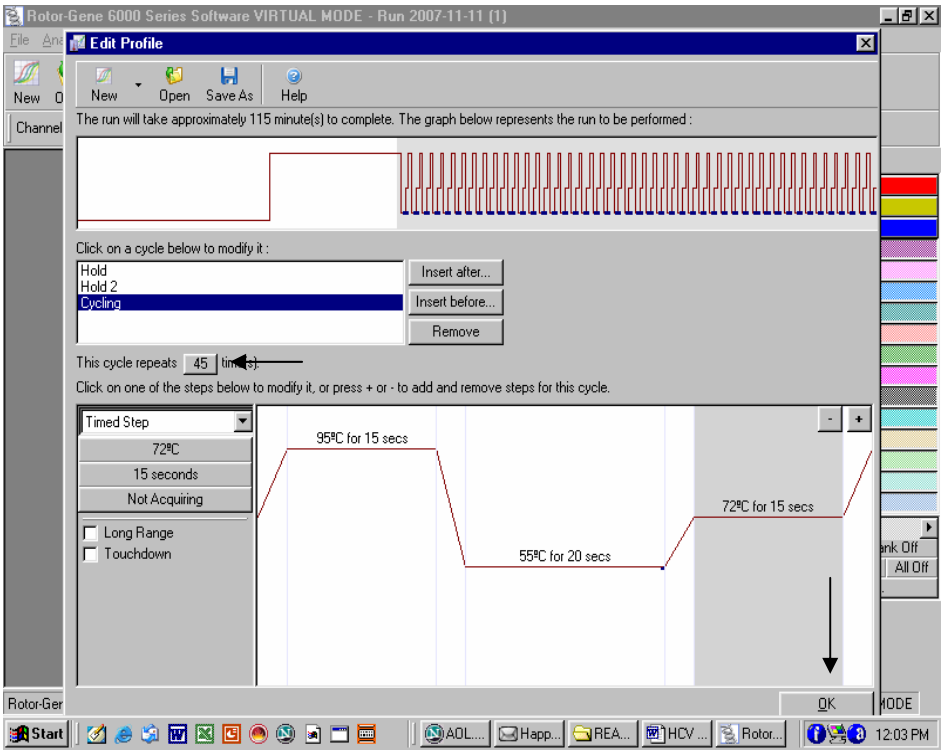


Fig. 34.

After setting the number of Cycles Press OK.

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

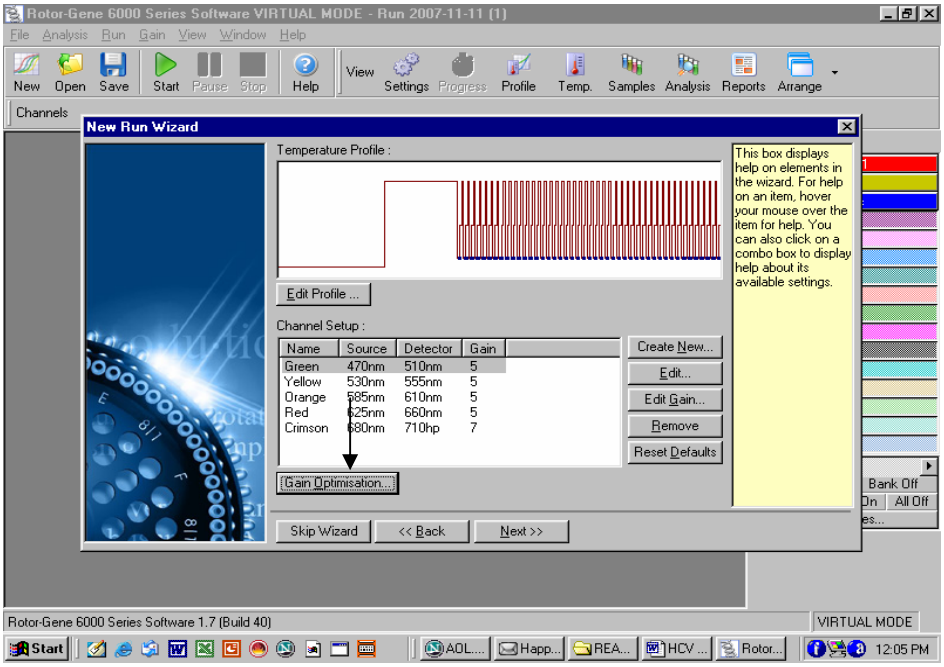


Fig. 35

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

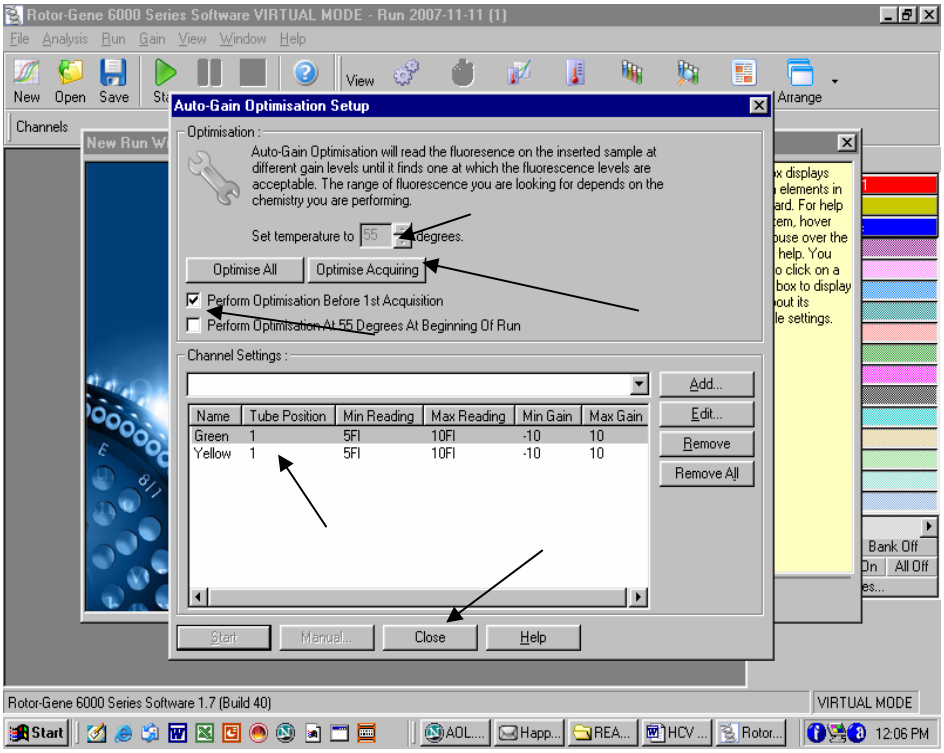


Fig. 36.

The following needs to be done.

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

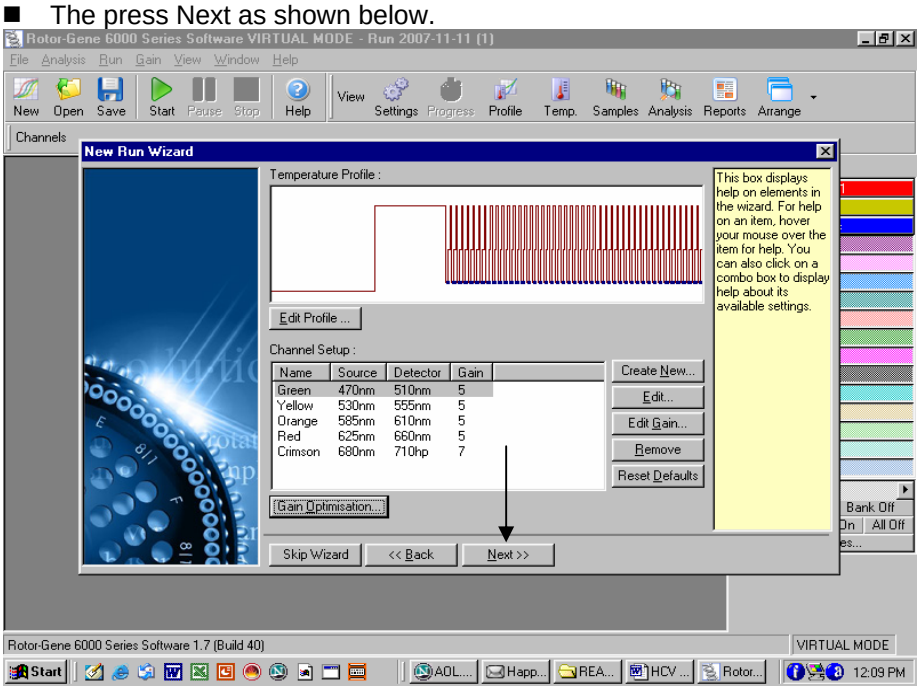


Fig. 37.

