Geno-Sen's

CHIKUNGUNYA VIRUS

Real Time PCR Kit

Quantitative

IVD

PACK INSERT

CE

Genome Diagnostics Pvt. Ltd.

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

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EMERGO EUROPE
Prinsessegracht 20,2514 AP , The Hague
The Netherlands

Table of Contents

1. Contents of the Kit:

Color Code	Contents	REF 9111074 100 rxns	REF 9111075 50 rxns	REF 9111076 25 rxns
R1 Blue	Chikungunya 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
CHIK-S1 Red	Chikungunya *Standard 1* *1 X 10^5 copies/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
CHIK-S2 Red	Chikungunya *Standard 2* *1 X 10^4 copies/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
CHIK-S3 Red	Chikungunya *Standard 3* *1 X 10^3 copies/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
CHIK-S4 Red	Chikungunya *Standard 4* *1 X 10^2 copies/µl*	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
CHIK-S5 Red	Chikungunya *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.

Chikungunya Information

Application

Chikungunya (in the Makonde language "that which bends up") virus (CHIKV) is an insect-borne virus, of the genus *Alphavirus*, that is transmitted to humans by virus-carrying *Aedes* mosquitoes. There have been recent breakouts of CHIKV associated with severe illness.

CHIKV infection causes an illness with symptoms similar to dengue fever, with an acute febrile phase of the illness lasting only two to five days, followed by a prolonged arthralgic disease that affects the joints of the extremities. The pain associated with CHIKV infection of the joints persists for weeks or months, or in some cases years.

Signs and symptoms

The incubation period of chikungunya disease ranges from one to twelve days, usually two to three. Its symptoms include a fever up to 40 °C (104 °F), a petechial or maculopapular rash of the trunk and occasionally the limbs, and arthralgia or arthritis affecting multiple joints. Other nonspecific symptoms can include headache, conjunctivitis, slight photophobia and partial loss of taste.

Typically, the fever lasts for two days and then ends abruptly. However, other symptoms—namely joint pain, intense headache, insomnia and an extreme degree of prostration—last for a variable period; usually for about five to seven days. Patients have complained of joint pains for much longer time periods; some as long as two years, depending on their age.

Diagnosis

Common laboratory tests for chikungunya include **Real Time PCR**, virus isolation, and serological tests.

- **Real Time PCR** is the best method to amplify several chikungunya-specific genes from serum/plasma/whole blood. Results can be determined in 2-3 hours.
- Virus isolation provides the most definitive diagnosis, but takes one to two weeks for completion and must be carried out in biosafety level 3 laboratories. The technique involves exposing specific cell lines to samples from whole blood and identifying chikungunya virus-specific responses.
- Serological diagnosis requires a larger amount of blood than the other methods, and uses an ELISA assay to measure chikungunya-specific IgM levels. Results require two to three days, and false positives can occur with infection via other related viruses, such as o'nyong'nyong virus and Semliki Forest virus.

Causes

Chikungunya virus is indigenous to tropical Africa and Asia, where it is transmitted to humans by the bite of infected mosquitoes, usually of the genus *Aedes*.

Chikungunya virus belongs to alphavirus genus of the Togaviridae family. It is an "Arbovirus" (Ar-arthropod, bo-borne).

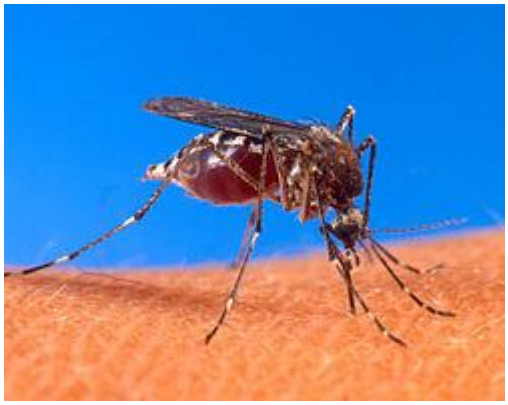
CHIK fever epidemics are sustained by human-mosquito-human transmission. The main virus reservoirs are monkeys, but other species can also be affected, including humans.

Pathophysiology

Human epithelial and endothelial cells, primarily fibroblasts and monocyte-derived macrophages, are susceptible to infection. Lymphoid and monocytoid cells, primary lymphocytes and monocytes and monocyte-derived dendritic cells are not susceptible to infection. Viral entry occurs through pH-dependent endocytosis. Infection is cytopathic and associated with the induction of apoptosis in the infected cell.

Infection is highly sensitive to the antiviral activity of type I and II interferon.

Prevention



The *Aedes aegypti* mosquito biting a person.

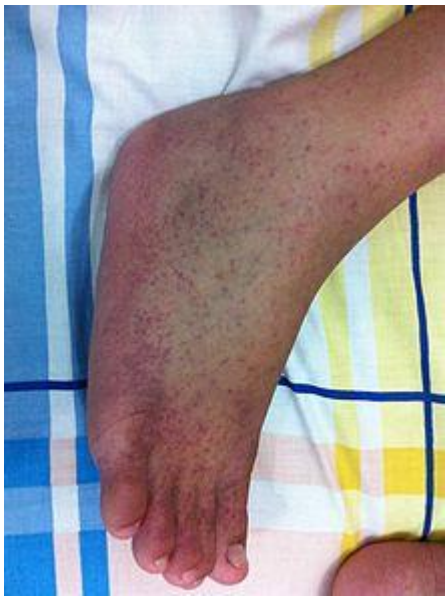
There currently is no licensed vaccine to protect against chikungunya virus. The most effective means of prevention are protection against contact with the disease-carrying mosquitoes and mosquito control. These include using insect repellents with substances such as DEET (N,N-diethyl-meta-toluamide; also known as N,N'-diethyl-3-methylbenzamide or NNDB), icaridin (also known as picaridin and KBR3023), PMD (p-menthane-3,8-diol, a substance derived from the lemon eucalyptus tree), or IR3535. Wearing bite-proof long sleeves and trousers (pants) also offers protection.

In addition, garments can be treated with pyrethroids, a class of insecticides that often has repellent properties. Vaporized pyrethroids (for example in mosquito coils) are also insect repellents. Securing screens on windows and doors will help to keep mosquitoes out of the house. In the case of the day-active *Aedes aegypti* and *Aedes albopictus*, however, this will have only a limited effect, since many contacts between the vector and the host occur outside.

Vaccine research

Early-stage (phases 1 and 2) clinical trials have provided evidence in humans for safety and prophylactic efficacy of candidate vaccines, but these have not been developed further due to shifting research priorities.

Treatment



Chikungunya on the right feet

There are no specific treatments for chikungunya, and no vaccine is currently available. A Phase II vaccine trial, sponsored by the US Government and published in the *American Journal of Tropical Medicine and Hygiene* in 2000, used a live, attenuated virus, developing viral resistance in 98% of those tested after 28 days and 85% still showed resistance after one year.

Chloroquine is gaining ground as a possible treatment for the symptoms associated with chikungunya, and as an anti-inflammatory agent to combat the arthritis associated with the virus. A University of Malaya study found that for arthritis-like symptoms not relieved by aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs), chloroquine phosphate (250 mg/day) has given promising results. Unpublished studies in cell culture and monkeys show no effect of chloroquine treatment on reduction of chikungunya disease. The fact sheet on chikungunya advises against using aspirin, ibuprofen, naproxen and other NSAIDs that are recommended for arthritic pain and fever.

Prognosis

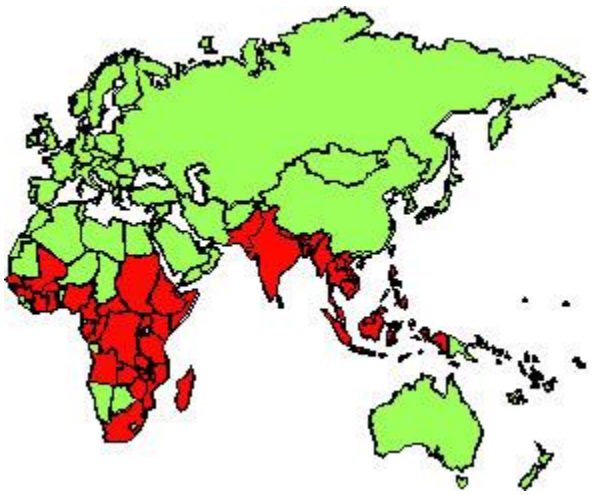
Recovery from the disease varies by age. Younger patients recover within 5 to 15 days; middle-aged patients recover in 1 to 2.5 months. Recovery is longer for the

elderly. The severity of the disease as well as its duration is less in younger patients and pregnant women. In pregnant women, no untoward effects are noticed after the infection.

