Geno-Sen's

MALARIA MULTIPLEX

(P.falciparum, P.vivax, P.Malariae & P.Ovale)

(Rotor Gene) Real Time PCR Kit

Qualitative

For use with the

Rotor Gene™ 2000/3000/6000

(Corbett Research Australia)

IVD

PACK INSERT

2015

Genome Diagnostics Pvt. Ltd.

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

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Table of Contents

MALARIA MULTIPLEX 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 9111434 100 rxns	REF 9111435 50 rxns	REF 9111436 25 rxns
R1 Blue	MALARIA MULTIPLEX Super mix.	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R2 Yellow	MALARIA MULTIPLEX Mg Sol. RT.	1 Vial	1 Vial	1 Vial
P. Falciparum PC Red	P.Falciparum PC	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
P. Vivax PC Red	P.Vivax PC	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
P. Ovale PC Red	P.Ovale PC	1 Vial of 300µl	1 Vial of 300µl	1 Vial of 300µl
P. Malariae PC Red	P.Malariae PC	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

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

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

2. Storage of the Kit.

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

3. MALARIA MULTIPLEX Information

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

Application

MALARIA is one the world's most prevalent diseases. Current estimates predict over 200 million cases of MALARIA annually. The number of clinical cases exceeds 150 million with approximately 2-3 million deaths per year. Most of these victims are infants, young children. Over half the world's population lives in malarious areas.

MALARIA is caused by a protozoan (*Plasmodium sp.*) which invades human red blood cells. The parasite is usually transmitted to humans by the bite of the female *Anopheles* mosquito. Eradication of the *Anopheles* vector has proven to be a very difficult and expensive process. Once introduced into the blood stream by the *Anopheles* mosquito, the newly released sporozoite form of the MALARIA parasite quickly invades the liver. During the next 2 weeks the intracellular parasite multiplies within a liver cell to form as many as 200,000 merozoites. On maturation the merozoites are released into the blood where they invade human red blood cells. In the red blood cell the parasite matures asexually to produce another 10-20 merozoites which are in turn released into the blood to invade still more red cells. The rupture and release of the merozoites is associated with the clinical symptoms of the disease: fever and chills. Depending on the species of parasites these cycles occur every 2-3 days. The diagram at left shows the life cycle of *Plasmodium falciparum*.

As the malaria parasites enter the blood stream they infect and destroy red blood cells. Destruction of these essential cells leads to fever and flu-like symptoms, such as chills, headache, muscle aches, tiredness, nausea, vomiting and diarrhea. These initial symptoms are non-specific: in other words, they are self-reported symptoms that do not indicate a specific disease process.

Uncomplicated malaria (can be caused by all strains of Plasmodium)

Malaria is considered uncomplicated when symptoms are present but there are no clinical or laboratory signs to indicate severity or vital organ dysfunction. The symptoms of uncomplicated malaria are non-specific and include fever.

Severe malaria (only caused by *P. falciparum*)

Infection with *P. falciparum*, if not promptly treated, can quickly progress to severe malaria. The main symptoms of severe malaria include: coma, severe breathing difficulties, low blood sugar, and low blood haemoglobin (severe anaemia). It is diagnosed on the basis of the presence *P. falciparum* parasites and one of the above symptoms with no other obvious cause. Children are particularly vulnerable since they have little or no immunity to the parasite. If untreated, severe malaria can lead to death.

Cerebral malaria (only caused by *P. falciparum*)

Malaria is classified as cerebral when it manifests with cerebral symptoms, such as coma.

Malaria life Cycle

Malaria parasites spread by successively infecting two types of hosts: female Anopheles mosquitoes and humans. See an animated PowerPoint slide of the malaria parasite lifecycle.