PRESS Start RUN

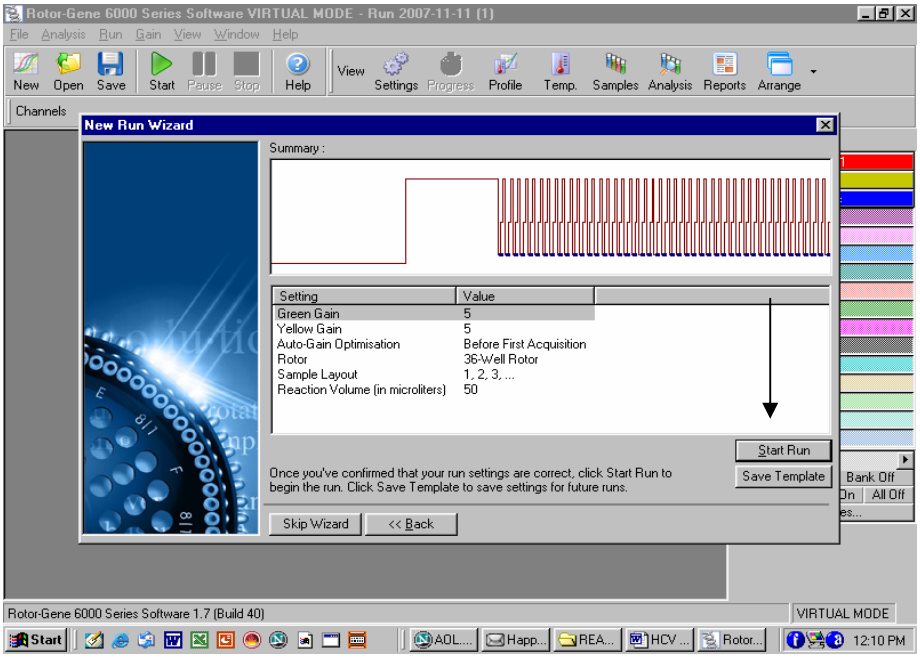


Fig. 38.

Saving the RUN File.

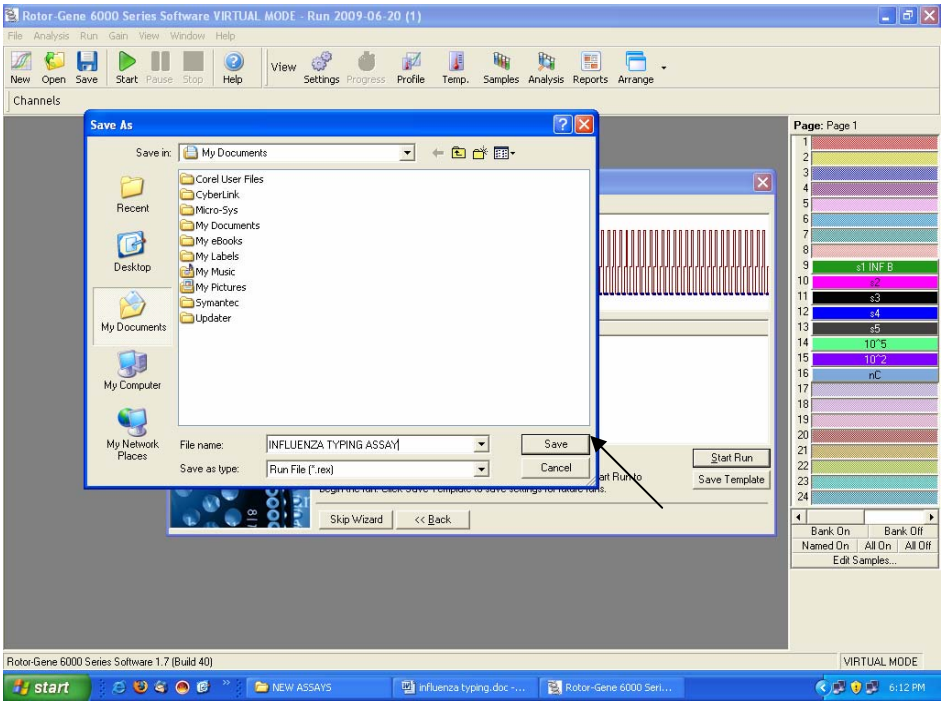


Fig. 39.

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*™ 2000/*3000 Operator's* Manual).

The results are summarized in the following table:

Sample form	H1	N1	Influenza A	Influenza B	H3	N2	Remarks
FAM	Signal Detected	Signal Detected	Signal Detected	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	+ Positive for H1N1 Virus & Influenza Group A Virus
FAM	Signal Detected	Signal Not Detected but signal	Signal Detected	Signal Not Detected but signal	Signal Not Detected but signal	Signal Detected	+ Positive for H1N2 Virus & Influenza

		detected in Joe		detected in Joe	detected in Joe		Group A Virus
FAM	Signal Detected	Signal Not Detected but signal detected in Joe	Signal Detected	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Positive for Influenza A Group Virus & H1 but not H1N1, H1N2
FAM	Signal Not Detected but signal detected in Joe	Signal Detected	Signal Detected	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Positive for Influenza A Group Virus & N1 but not H1N1, H3N1
FAM	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Signal Detected	Signal Not Detected but signal detected in Joe	Signal Detected	Signal Detected	+ Positive for H3N2 Virus & Influenza Group A Virus
FAM	Signal Not Detected but signal detected in Joe	Signal Detected	Signal Detected	Signal Not Detected but signal detected in Joe	Signal Detected	Signal Not Detected but signal detected in Joe	+ Positive for H3N1 Virus & Influenza Group A Virus
FAM	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Signal Detected	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Signal Detected	Positive for Influenza A Group Virus & N2 but not H3N2, & H1N2
FAM	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Signal Detected	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Positive for Influenza A Group Virus but not H1N1, H3N2, H1N2, H3N1.
FAM	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Signal Detected	Signal Not Detected but signal detected in Joe	Signal Not Detected but signal detected in Joe	Positive for Influenza B Group Virus but not Influenza A Group, H1N1, H3N1, H3N2, H1N2.

FAM	**Signal Not Detected but signal detected in Joe**	**Signal Not Detected but signal detected in Joe**	**Signal Not Detected but signal detected in Joe**	**Signal Not Detected but signal detected in Joe**	**Signal Not Detected but signal detected in Joe**	**Signal Not Detected but signal detected in Joe**	**-Ve Negative for Influenza A & B Group Virus & H1N1, H1N2, H3N1 & H3N2.**
Joe	**Signal Not Detected**	**Signal Not Detected**	**Signal Not Detected**	**Signal Not Detected**	**Signal Not Detected**	**Signal Not Detected**	**Possible PCR Inhibition.**

Example of the Curves generated in H1 Gene Samples

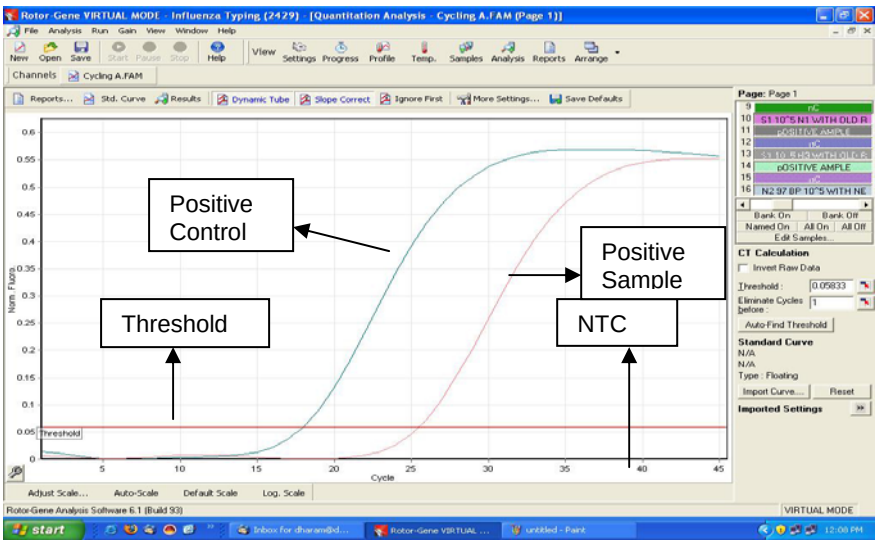


Fig. 40: Detection of the Positive Control *(***H1** *PC)* in fluorescence channel Cycling A.FAM. NTC: non-template control.