Ocular inflammation from Chikungunya may present as iridocyclitis, and have retinal lesions as well.

Pedal oedema (swelling of legs) is observed in many patients, the cause of which remains obscure as it is not related to any cardiovascular, renal or hepatic abnormalities.

Epidemiology



Cases of chikungunya fever (between 1952-2006) have been reported in the countries depicted in red on this map.

Chikungunya virus is an alphavirus closely related to the o'nyong'nyong virus, the Ross River virus in Australia, and the viruses that cause eastern equine encephalitis and western equine encephalitis.

Three genotypes of this virus have been described: West African, East/Central/South African and Asian genotypes.

Chikungunya is generally spread through bites from *Aedes aegypti* mosquitoes, but recent research by the Pasteur Institute in Paris has suggested chikungunya virus strains in the 2005-2006 Reunion Island outbreak incurred a mutation that facilitated transmission by Asian tiger mosquito (*Aedes albopictus*).

Concurrent studies by arbovirologists at the University of Texas Medical Branch in Galveston, Texas, confirmed definitively that enhanced chikungunya virus infection of *A. albopictus* was caused by a point mutation in one of the viral envelope genes (E1). Enhanced transmission of chikungunya virus by *A. albopictus* could mean an increased risk for chikungunya outbreaks in other areas where the Asian tiger mosquito is present. A recent epidemic in Italy was likely perpetuated by *A. albopictus*.

In Africa, chikungunya is spread via a sylvatic cycle in which the virus largely resides in other primates in between human outbreaks.

On 28 May 2009 in Changwat Trang of Thailand where the virus is endemic, the provincial hospital decided to deliver by Caesarean section a male baby from his chikungunya-infected mother, Khwanruethai Sutmueang, 28, a Trang native, to prevent mother-fetus virus transmission. However, after delivering the baby, the physicians discovered the baby was already infected with the virus, and put him into intensive care because the infection had left the baby unable to breathe by himself or to drink milk. The physicians presumed the virus might be able to be transmitted from a mother to her fetus, but without laboratory confirmation.

Viral evolution

This virus was first identified in Tanzania in the 1953 appears to have evolved from an alphavirus ~1713 AD (95% credible interval: 1573 - 1843) in Africa. The East/Central/South African (ECSA) and Asian strains diverged within the last 150 years (95% HPD: 1879 to 1927). The extant ECSA strains - which include the first isolate of this virus - evolved between 1924 and 1943.

The Asian group split into two clades: an Indian lineage which has probably become extinct and a Southeast Asian lineage. The Southeast Asian lineage spread from Thailand to Indonesia; and then to the Philippines and more recently Malaysia. The recent Indian Ocean basin outbreak that began in 2004 appears to have originated from the ECSA group back in 2002 (95% credible interval: December 2001 to December 2003).

The estimated overall mutation rate is 4.33×10^{-4} nucleotide substitutions per site per year - a rate similar to those found in other viruses with RNA genomes.

History

The word *chikungunya* is thought to derive from a description in the local Makonde dialect, meaning "that which bends up", of the contorted posture of patients afflicted with the severe joint pain and arthritic symptoms associated with this disease. The disease was first described by Marion Robinson and W.H.R. Lumsden in 1955, following an outbreak in 1952 on the Makonde Plateau, along the border between Mozambique and Tanganyika (the mainland part of modern day Tanzania).

According to the initial 1955 report about the epidemiology of the disease, the term *chikungunya* is derived from the Makonde root verb *kungunyala*, meaning to dry up or become contorted. In concurrent research, Robinson glossed the Makonde term more specifically as "that which bends up". Subsequent authors apparently overlooked the references to the Makonde language and assumed the term derived from Swahili, the *lingua franca* of the region. The erroneous attribution of the term as a Swahili word has been repeated in numerous print sources. Many other erroneous spellings and forms of the term are in common use including "chicken guinea", "chicken gunaya," and "chickengunya".

Since its discovery in Tanganyika, Africa, in 1952, chikungunya virus outbreaks have occurred occasionally in Africa, South Asia, and Southeast Asia, but recent outbreaks have spread the disease over a wider range.

The first recorded outbreak of this disease may have been in 1779. This is in agreement with the molecular genetics evidence that suggests it evolved around the year 1700.

The *Geno-Sen's* ***C***hikungunya 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: Serum, plasma, whole blood, C.S.F, Cell cultures.

3. 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

4. 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)

5. 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.

6. Description Of the Product.

The ***Geno-Sen's C*hikungunya** *PCR* Reagents constitute a ready to use system for detection and quantification of ***C*hikungunya** 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 ***C*hikungunya** Structural polyprotein gene which covers gene sequences for both the African and Asian lineage strains. A part of the structural polyprotein gene is amplified to generate 87bp amplified product and for the direct detection of the specific amplicon in fluorescence channel Cycling A.FAM/GREEN of the *Rotor Gene 2000/3000/6000 & the Reference gene on* Cycling A. JOE/YELLOW External positive Standards (CHIK S 1-5) are supplied which allow the determination of the gene load. For further information, please refer to section 8.c Quantitation.

7. 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	***Geno Sen's***® Viral RNA Extraction Mini Kit (Columns based)	98001 or 98002	Genome Diagnostics Pvt. Ltd. India.

Culture supernatants.			

The ***Geno Sen's***® ChikunGunya Real Time PCR kits 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.

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 **Chikungunya** *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 *(***Chikungunya** *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

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 *(CHIKUNGUNYA 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

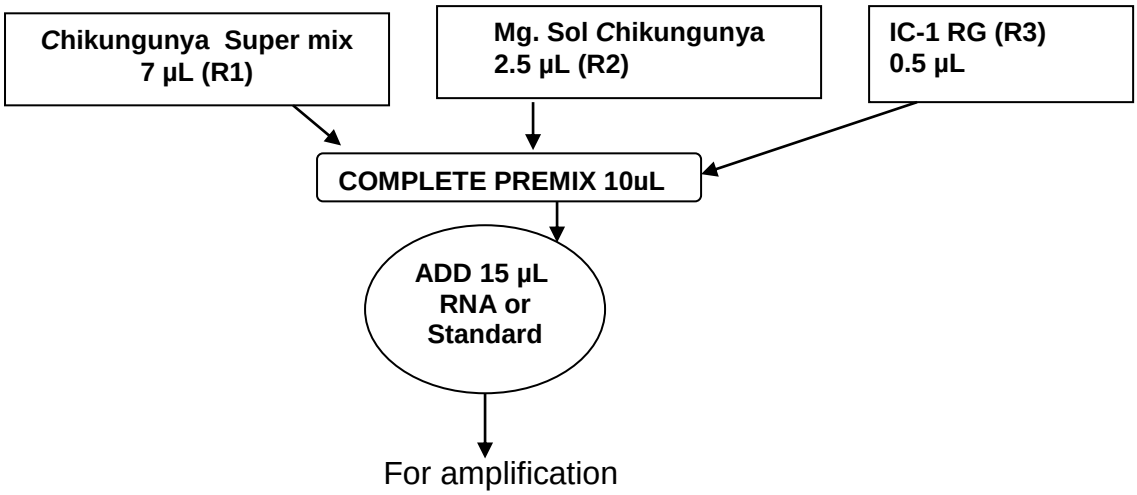


Fig. 4.

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

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

Fig. 5.

Pipette 10 µl of the Master Mix into each labelled PCR tube. Then add 15 µl of the earlier extracted RNA to each sample tube and mix well by pipeting up and down. Correspondingly, 15 µl of the Standards (CHIKUNGUNYA *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 instrument

The program for the detection of ChikunGunya Virus 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*™ 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.