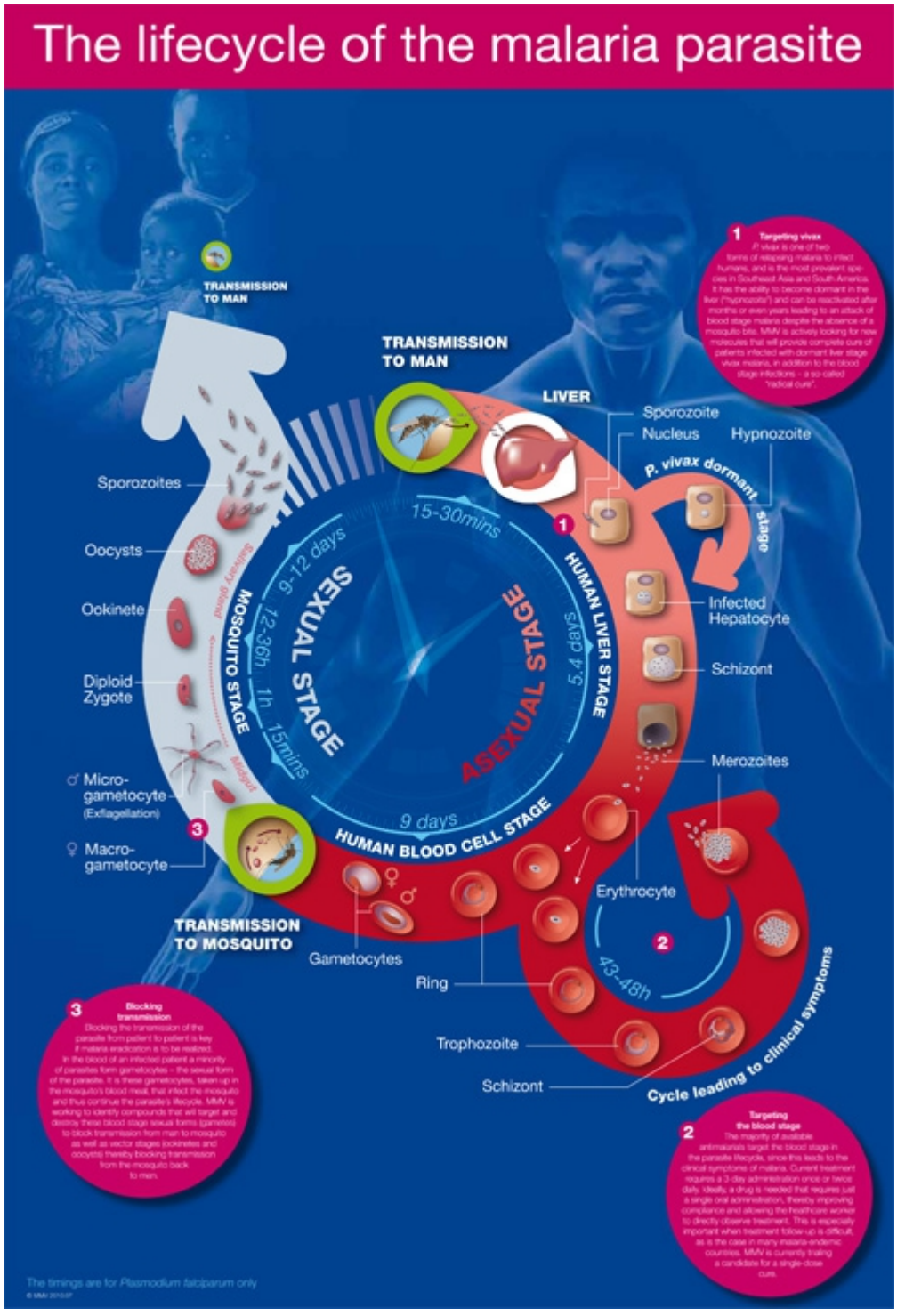

Fig.2

At the same time she takes a blood meal to nourish her eggs, the female *Anopheles mosquito* injects sporozoites into the blood stream of malaria's next victim.

- The sporozoites are rapidly taken up by the liver cells.