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

Example of analysed data for H1 where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standrads have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure

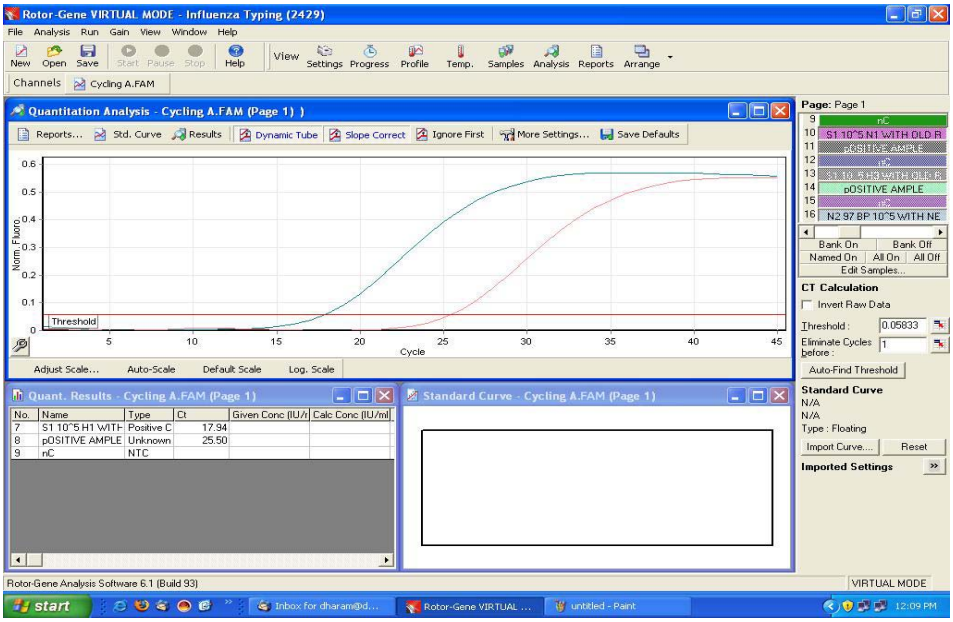


Fig. 41.

Example of the Curves generated in N1 Gene Samples

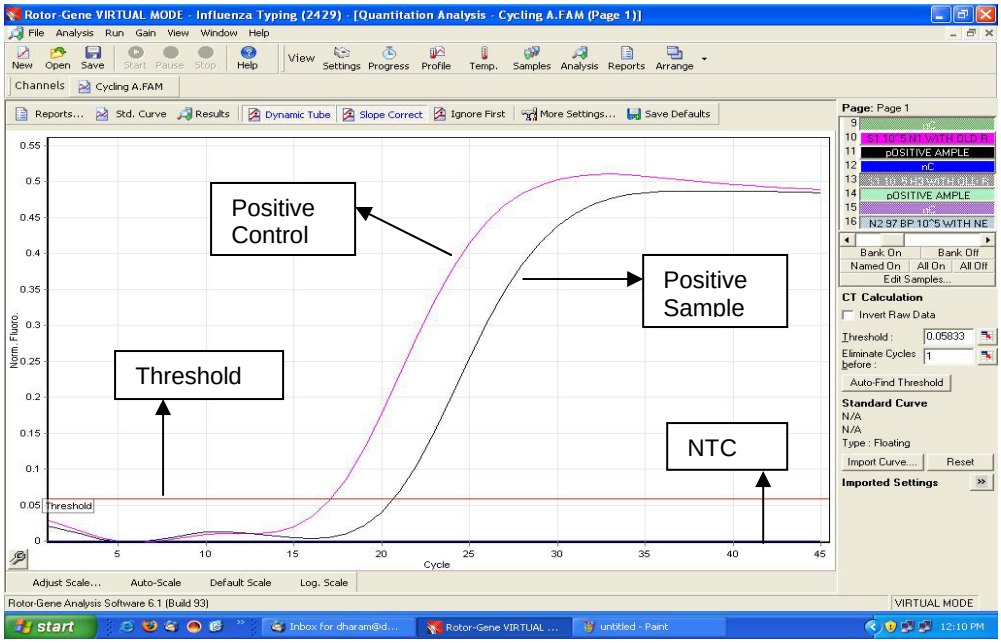


Fig. 42: Detection of the Positive Control *(***N1** *PC)* in fluorescence channel Cycling A.FAM. NTC: non-template control.

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

Example of analysed data for N1 where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standards have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure.

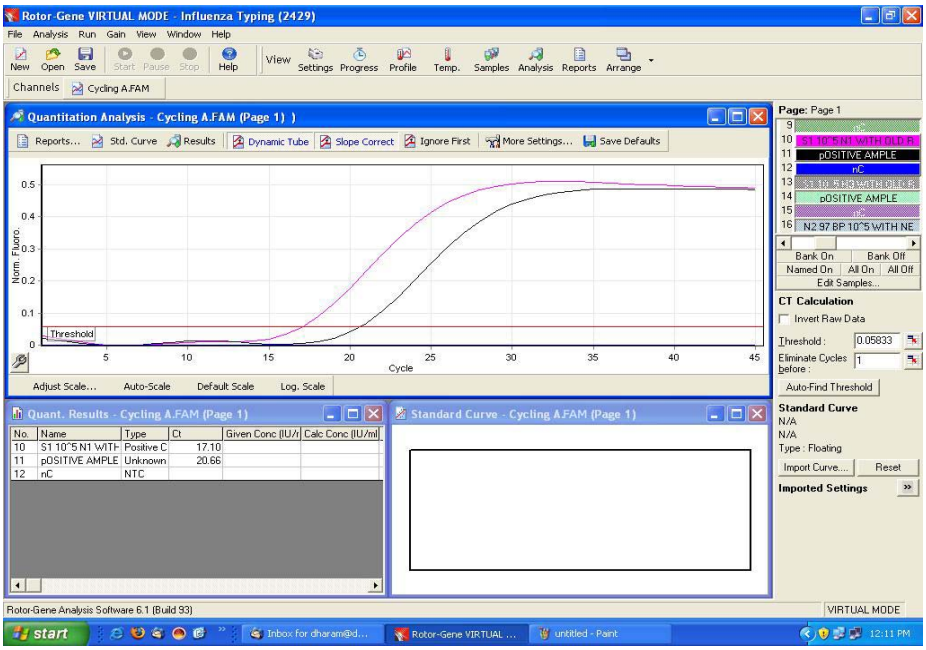


Fig. 43.

Example of the Curves generated in H3 Gene Samples

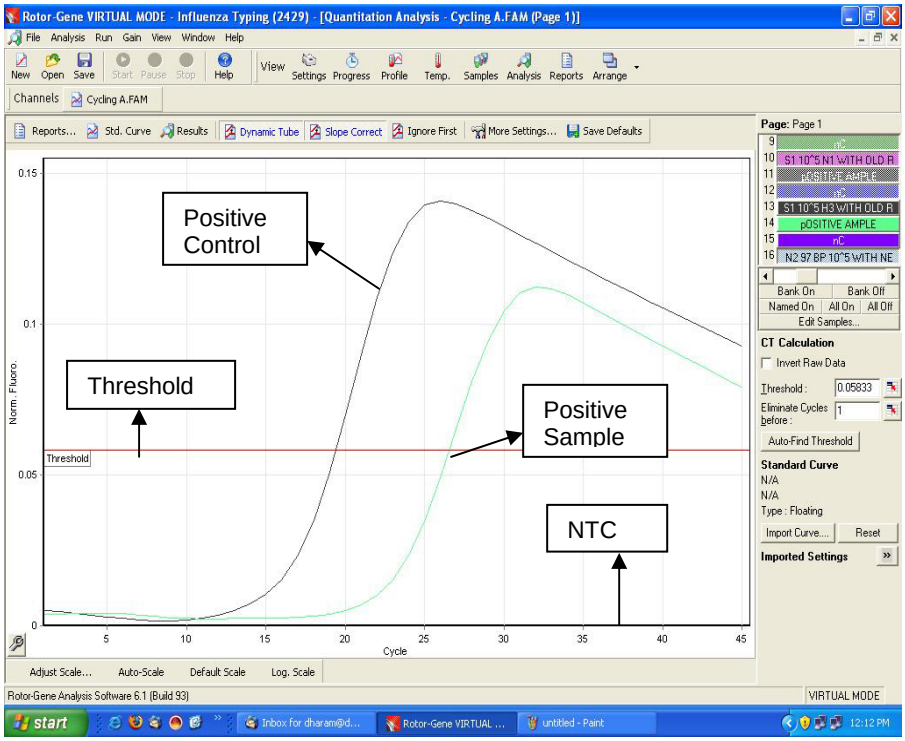


Fig. 44: Detection of the Positive Control *(H3 PC)* in fluorescence channel Cycling A.FAM. NTC: non-template control.

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

Example of analysed data for H3 where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standards have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure.