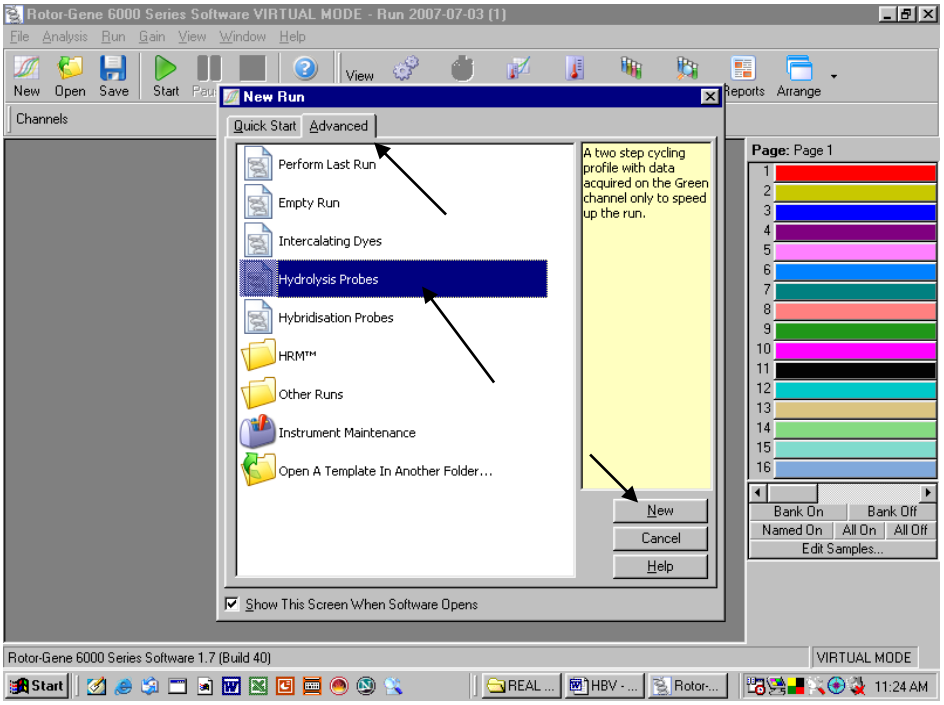


Fig. 22.

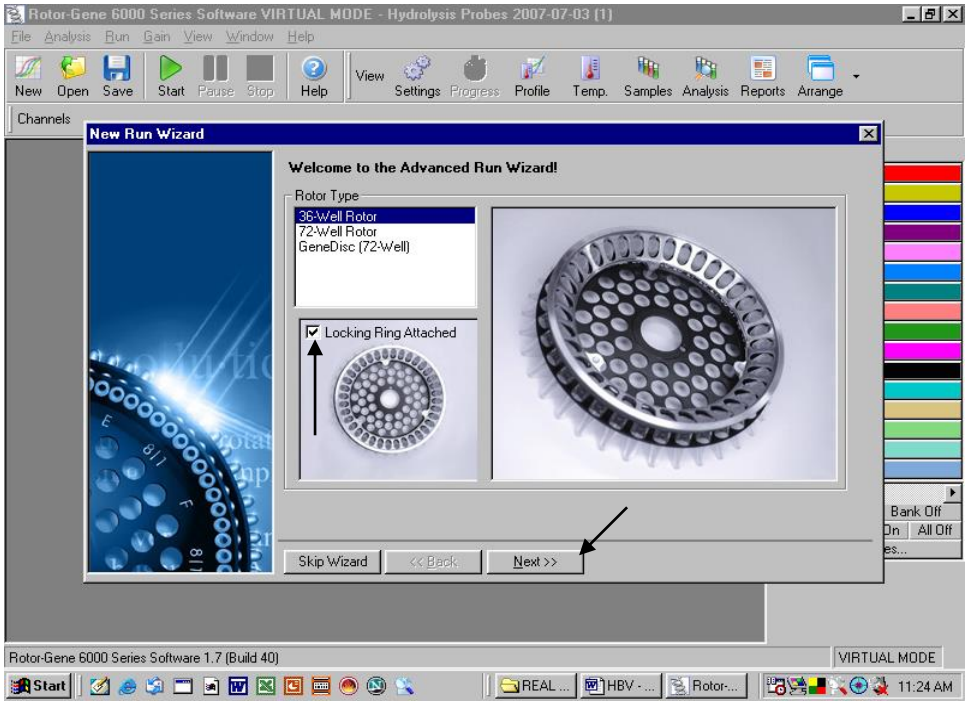


Fig. 23.

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.

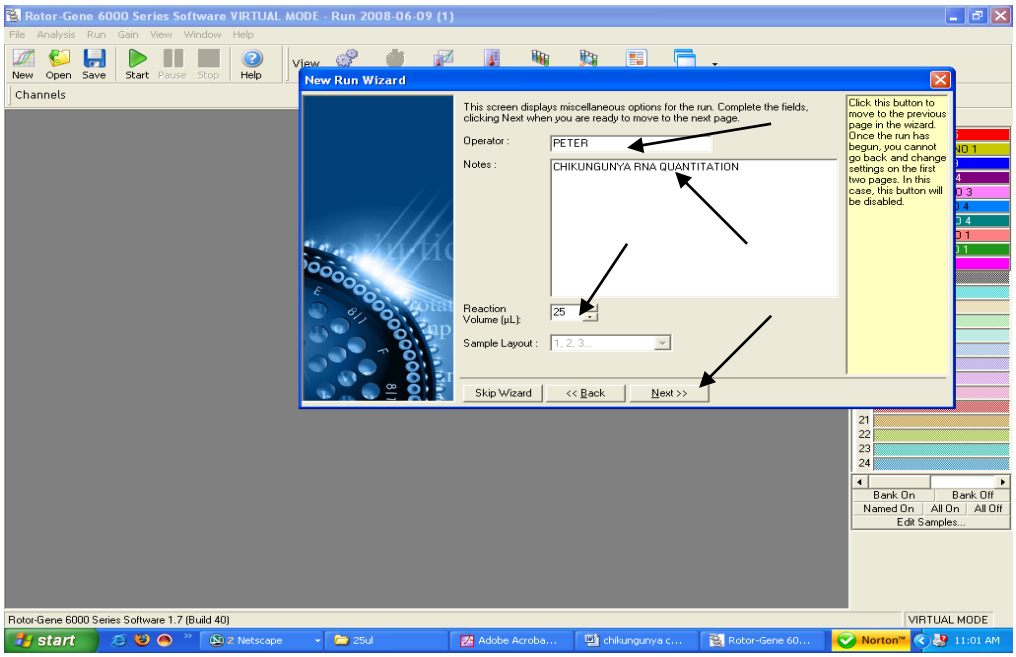


Fig. 24.

- 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.

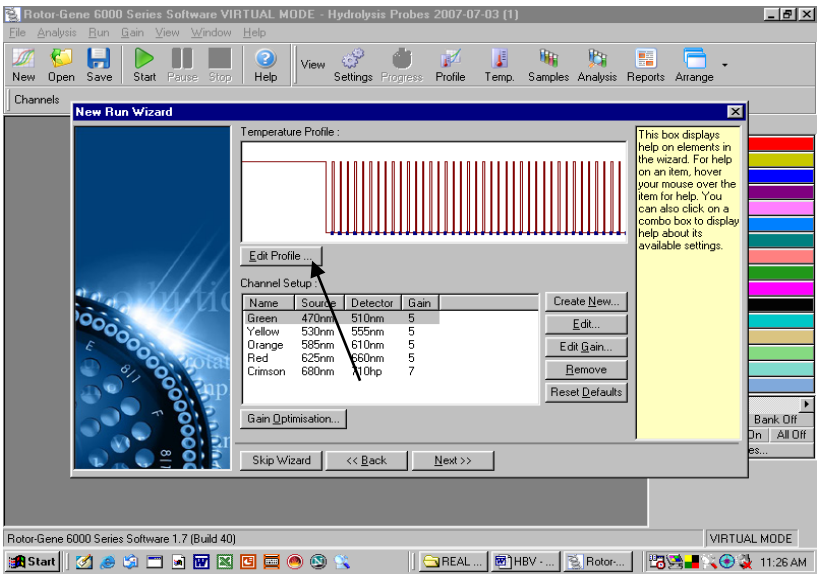


Fig. 25.