- In all species of *Plasmodium*, these parasites develop to form schizonts (the multinucleate stage of the cell during asexual reproduction), from which several thousand merozoites develop.
- In *Plasmodium vivax* and *Plasmodium ovale* only, a proportion of the liver-stage parasites (known as hypnozoites) remain dormant in the hepatocytes. In this stage the parasite can remain dormant for months or several years. These two species of parasite can therefore initiate a cycle of asexual reproduction causing clinical symptoms in the absence of a new mosquito bite, giving *P. vivax* infection the name relapsing malaria.
- When the liver cells rupture, the merozoites are released into the bloodstream where they rapidly invade the red blood cells. These blood-stage parasites replicate asexually – rapidly attaining a high parasite burden and destroying each red blood cell they infect, leading to the clinical symptoms of malaria.
- The trigger is as yet unknown, but a small percentage of merozoites, differentiate into male and female gametocytes, which are taken up by the mosquito in her blood meal. It is these gametocytes that cause the cycle of transmission to continue back to the mosquito.
- Male and female gametocytes fuse within the mosquito forming diploid zygotes, which in turn become ookinetes.
- These ookinetes migrate to the midgut of the insect, pass through the gut wall and form the oocysts.
- Meiotic division of the oocysts occur and sporozoites are formed, which then migrate to the salivary glands of the female *Anopheles* mosquito ready to continue the cycle of transmission back to man.

There are four species of *Plasmodium* which infect humans. *P. falciparum*, *P.vivax*, *P. MALARIA* , and *P.ovale*. *P. falciparum* is the most virulent species of MALARIA since it can cause the death of the host as a result of: cerebral MALARIA, pulmonary or renal failure. In *falciparum* MALARIA the infected red blood cells become sticky and adhere to the small vessels in the brain severely restricting the flow of essential nutrients to the brain.

P. vivax is the most common cause of human MALARIA , especially in South East Asia and in Central and South America. Though *P. vivax* rarely causes death, patients who contract this form of MALARIA may become very ill, with severe clinical symptoms. Sometimes these patients also may become anemic.

The other two forms of human MALARIA account for about 5-10 % of the cases of MALARIA Both *P. vivax* and *P. ovale* are associated with relapsing MALARIA, as both these parasites are able to deposit a dormant form in the hosts liver. This form is called the hypnozoite. The hypnozoite may awake at anytime, and begin to divide and to produce merozoites that invade the blood and cause clinical symptoms. Consequently, after the initial blood stages of *P. vivax* and *P.ovale* have been effectively treated, the liver stage must then be eliminated in order to cure the patient. The treatment of the liver stage requires the use of an anti MALARIA drug such as primaquine. Since primaquine is well known to hemolyzes red blood cells from patients with glucose phosphate dehydrogenase deficiency, it is

absolutely essential that before primaquine is administered to a patient with either *P.vivax* or *P. ovale* the patient should be tested for the presence of this enzyme in their red blood cells. If there is no enzyme present then other medications, or different approaches should be considered. It is rare for Africans, or African descendants to contract *P. vivax* as their red cells do not contain a protein on their surface that permits vivax to enter the red blood cells. This glycoprotein is called the Duffy antigen.

Almost 85% of the world's MALARIA occurs in sub-Saharan Africa. The vast majority of these cases are *P. falciparum* MALARIA, though *P.ovale* and *P. MALARIA* are also present. MALARIA is also prevalent in Southeast Asia, India, South and Central America.

Samples which can be used for Extraction: Whole blood.

4. Precautions for PCR

The following aspects should always be taken care of:

- Store positive material (Specimens, PC 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 PC's & specimens) should be mixed & dispensed in pre-mix area.
- All the standards & specimens should be mixed & dispensed in extraction area.
- Use pipette tips with filters only.
- Always use disposable powder-free gloves.