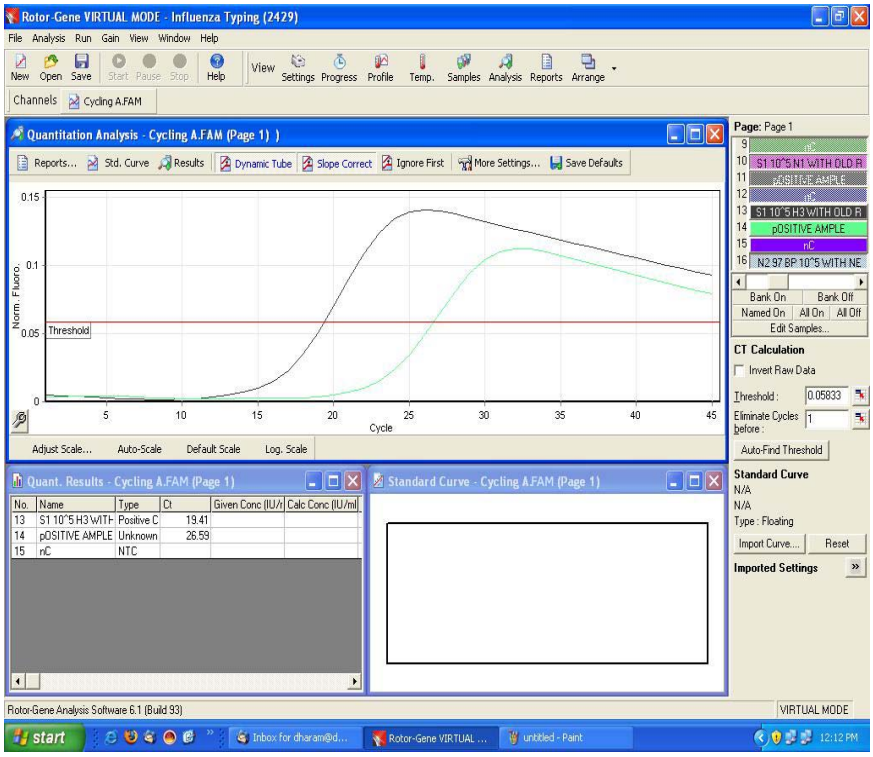


Fig. 45.

Example of the Curves generated in N2 Gene Samples

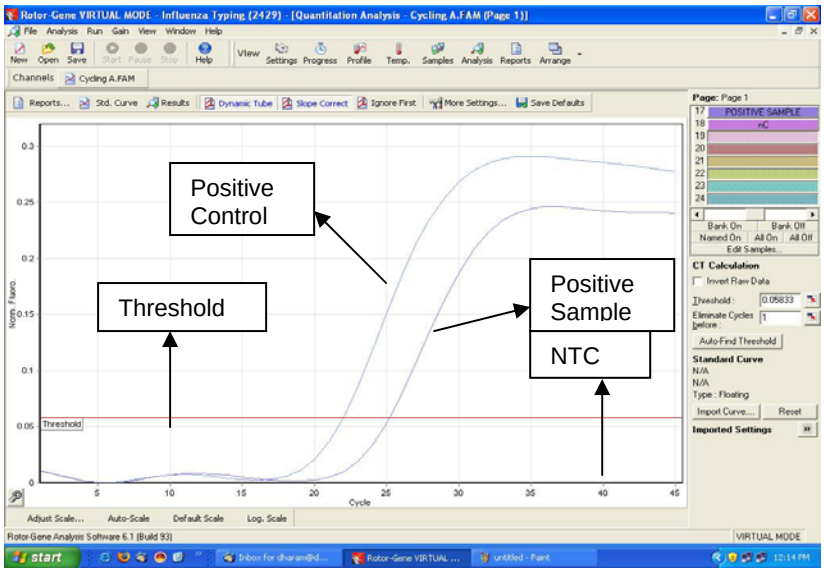


Fig. 46: Detection of the Positive Control *(N2 PC)* in fluorescence channel Cycling A.FAM. NTC: non-template control.

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

Example of analysed data for N2 where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standards have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure.

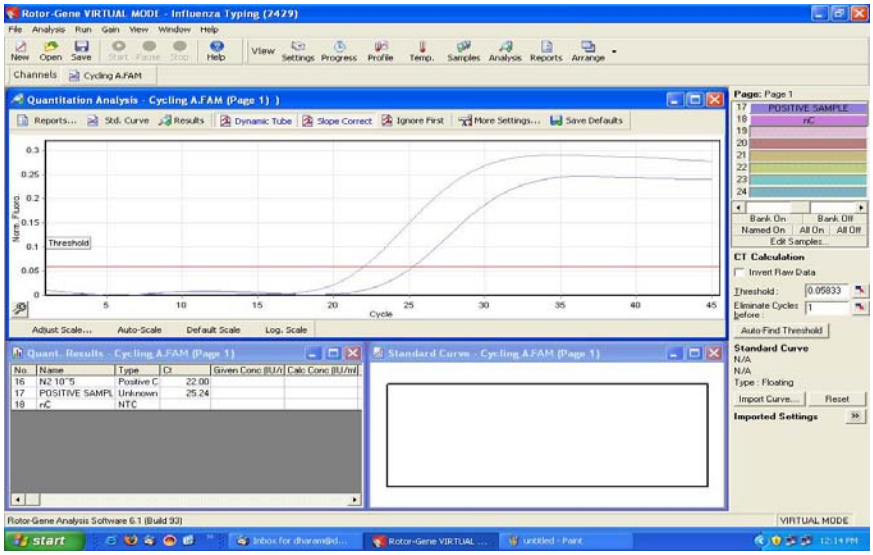


Fig. 47.

Example of the Curves generated in Infi A Gene Samples

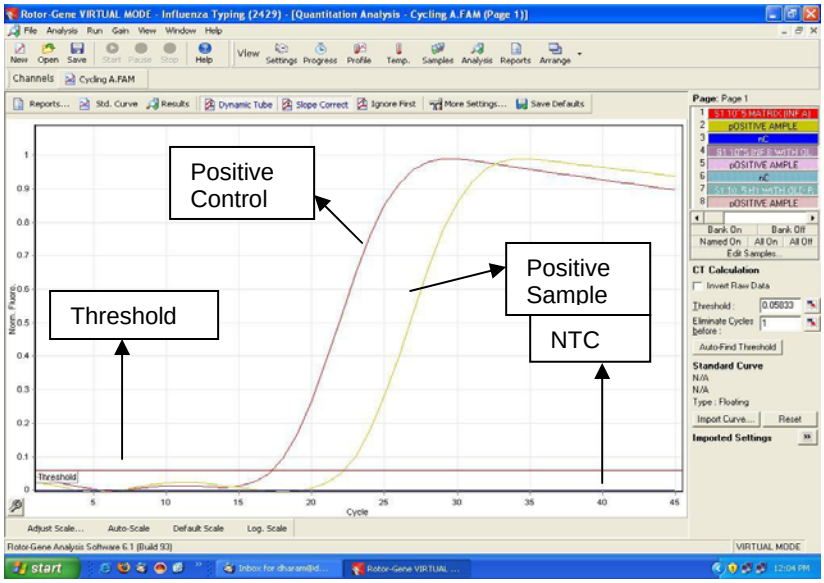


Fig. 48: Detection of the Positive Control *(Infi A PC)* in fluorescence channel Cycling A.FAM. NTC: non-template control.

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

Example of analysed data for Infi A where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standards have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure.

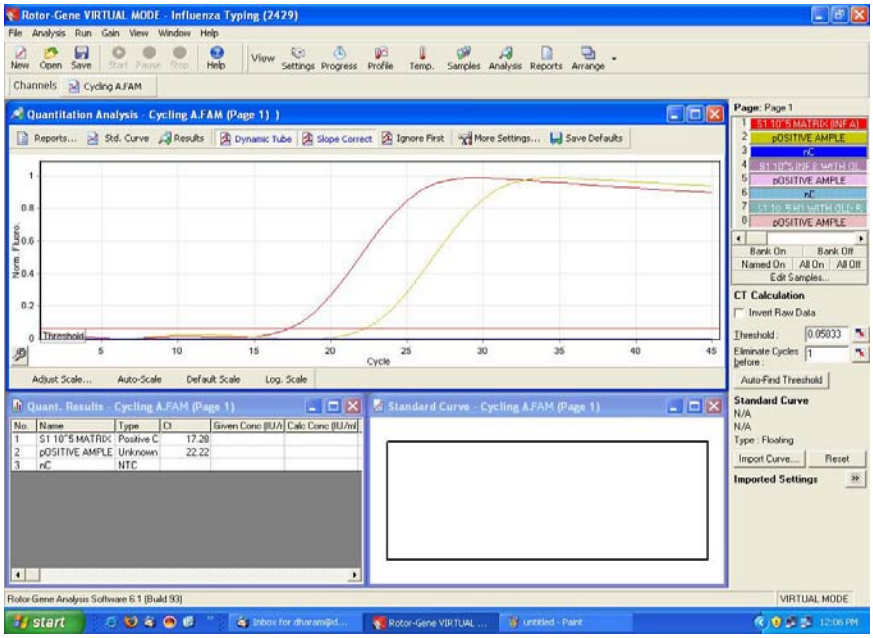


Fig. 49.