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

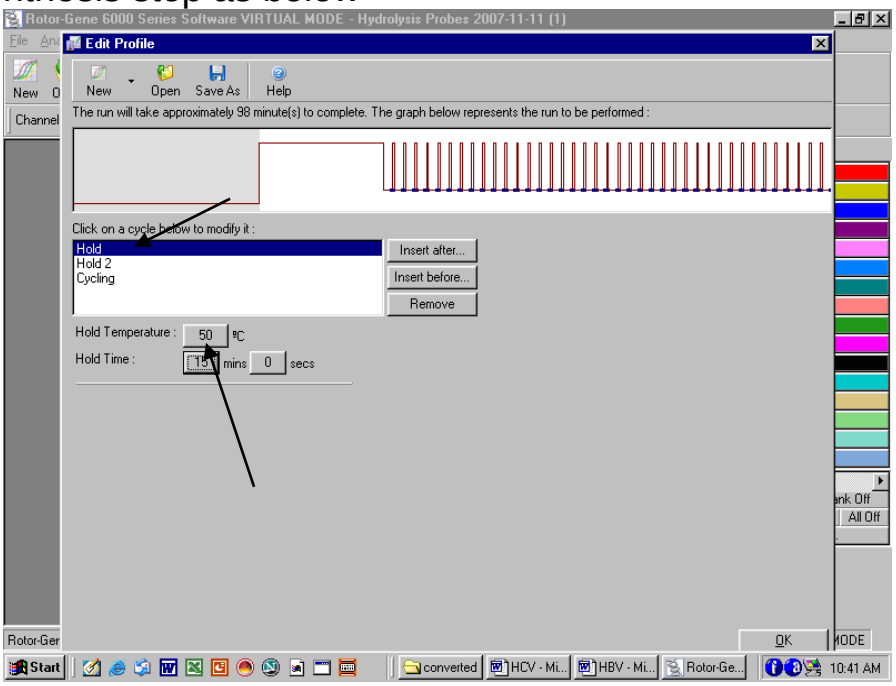


Fig. 26.

Second hold 95°C for 10 minutes as below

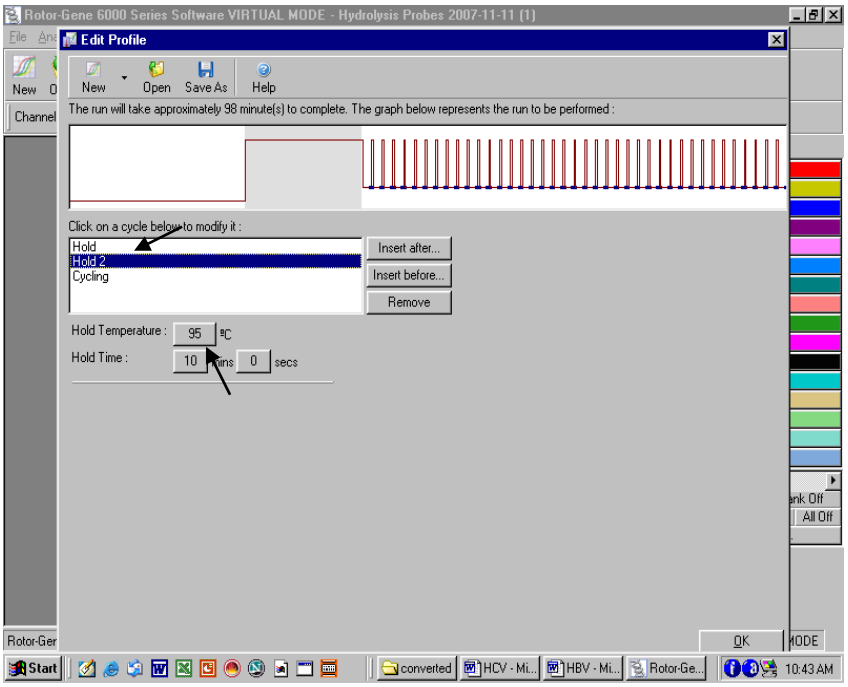


Fig. 27. Initial activation of the Hot Start enzyme. Generally the window will open with the Hold Temp as 95°C and the Hold Time as 10 minutes. In case it is different then set the same to as shown above.

j) Setting the Cycling and acquisition.

When clicked on Cycling the window will open as below.

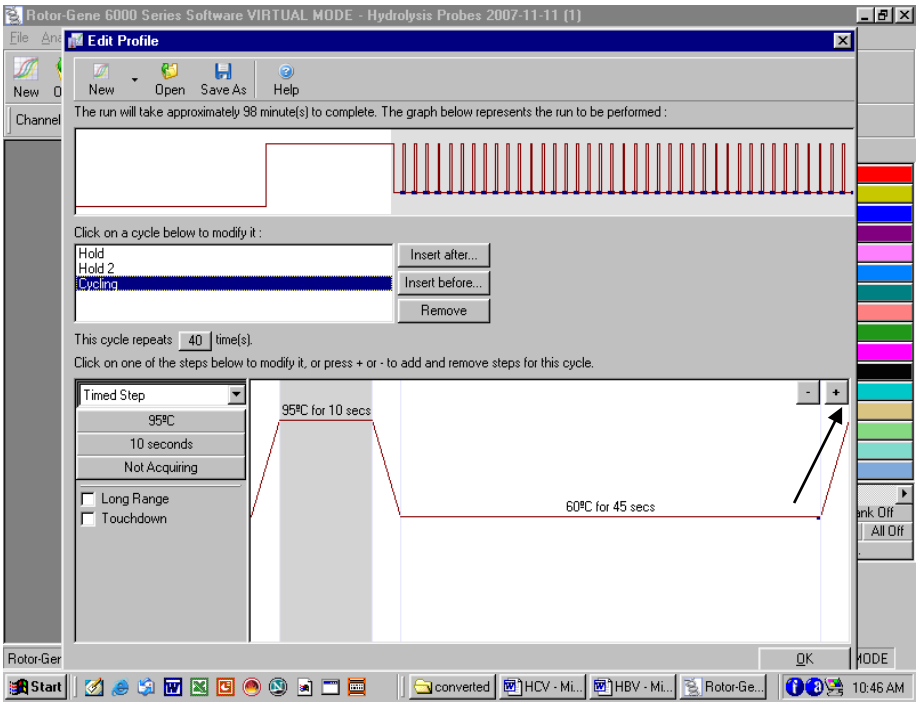


Fig. 28.

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.

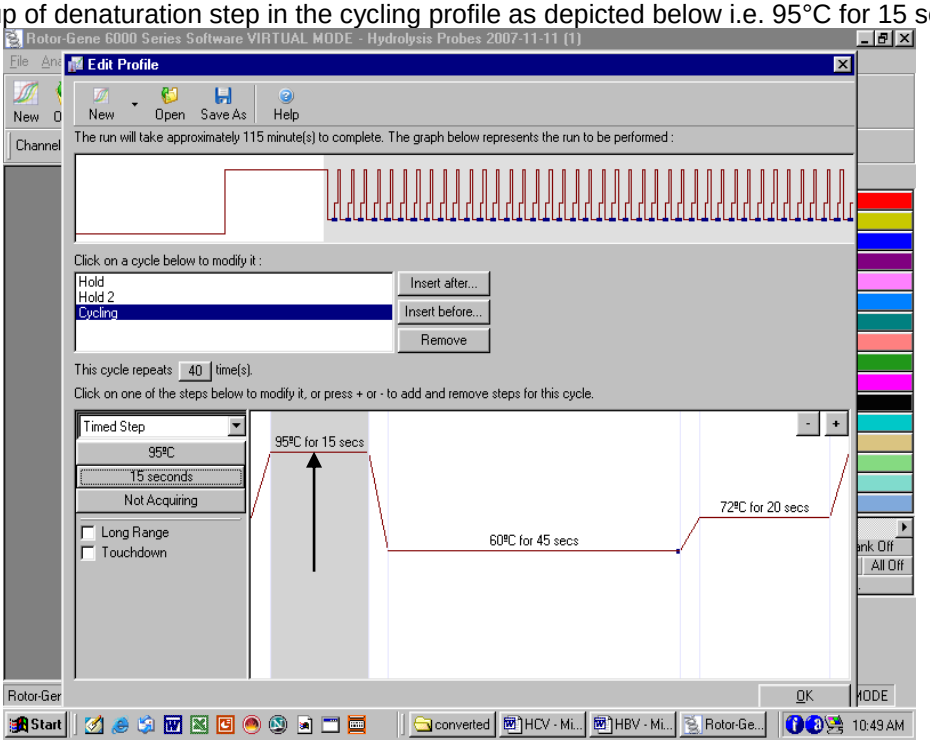


Fig. 29.