5. Additionally Required Materials and Devices

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

- Centrifuge Desktop with rotor for 1.7 ml reaction tubes
- *Rotor Gene™ 2000/3000* or *Rotor Gene™ 6000*, Corbett Research (The Real time PCR Instrument)

6. Principle of Real-Time PCR

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

7. Description of the Product.

The ***Geno-Sen's MALARIA MULTIPLEX (P.falciparum, P.vivax,P.ovale & P.malariae)*** *PCR* Reagents constitute a ready to use system for differentiation and detection of **P.falciparum, P.vivax,P.ovale & P.malariae** 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 ***MALARIA MULTIPLEX (*P.falciparum, P.vivax, P.ovale & P.malariae)** and for the direct detection of the specific amplicon in fluorescence channel Cycling A.**FAM/Green (P.falciparum), Cy5/Red (P.vivax), ROX/Orange (P.ovale) & Joe/Yellow (P.malariae)** (of the *Rotor Gene 2000/3000/6000.*External positive Control **(PC Multiplex)** are supplied which allow the determination of the gene Present. For further information, please refer to section 8.3.

8. Procedure

8.a DNA Extraction

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

Sample Material	**Nucleic Acid Isolation Kit**	**REF Cat. Num.**	
Whole blood.	**Geno Sen's® Whole Blood DNA Extraction Mini Kit — for DNA purification**	98027 98028	Genome Diagnostics Pvt Ltd.

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

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

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

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

8.b Inhibition Control.

There is no Inhibition in the kit as all the four channels are used for Gene of Interest. Besides which the assay has to be performed on the Known Malaria positive samples.

8.c Positive Control

The Positive controls provided in the kit are treated in the same way as extracted samples and the same volume is used i.e.(10µl) instead of the sample. The same should also be defined as Positive Control. (See *Rotor Gene*™ Manual). The positive controls provided in the kit are for ***(P.falciparum*, P.vivax, and P.ovale & P.malariae** separately).

Preparation for PCR

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 Positive Control & 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

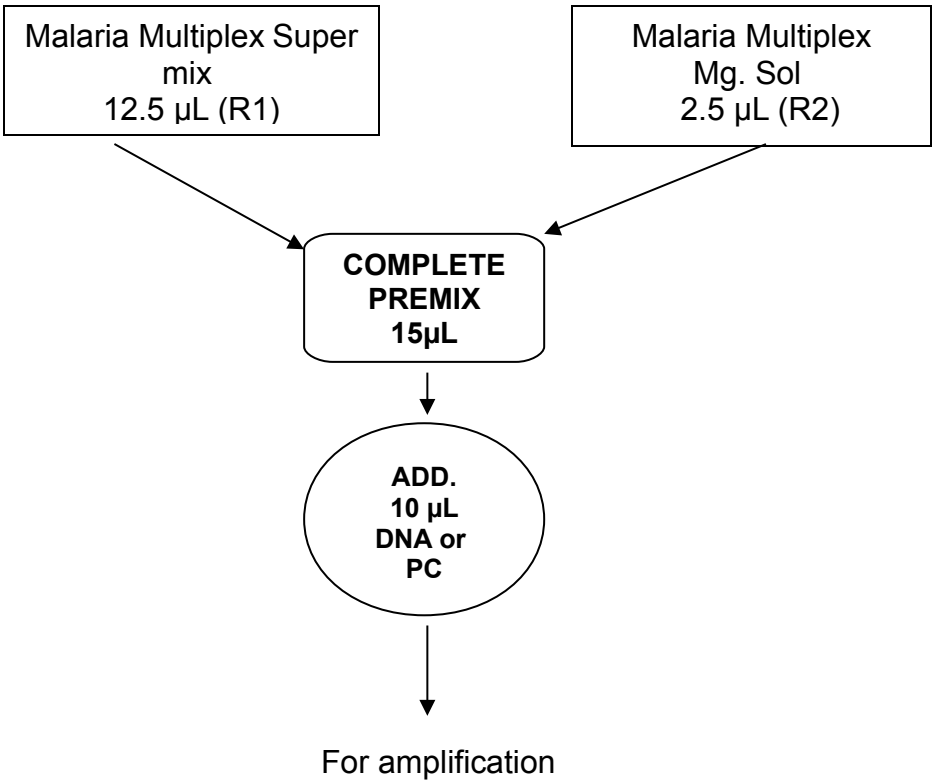

Fig. 3.