Example of the Curves generated in Infi B Gene Samples

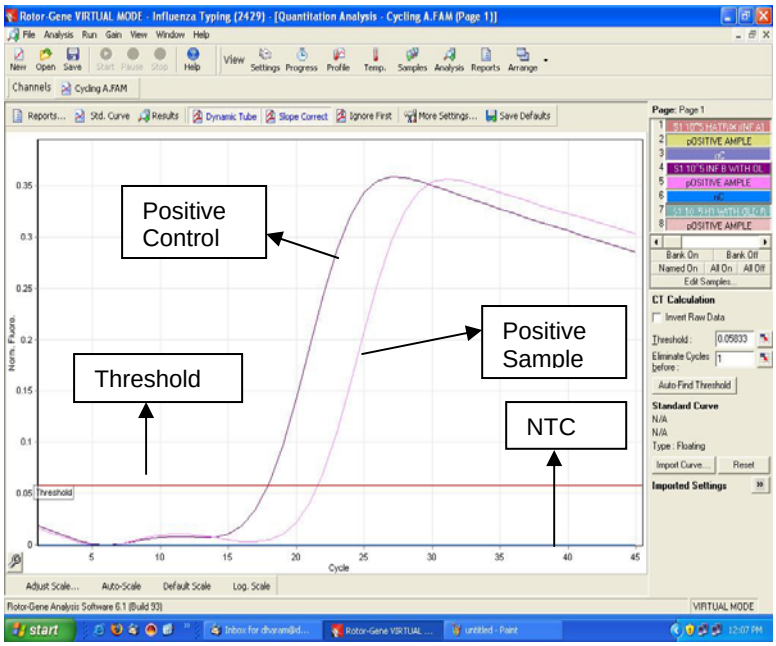


Fig. 50: Detection of the Positive Control *(Infi B PC)* in fluorescence channel Cycling A.FAM. NTC: non-template control.

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

Example of analysed data for Infi B where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standards have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure.

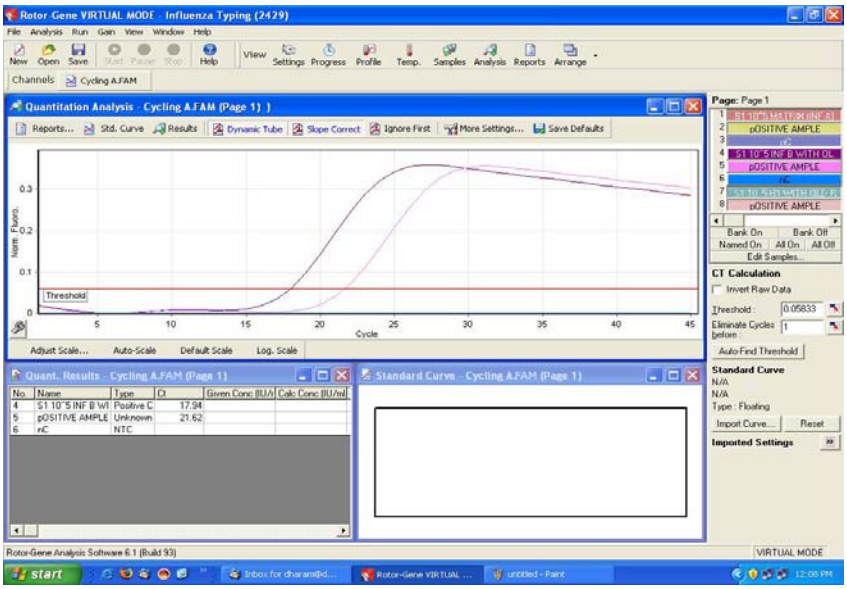


Fig. 51.

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 can be interpreted as per the table provided earlier.

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 results are summarized in the following table:

Sample form	H1	N1	Influenza A	Influenza B	H3	N2	Remarks
GREEN	Signal Detected	Signal Detected	Signal Detected	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	+ Positive for H1N1 Virus & Influenza Group A Virus
GREEN	Signal Detected	Signal Not Detected but signal detected in YELLOW	Signal Detected	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Detected	+ Positive for H1N2 Virus & Influenza Group A Virus
GREEN	Signal Detected	Signal Not Detected but signal detected in YELLOW	Signal Detected	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Positive for Influenza A Group Virus & H1 but not H1N1, H1N2
GREEN	Signal Not Detected but signal detected in YELLOW	Signal Detected	Signal Detected	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Positive for Influenza A Group Virus & N1 but not H1N1, H3N1
GREEN	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Detected	Signal Not Detected but signal detected in YELLOW	Signal Detected	Signal Detected	+ Positive for H3N2 Virus & Influenza Group A Virus
GREEN	Signal Not Detected but signal detected in YELLOW	Signal Detected	Signal Detected	Signal Not Detected but signal detected in YELLOW	Signal Detected	Signal Not Detected but signal detected in YELLOW	+ Positive for H3N1 Virus & Influenza Group A Virus
GREEN	Signal Not Detected	Signal Not Detected	Signal Detected	Signal Not Detected	Signal Not Detected	Signal Detected	Positive for Influenza A

	but signal detected in YELLOW	but signal detected in YELLOW		but signal detected in YELLOW	but signal detected in YELLOW		Group Virus & N2 but not H3N2, & H1N2
GREEN	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Detected	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Positive for Influenza A Group Virus but not H1N1, H3N2, H1N2, H3N1.
GREEN	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Detected	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Positive for Influenza B Group Virus but not Influenza A Group, H1N1, H3N1, H3N2, H1N2.
GREEN	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	Signal Not Detected but signal detected in YELLOW	-Ve Negative for Influenza A & B Group Virus & H1N1, H1N2, H3N1 & H3N2.
YELLOW	Signal Not Detected	Signal Not Detected	Signal Not Detected	Signal Not Detected	Signal Not Detected	Signal Not Detected	Possible PCR Inhibition.

Example of the Curves generated in H1 Gene Samples

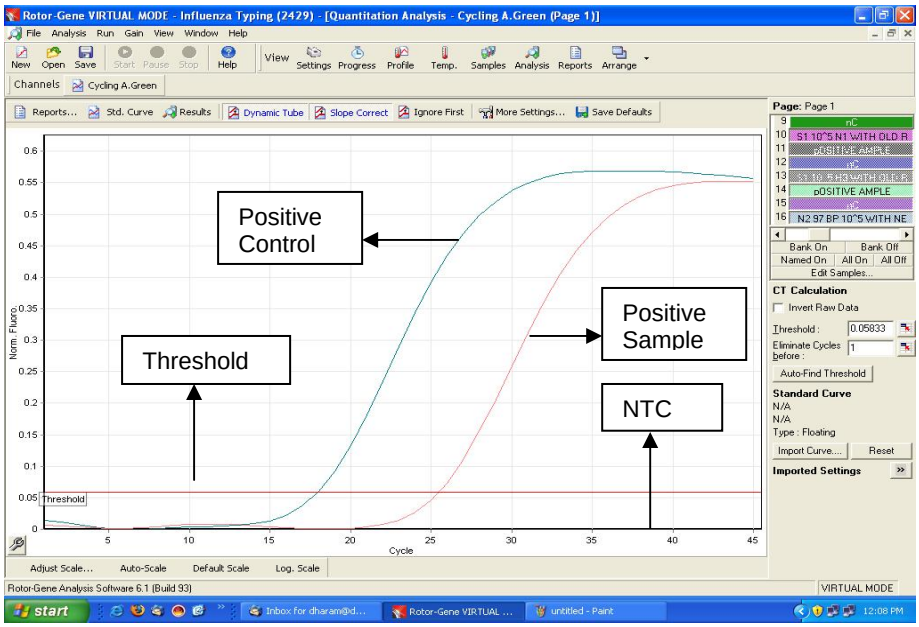


Fig. 52: Detection of the Positive Control *(***H1** *PC)* in fluorescence channel Cycling A.Green. NTC: non-template control.

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

Example of analysed data for H1 where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standrads have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure

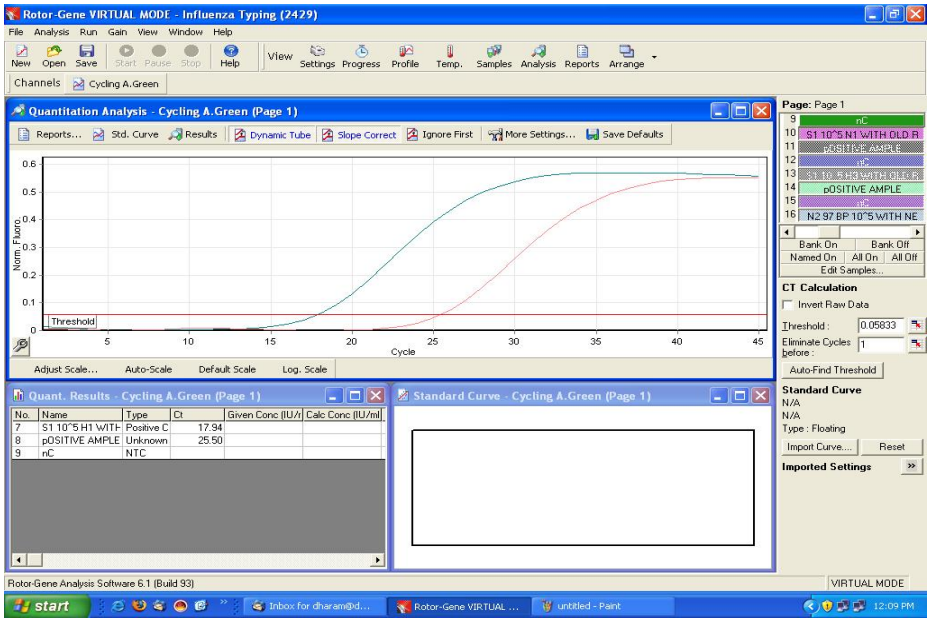


Fig. 53.