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

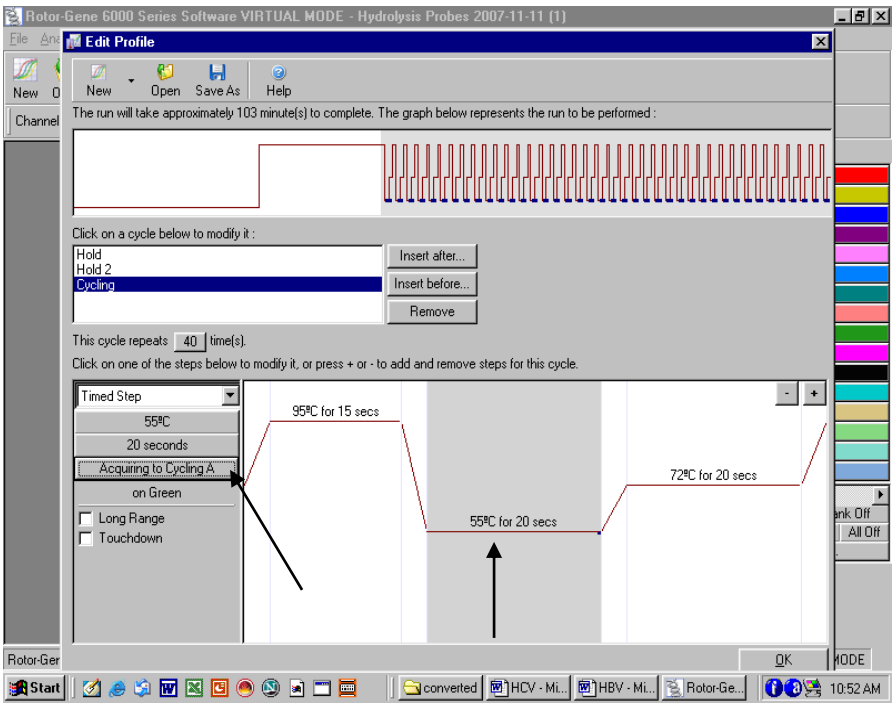


Fig. 30.

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

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

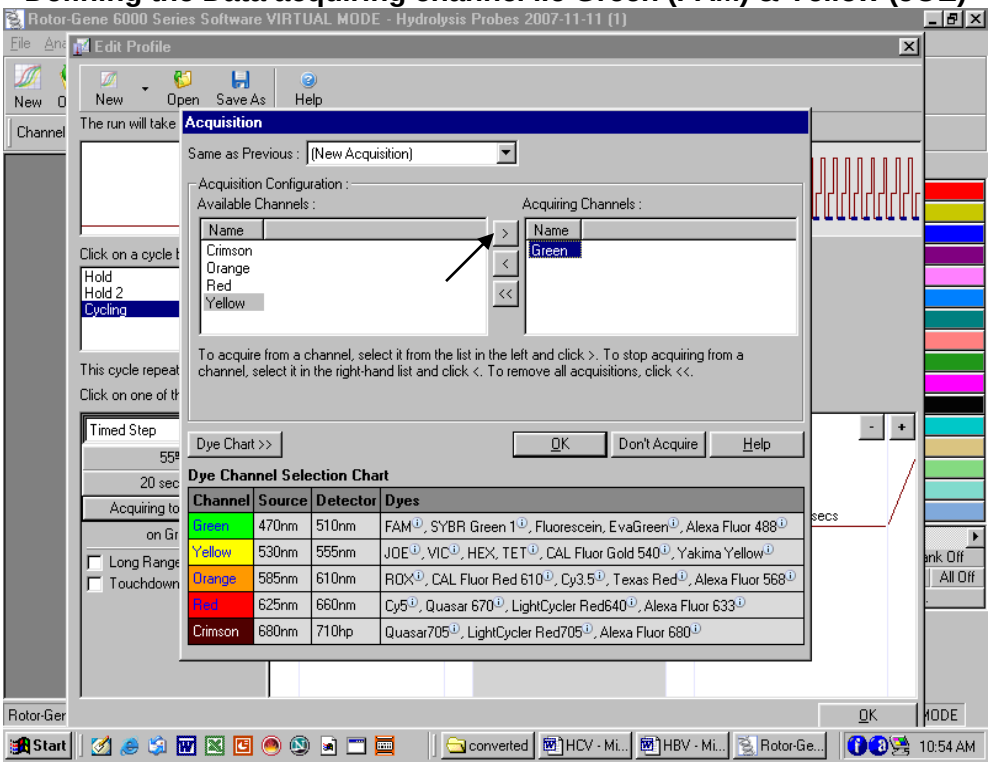


Fig. 31.

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

Confirmation of Channels as shown below.

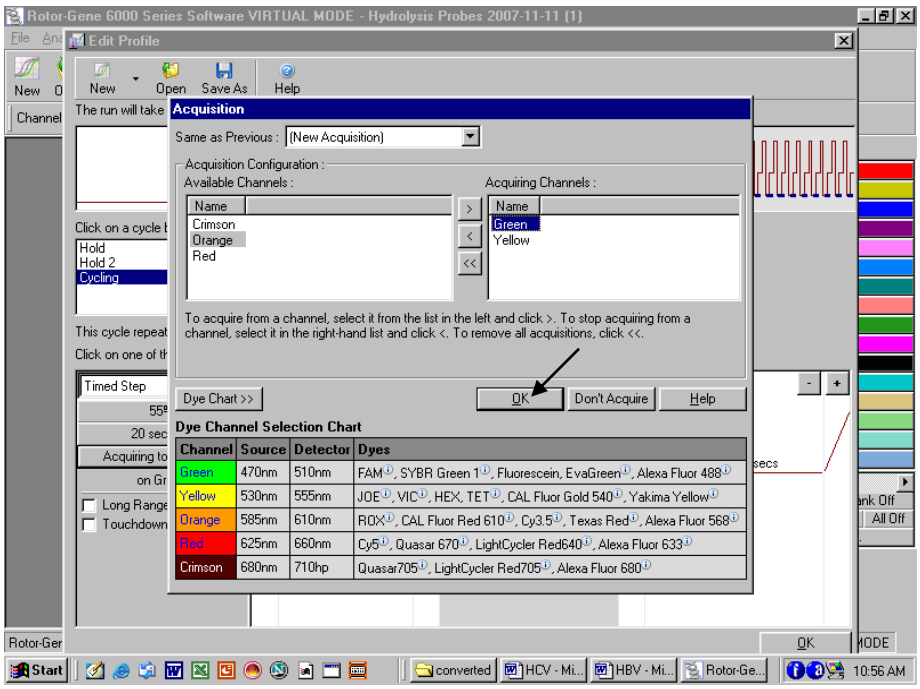


Fig. 32.

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

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

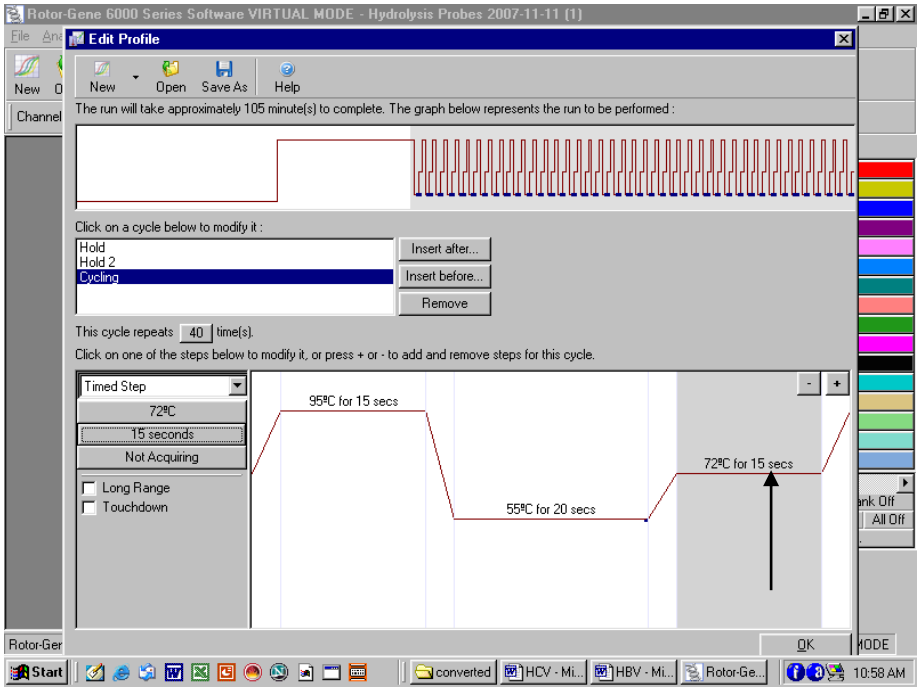


Fig. 33.