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

MALARIA MULTIPLEX MASTER MIX	**1 rxns.**	**10 rxns.**
MALARIA MULTIPLEX Super Mix (R1)	12.5 µL	125 µL
MALARIA MULTIPLEX Mg Sol. (R2)	2.5 µL	25 µL
Total	15µL	150µL

Fig. 4.

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

8.e. Programming the *Rotor Gene*™ 2000/3000

The *Rotor Gene™ 2000/3000* PCR program for the detection of Malaria Multiplex can be divided into following steps:

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

Program the *Rotor Gene™ 2000/3000* for these 5 steps according to the parameters shown in Fig. 5-21. All specifications refer to the *Rotor Gene™* software version 6.0.33. Please find further information on programming the *Rotor Gene™* in the *Rotor Gene™ 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.

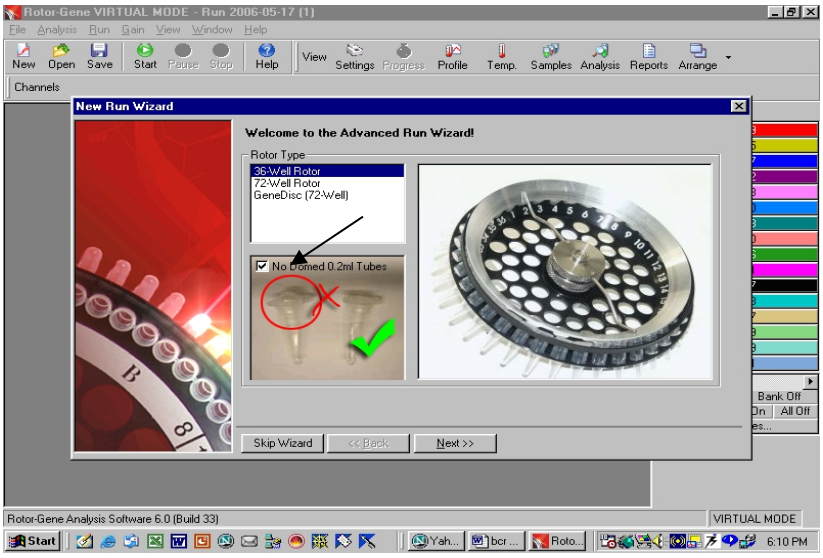

Fig. 5.

Confirmation of Reaction Volume as follows.