Example of the Curves generated in N1 Gene Samples

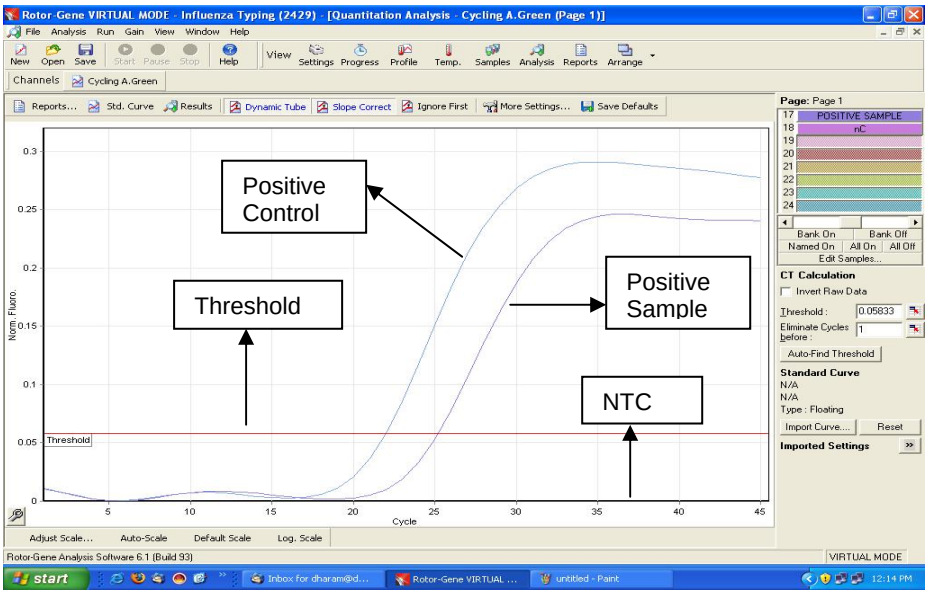


Fig. 54: Detection of the Positive Control *(**N1** PC)* in fluorescence channel Cycling A.Green. NTC: non-template control.

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

Example of analysed data for N1 where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standards have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure.

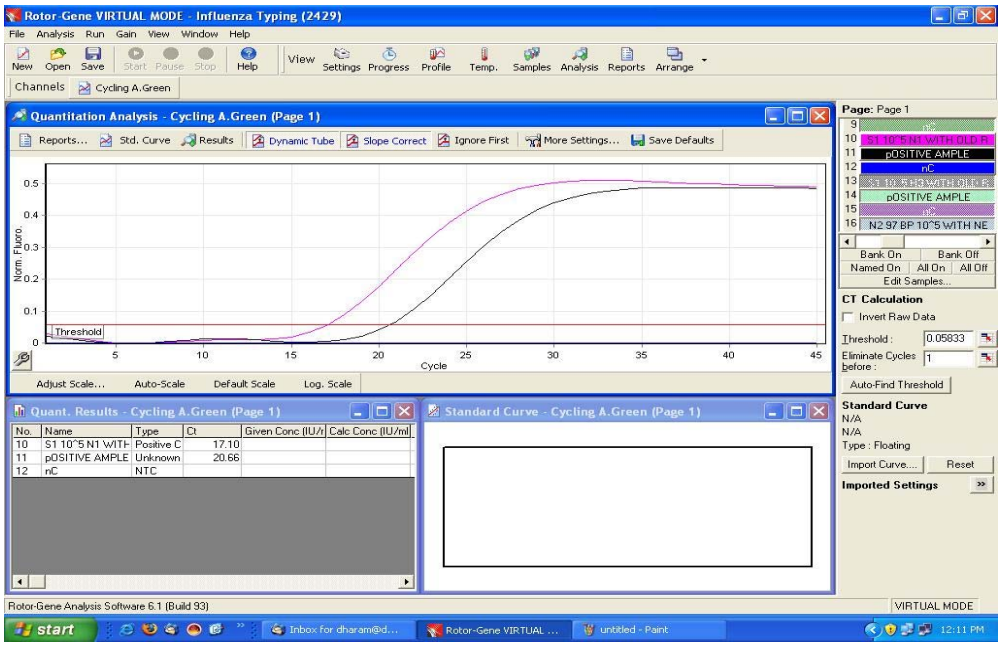


Fig. 55.

Example of the Curves generated in H3 Gene Samples

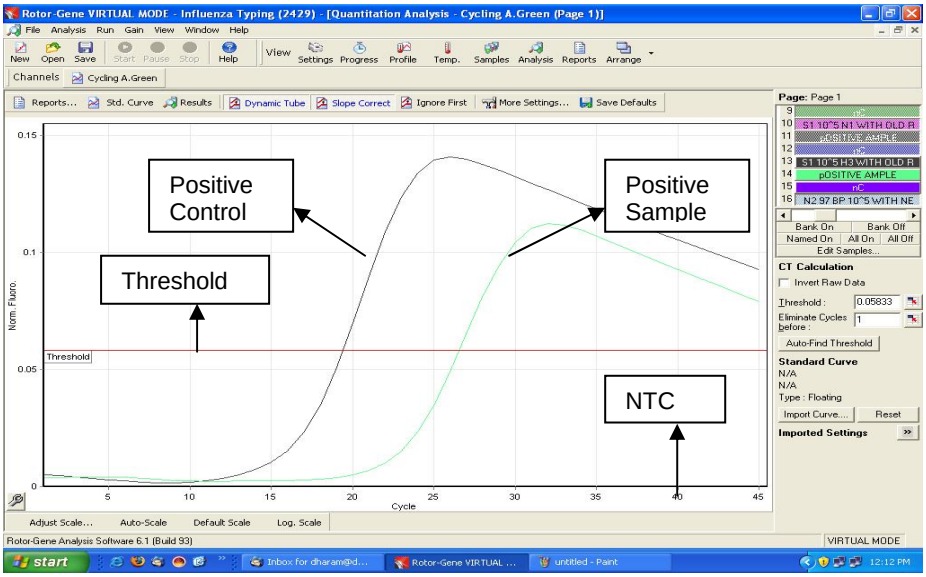


Fig. 56: Detection of the Positive Control *(H3 PC)* in fluorescence channel Cycling A.Green. NTC: non-template control.

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

Example of analysed data for H3 where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standards have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure.

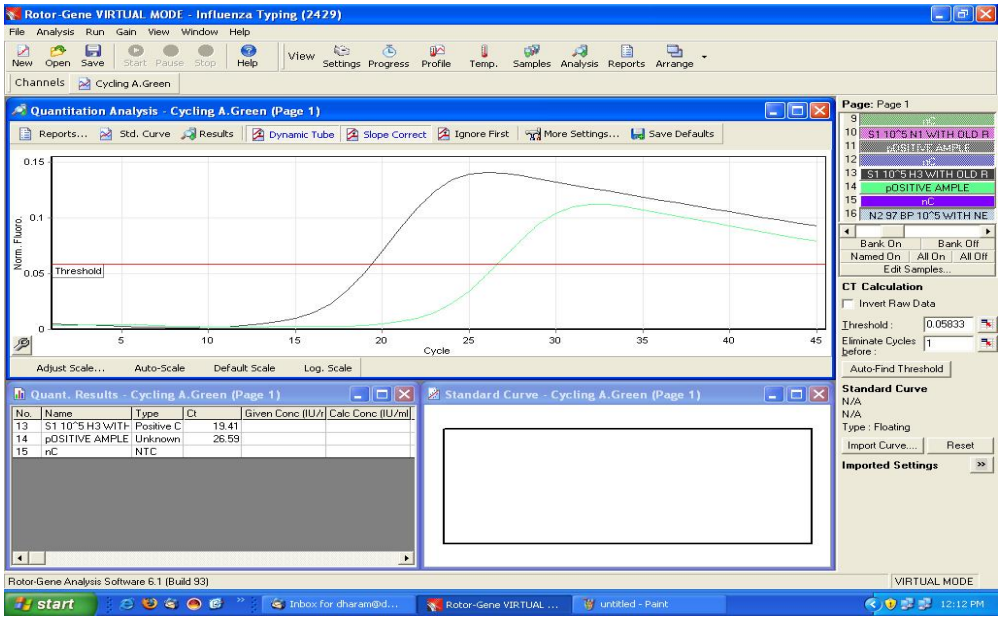


Fig. 57.