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

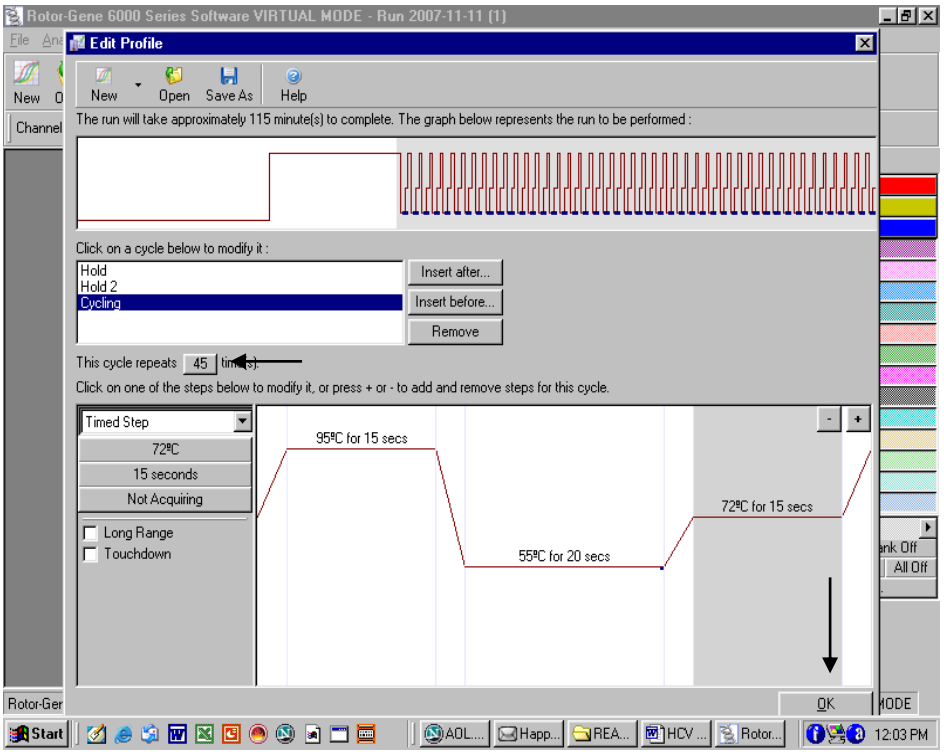


Fig. 34.

After setting the number of Cycles Press OK.

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

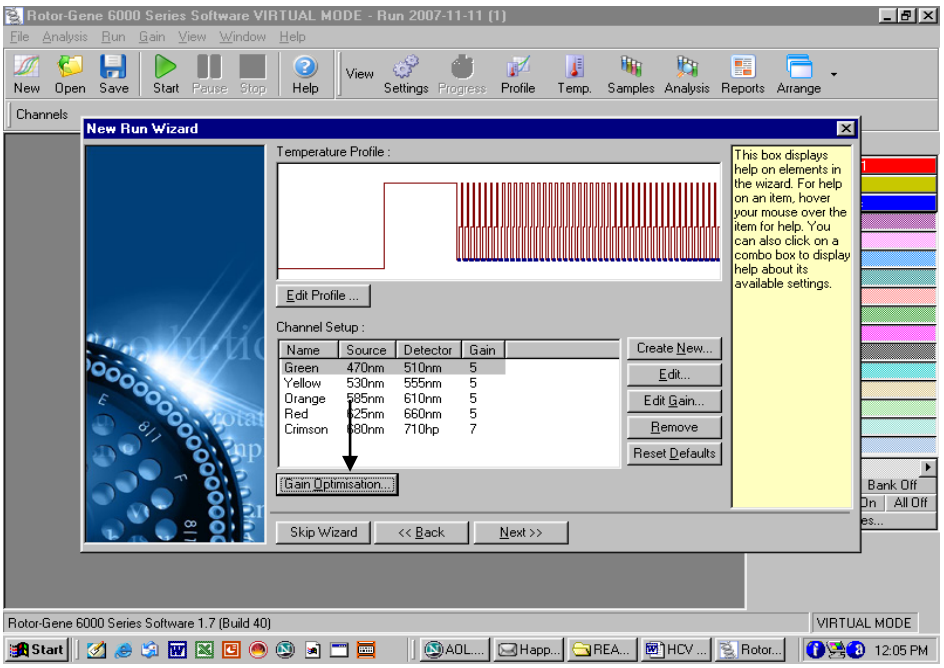


Fig. 35

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

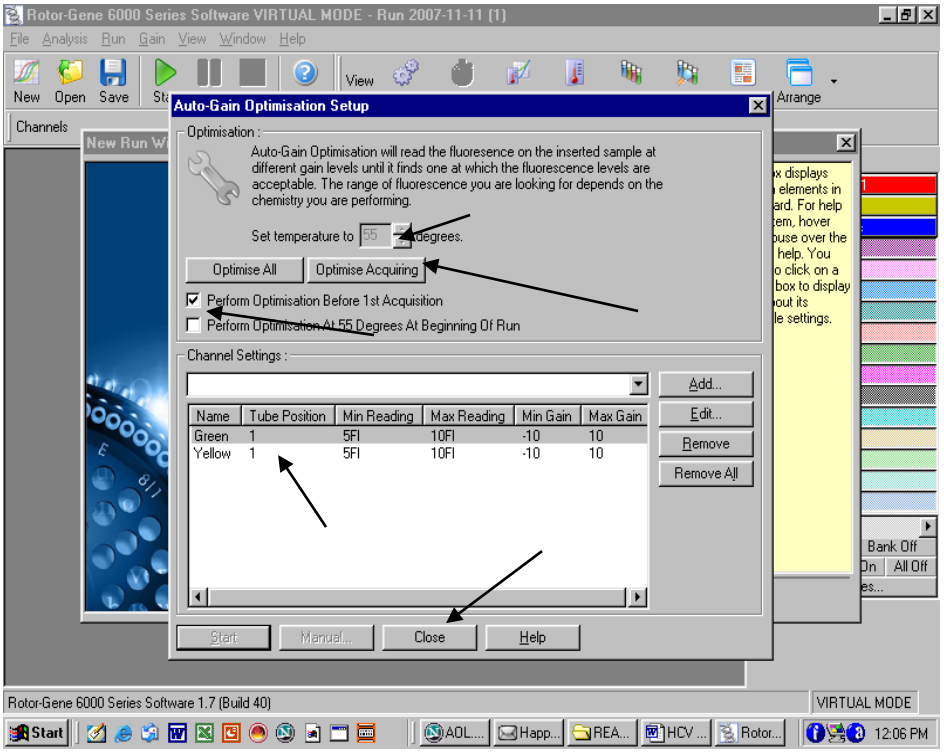


Fig. 36.

The following needs to be done.

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

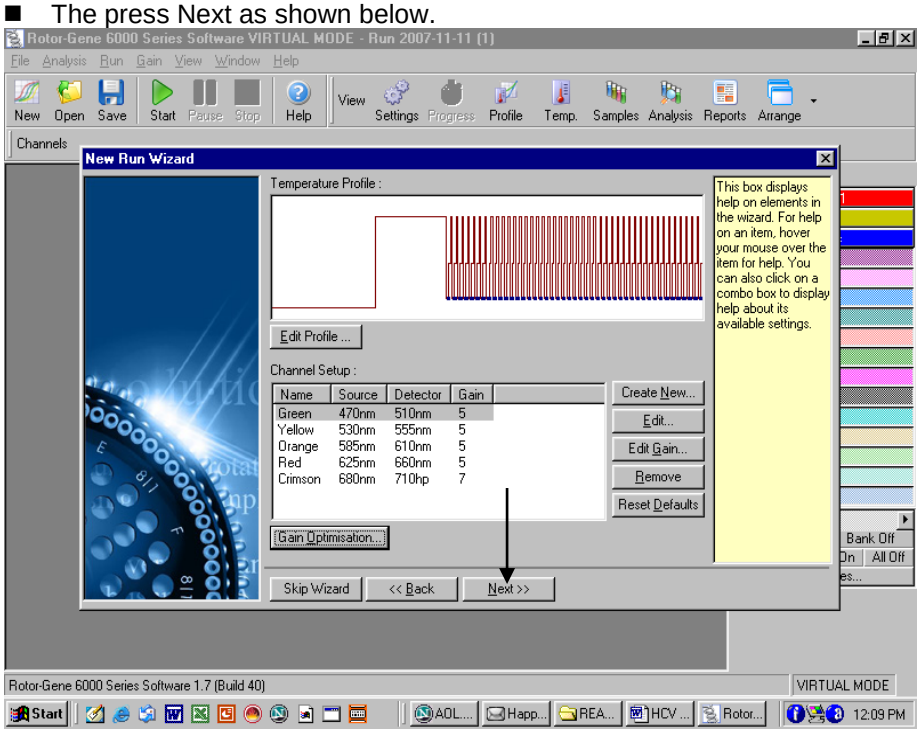


Fig. 37.