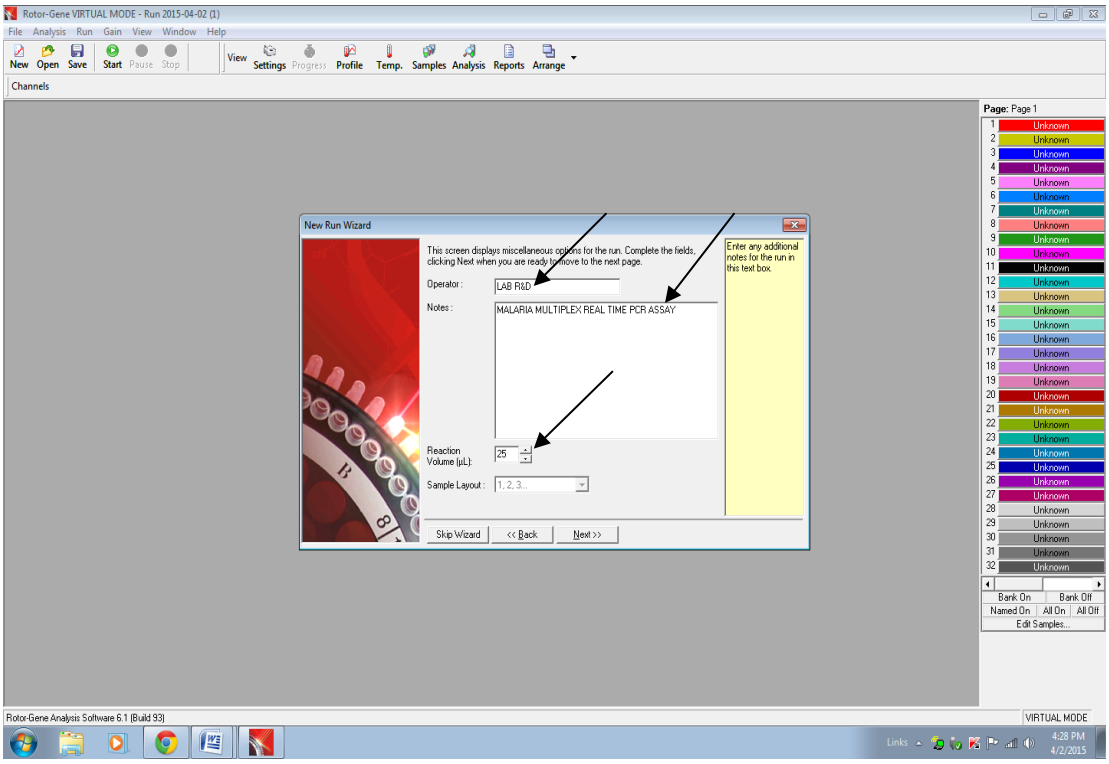

Fig. 6. Setting of general assay parameters.

- 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.

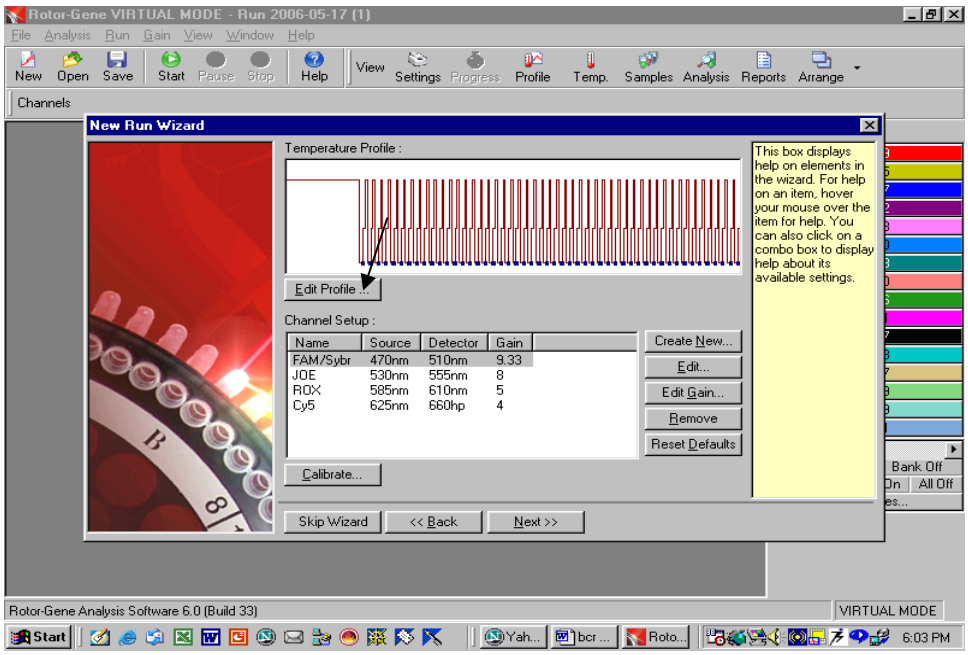

Fig. 7.