Example of the Curves generated in N2 Gene Samples

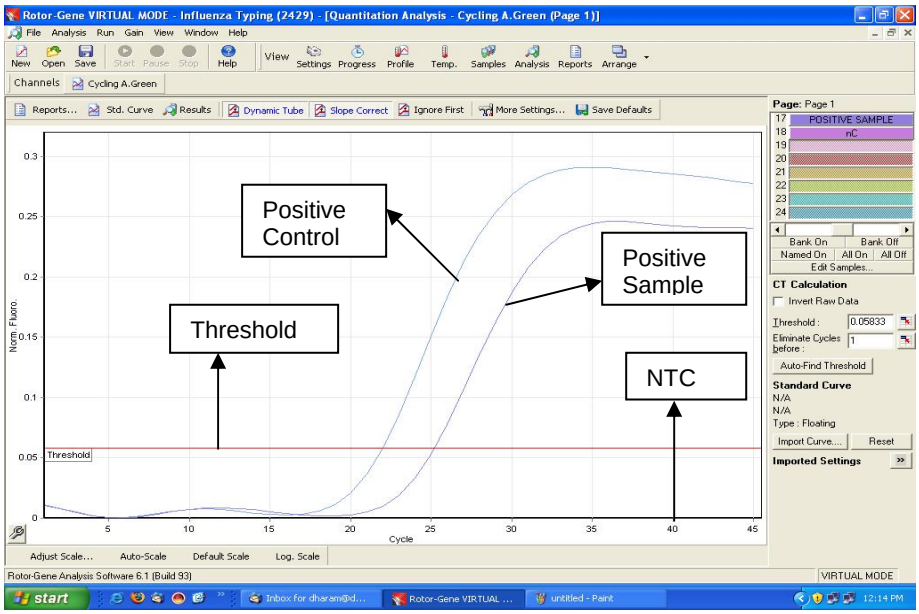


Fig. 58: Detection of the Positive Control *(N2 PC)* in fluorescence channel Cycling A.Green. NTC: non-template control.

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

Example of analysed data for N2 where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standards have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure.

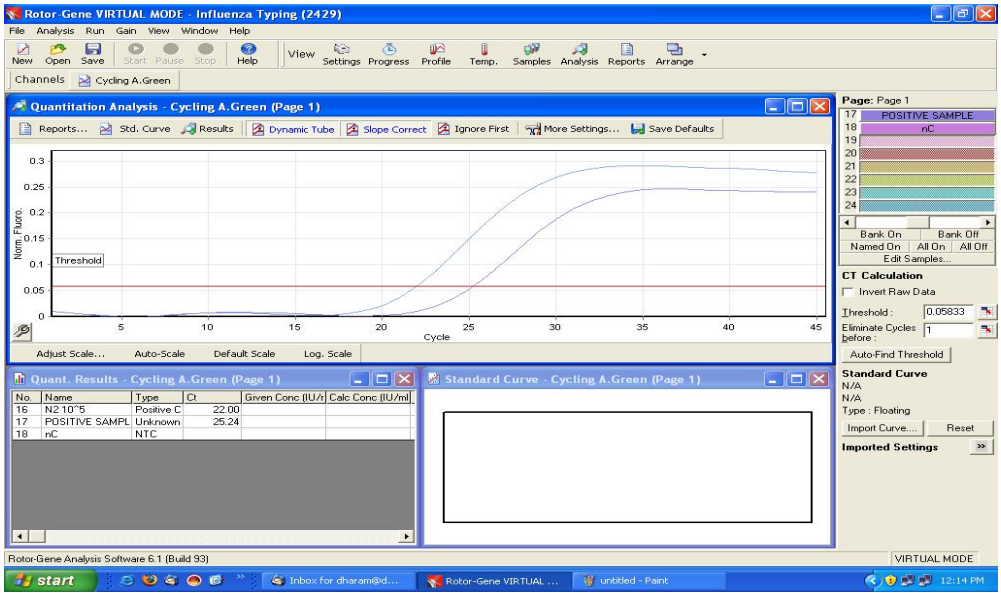


Fig. 59.

Example of the Curves generated in Infi A Gene Samples

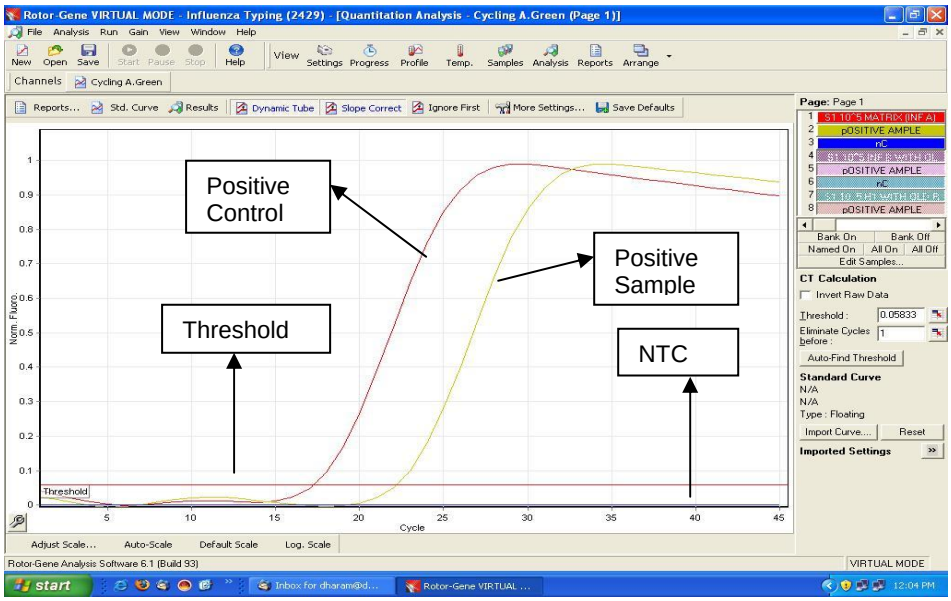


Fig. 60: Detection of the Positive Control *(Infi A PC)* in fluorescence channel Cycling A.Green. NTC: non-template control.

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

Example of analysed data for Infi A where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standards have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure.

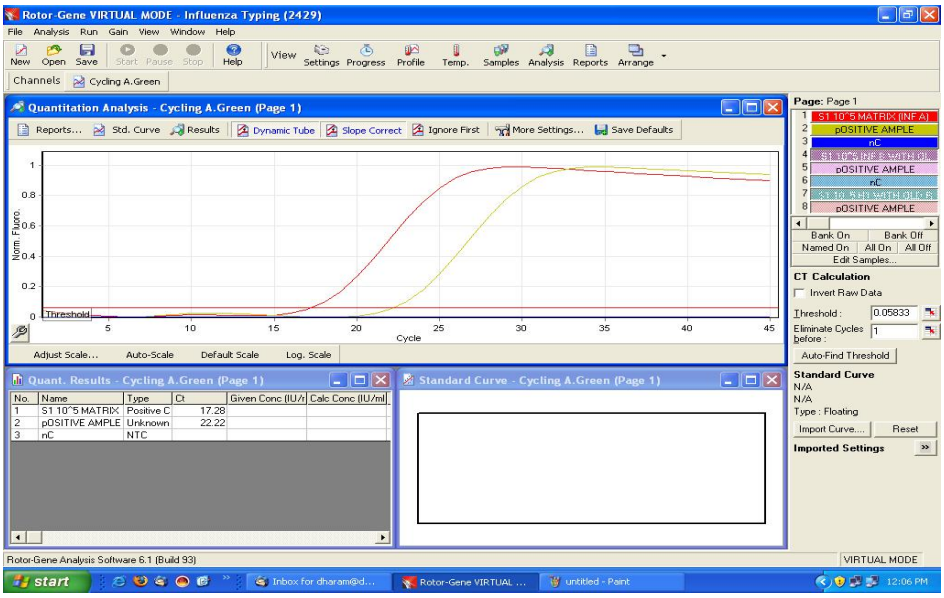


Fig. 61.

Example of the Curves generated in Infi B Gene Samples

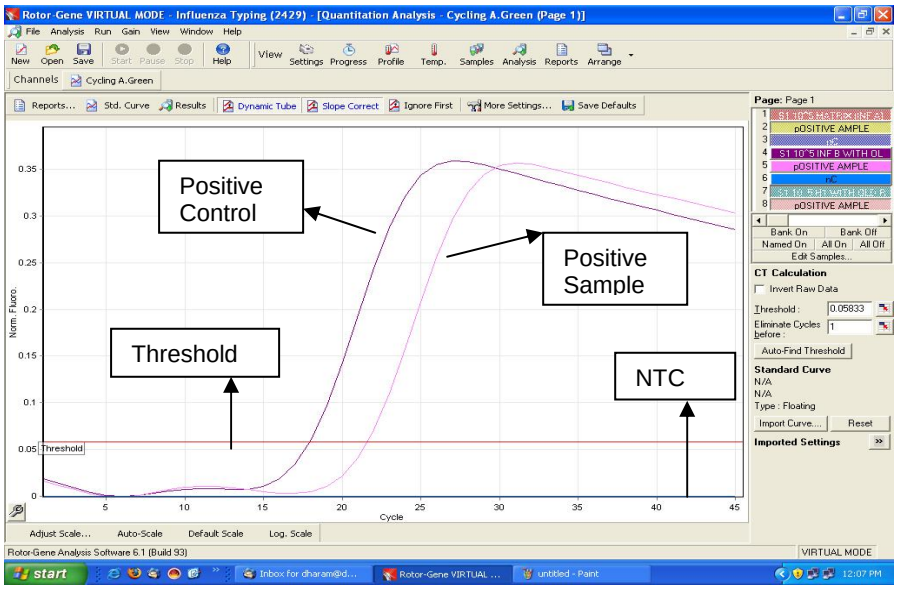


Fig. 62: Detection of the Positive Control *(Infi B PC)* in fluorescence channel Cycling A.Green. NTC: non-template control.