L) PRESS Start RUN

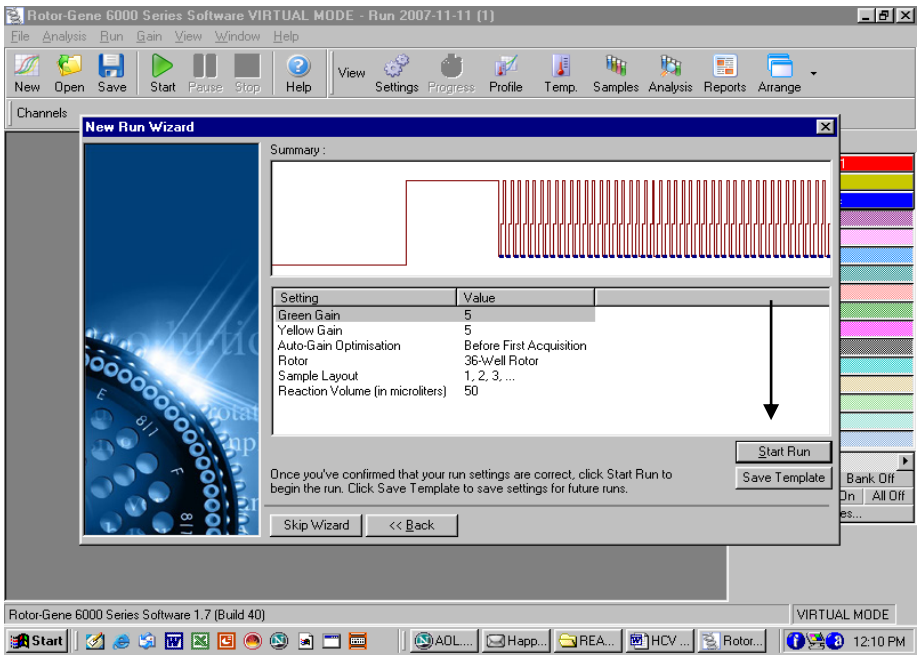


Fig. 38.

Saving the RUN File.

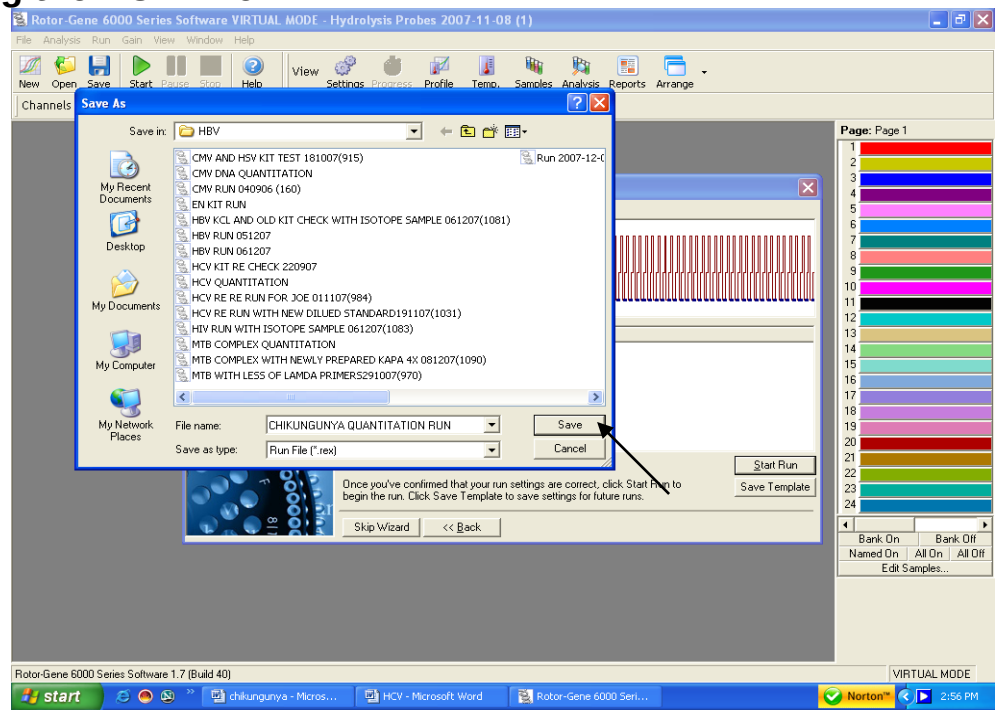


Fig. 39.

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

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

9. Generated Data Interpretation & Analysis

For Rotor Gene 6000

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

The following results are possible:

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

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

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

No Chikungunya 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.

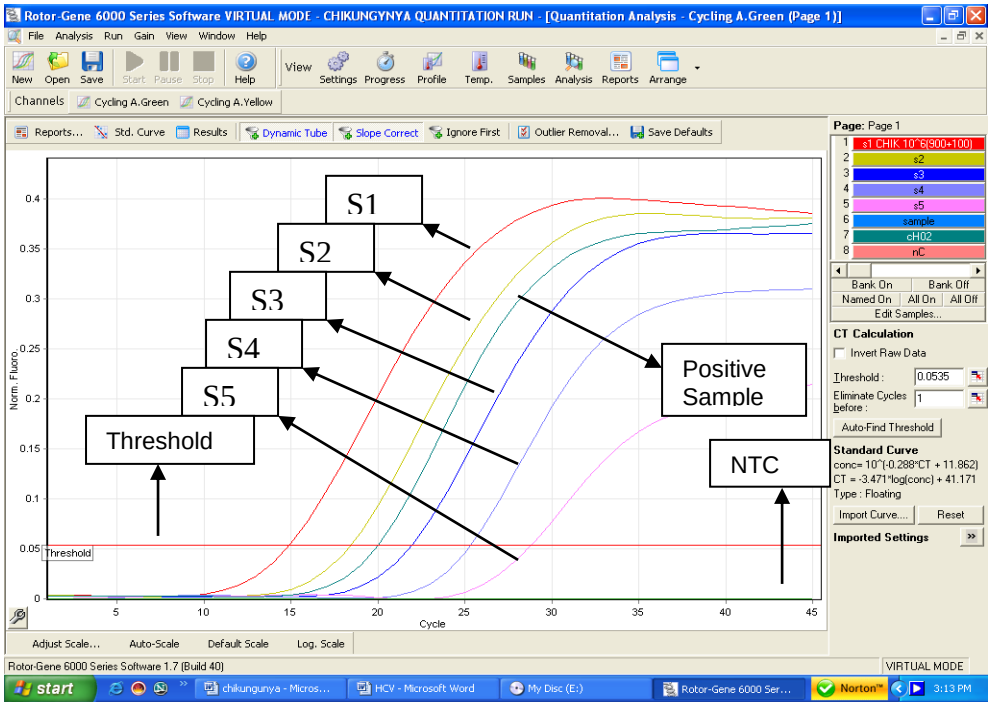


Fig. 42: Detection of the quantitation standards *(***Chikungunya** *S 1-5)* in fluorescence channel Cycling A.Green. NTC: non-template control.