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

Example of analysed data for Infi B where all the amplification curves can be seen. However in the standard Curve no data will be visible as Standards have not been run hence the reaction efficiency, M value etc. cannot be seen in the below figure.

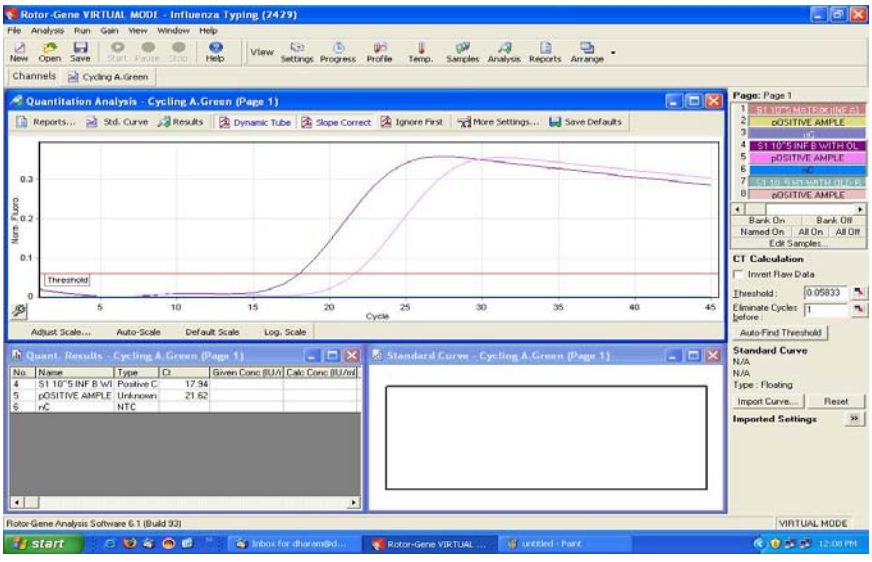


Fig. 63.

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 can be interpreted as per the table provided earlier.

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 (H1, N1, H3, N2, Infi A, & Infi B PC) 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 H1, N1, H3, N2, Infi A & Infi B Super Mix *R1, R2, R3, R4, R5 & R6* has been thawed and frozen too often.
- The H1,N1, H3, N2, Infi A & Infi B Super Mix R1, R2, R3, R4, R5 & R6 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 **H1,N1, H3, N2, Infi A & Infi B** super mix (R1, R2, R3, R4, R5 & R6).

- **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 (H1, N1, H3, N2, Infi A, & Infi B PC) 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 H1, N1, H3, N2, Infi A & Infi B Super Mix *R1, R2, R3, R4, R5 & R6* has been thawed and frozen too often.
- The H1,N1, H3, N2, Infi A & Infi B Super Mix R1, R2, R3, R4, R5 & R6 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 **H1,N1, H3, N2, Infi A & Infi B** super mix (R1, R2, R3, R4, R5 & R6).

- **The PCR was inhibited.**

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

11. Specifications

11.a Sensitivity and Reproducibility

In order to determine the sensitivity of the ***Geno-Sen's*** *Influenza Typing Real Time PCR Kit*, a dilution series has been set up of **H1, N1, H3, N2, Infi A & Infi B** RNA and analyzed with the ***Geno-Sen's*** *Influenza typing Real Time PCR Kit*. The assays were carried out on three different days in the form of 8-fold determinations. The results were determined by a probit analysis. The detection limit as determined for ***Geno-Sen's*** *Influenza Typing Real Time PCR Kit* is consistently 110 copies/ml for **H1, N1, H3, N2, Infi A & Infi B** This means that there is 95% probability that 110 copies/ml will be detected.

11.b Specificity:

In order to check the specificity of the ***Geno-Sen's*** Influenza Typing Real Time PCR kit, different RNA & DNA listed below were analyzed with ***Geno-Sen's*** Influenza Real Time PCR Kit. None of these led to a positive signal with the ***Geno-Sen's*** Influenza Typing Real Time PCR kit. Gene sequence analysis of the amplified region of **H1,N1, H3, N2, Infi A** & **Infi B** shows a pronounced homology among the various subtypes, and no homology with other RNA. Besides which utmost care has been taken in selection of the primers & probes being used in the kit.

Cytomagalovirus	Hepatitis B Virus	N. Meningitis
C. pneumonia	Hepatitis A Virus	S. Pneumonia
Dengue Virus 1-4	Hepatitis E Virus	JEV
HCV	HIV-1	West Nile Virus
HSV 1 & 2	HIV 2	H3N2
ENTEROVIRUS	H. Influenza	Influenza B

Further studies are underway on this aspect.

12. Warranty::

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

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

13. Limitations of product use:

a.) All reagents may exclusively be used for *in vitro* diagnostics.
b.) The product is to be used by personnel specially instructed and trained in for the *in-vitro* diagnostics procedures only.
c.) It is important to pipet the indicated quantities, and mix well after each reagent addition. Check pipettes regularly.
d.) Instructions must be followed Correctly in order to obtain correct results. If the user has any questions, please contact our Technical Dept. (tech@genomediagnostics.co.in or at tech@diagnosticsgenome.com).
e.) This test has been validated for use with the reagents provided in the kit. The use of other Reagents or methods, or the use of equipment not fulfilling the specifications, may render equivocal results. User is responsible for any modifications done in any of the indicated parameters. Compliance with the kit protocol is required.
f.) Detection of Viral RNA depends on the number of Viruses present in the sample, and can be affected by sample collection methods, patient-related factors (e.g. age, symptoms), or for infection stage and sample size.
g.) False negative results may be obtained due to polymerase inhibition. It is recommended to perform control reactions to distinguish between inhibition and true negatives. This is achieved by the inhibition Control included in the kit.
h.) Cross contamination between samples and exogenous DNA can only be avoided by following good laboratory practice. Instructions in this document must be strictly followed.
i.) Attention should be paid to expiration dates printed on the kit box and labels of all components. Do not use expired components.

If you have any further questions or problems, please contact our technical support at tech@genomediagnostics.co.in or at tech@diagnosticsgenome.com

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

S.NO.	PRODUCT
1	HIV-1 RG quantitative Real time PCR kit.
2	HBV RG quantitative Real time PCR kit.
3	HCV RG quantitative Real time PCR kit.
4	HCV Genotyping 1/2/3/4 RG qualitative Real time PCR kit.
5	HEV RG quantitative Real time PCR kit.
6	HAV RG quantitative Real time PCR kit.
7	JEV RG quantitative Real time PCR kit.
8	ENTEROVIRUS RG quantitative Real time PCR kit.
9	DENGUE RG quantitative Real time PCR KIT
10	HSV 1 & 2 RG quantitative Real time PCR kit.
11	CMV RG quantitative Real time PCR kit.
12	Hanta Virus RG quantitative Real time PCR kit.
13	Measles Virus RG quantitative Real time PCR kit.
14	West Nile Virus RG quantitative Real time PCR kit.
15	H5 N1 (Bird Flu) RG quantitative Real time PCR kit.
16	Chikungunya RG quantitative Real time PCR kit.
17	TTV RG quantitative Real time PCR kit.
18	SARS RG quantitative Real time PCR kit.
19	JC/BK Virus RG quantitative Real time PCR kit.
20	MTb Complex RG quantitative Real time PCR kit.
21	MTb Complex /MOTT RG qualitative 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.**
44.	**Her-2 neu DNA detection & quantification Real Time PCR Kit.**
45.	**Her-2 neu RNA detection & quantification Real Time PCR Kit.**
46.	**H1N1 detection & quantification Real Time PCR Kit.**
47.	**H1N1 & Infi A detection & quantification Real Time PCR Kit.**

48.	**Infi A & Infi B detection & quantification Real Time PCR Kit.**
49.	**H1, N1, H3 N2, Infi A & Infi B detection & Typing Real Time PCR Kit.**
50.	**H3N2 detection & quantification Real Time PCR Kit.**
51.	**H3N2, Infi A, detection & quantification Real Time PCR Kit.**
52.	**H1N1, Mexican H1 & Infi A detection & quantification Real Time PCR Kit.**
53.	**EBV detection & quantification Real Time PCR Kit.**
54.	**Jak-2 mutation detection Real Time PCR Kit.**
55.	**h-Tert quantification Real Time PCR Kit.**

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