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

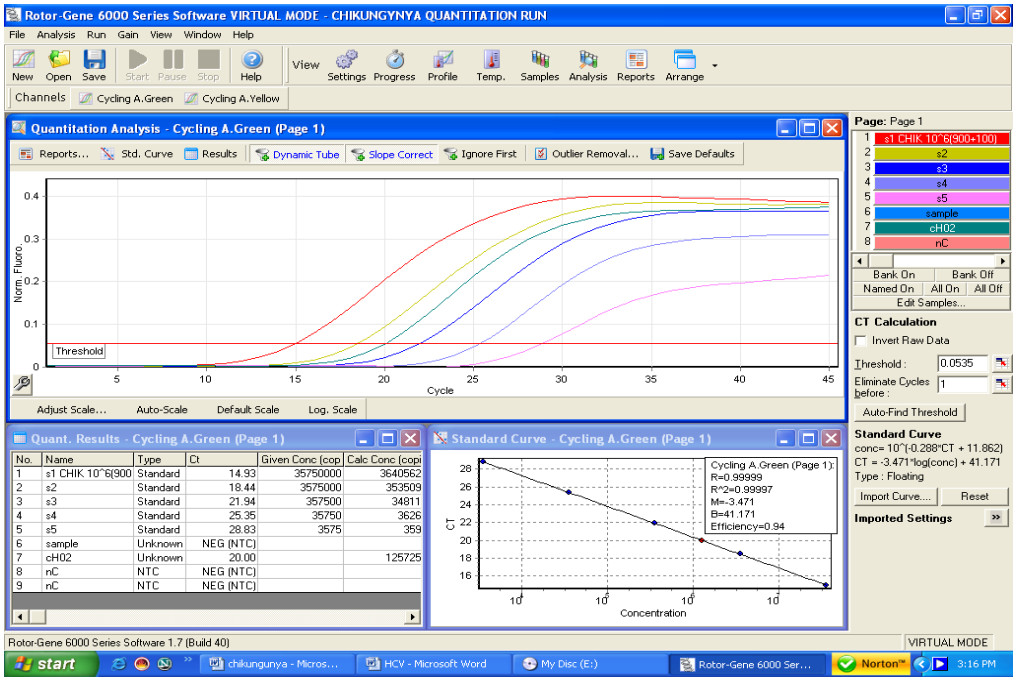


Fig. 43.

Example of analysed data for Chikungunya 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 Chikungunya RNA.

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

No signal detected in Green as well: A possible PCR inhibition has occurred.

10. Troubleshooting

For Rotor Gene 6000

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

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

➔ Repeat the PCR with corrected settings.

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

- The PCR conditions do not comply with the protocol.

➔ Repeat the PCR with corrected settings.

- The CHIKUNGUNYA Super Mix *R1* has been thawed and frozen too often.
- The CHIKUNGUNYA 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 **Chikungunya** 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®*** ChikunGunya *Real Time PCR RG Kit*, a dilution series has been set up from 10^7 copies/ul down to 10^0 Copies/µl of ChikunGunya In-Vitro Transcription and analyzed with the ***Geno Sen's®*** ChikunGunya Real Time PCR RG kit ***using*** *ROTOR GENE 3000/6000™. T*he assays were carried out in triplicate except for the last dilution run in 5 tubes. The results were determined by a probit analysis. The detection limit as determined for ***Geno Sen's®*** ChikunGunya *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

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®*** *ChikunGunya Real Time PCR RG Kit on ROTOR GRNR 3000/6000 was determined by Spiking a known negative Serum to a nominal 80 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 150µl & elution volume of 60µl.

All the Eight extractions were then analyzed with the *Geno Sen's*® ChikunGunya *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 78 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*®** ChikunGunya *Real Time PCR RG was determined to be 80 copies/ml.*

11.b Specificity

The specificity of the ***Geno Sen's*®** ChikunGunya *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 genotypes of ChikunGunya.*

To further Validate the stringent data In order to check the specificity of the ***Geno Sen's*®** ChikunGunya *Real Time PCR RG Kit*, different RNA & DNA listed below were analyzed with ***Geno Sen's*®** ChikunGunya *Real Time PCR RG Kit*. None of these led to a positive signal with the ***Geno Sen's*®** ChikunGunya *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	EnteroVirus	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*®** ChikunGunya Real Time PCR RG kit on ROTOR GENE 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 quantitate the ChikunGunya RNA Gene.

Sample Type	Serum	Plasma	CSF
High +ve's	8	4	0
Medium +ve's	13	8	3
Low +ve's	11	5	3
Extremely low +ve's	4	2	0
Negative samples.	5	6	4
	41	25	10

All the above samples were correctly identified by the ***Geno Sen's®*** ChikunGunya Real Time PCR RG kit & all the 6 extremely low samples were accurately detected by the ***Geno Sen's®*** ChikunGunya Real Time PCR RG kit & exhibited copies around 80 copies /ml or less than 80 copies/ml.

External Evaluation results:

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

11.C Linear Range:

The Linear range of the ***Geno Sen's®*** ChikunGunya Real Time PCR RG kit was determined by analyzing a dilution series of the ChikunGunya In-Vitro Transcription ranging from 10^7copies/µl down to 10^{-1} Copies/µl of ChikunGunya In-Vitro Transcription and analyzed with the ***Geno Sen's®*** ChikunGunya *Real Time PCR RG Kit*.

All the Dilutions were run in triplicate except the lowest dilution which was run in 5 wells using the ***Geno Sen's®*** ChikunGunya *Real Time PCR RG* kit & ROTOR GENE 3000/6000 SYSTEM.

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

In terms of copies per ml this translates to 4 x 10^9 copies/ml down to 40 copies/ml.

Further studies are underway on this aspect.

12. Warranty::

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

In Vitro Diagnostic Medical device, as per the Directive 98/79/EC specifications. This product must be used by qualified professionals only. It is the responsibility of the user to ascertain that a given product is adequate for a given application. Any product not fulfilling the specifications included in the product sheet will be replaced. This warranty limits our responsibility to the replacement of the product. No other warranties, of any kind, express or implied, including without limitation, implicit warranties of commercialization ability or adequacy for a given

purpose, are provided by Genome Diagnostics Pvt. Ltd. Genome Diagnostics Pvt. Ltd will not be responsible for any direct, indirect, consequential or incidental damage resulting of the use, misuses, results of the use or inability to use any product.

13. Limitations of product use:

a.) All reagents may exclusively be used for *in vitro* diagnostics.
b.) The product is to be used by personnel specially instructed and trained in for the *in-vitro* diagnostics procedures only.
c.) It is important to pipet the indicated quantities, and mix well after each reagent addition. Check pipettes regularly.
d.) Instructions must be followed Correctly in order to obtain correct results. If the user has any questions, please contact our Technical Dept. dharam.vsnl@gmail..com or at genome24@rediffmail.com. e.) This test has been validated for use with the reagents provided in the kit. The use of other Reagents or methods, or the use of equipment not fulfilling the specifications, may render equivocal results. User is responsible for any modifications done in any of the indicated parameters. Compliance with the kit protocol is required.
f.) Detection of Viral RNA depends on the number of Viruses present in the sample, and can be affected by sample collection methods, patient-related factors (e.g. age, symptoms), or for infection stage and sample size.
g.) False negative results may be obtained due to polymerase inhibition. It is recommended to perform control reactions to distinguish between inhibition and true negatives. This is achieved by the inhibition Control included in the kit.
h.) Cross contamination between samples and exogenous DNA can only be avoided by following good laboratory practice. Instructions in this document must be strictly followed.
i.) Attention should be paid to expiration dates printed on the kit box and labels of all components. Do not use expired components.

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

15. Publication and Reference

Chikungunya virus iridocyclitis in Fuchs' heterochromic iridocyclitis

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

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

22	**Chlamydia pneumonia RG quantitative Real time PCR kit.**
23	**Streptococcous pneumonia RG quantitative Real time PCR kit.**
24	**N. Meningitis RG quantitative Real time PCR kit.**
25	**H. Influenza RG quantitative Real time PCR kit.**
26	**Leprosy RG quantitative Real time PCR kit.**
27	**Helicobacter Pylori RG quantitative Real time PCR kit.**
28	**Scrub Typhus RG quantitative Real time PCR kit.**
29	**B. Pseudomalie RG quantitative Real time PCR kit.**
30	**Filaria RG quantitative Real time PCR kit.**
31	**Leptospira(pathogenic) RG quantitative Real time PCR kit.**
32	**CCL3-L1 RG quantitative Real time PCR kit.**
33	**Malaria (P. Vivax) RG quantitative Real time PCR kit.**
34	**Bcr/abl Major RG quantitative Real time PCR kit.**
35	**Bcr/abl Minor RG quantitative Real time PCR kit.**
36	**PML/RARA RG quantitative Real time PCR kit.**
37	**RARA/PML RG quantitative Real time PCR kit.**
38	**GAPDH RG quantitative Real time PCR kit.**
39	**β-Actin RG quantitative Real time PCR kit.**
40	**β-Globin RG quantitative Real time PCR kit.**
41	**Abl gene RG quantitative Real time PCR kit.**
42	**Rabies RG quantitative Real time PCR kit.**
43	**Factor V Leiden detection RG Real time PCR kit.**

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