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 95°C for 10 minutes as below

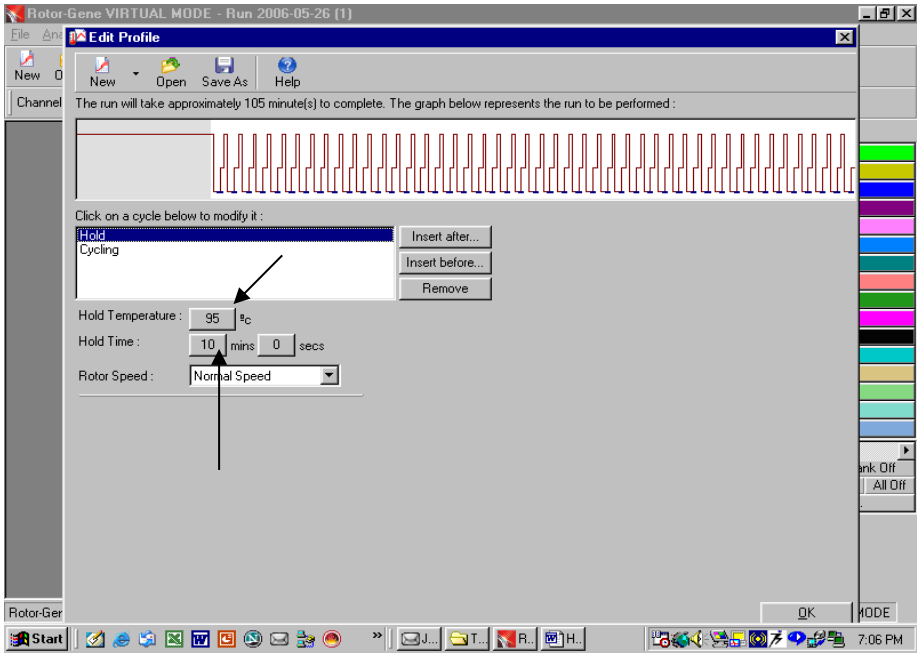

Fig. 8. Initial activation of the Hot Start enzyme.

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

95°C for 15 seconds.

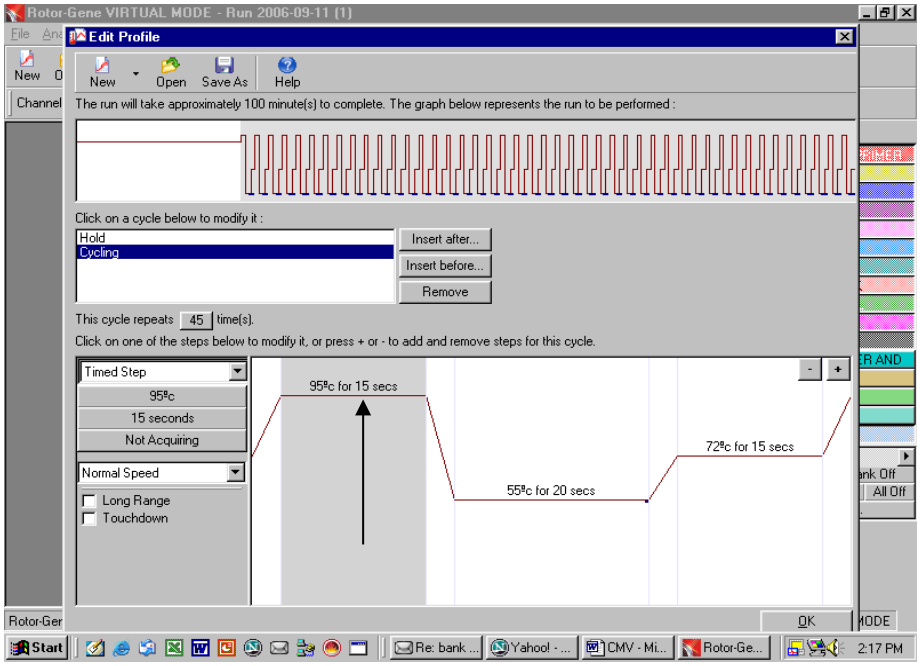

Fig. 9.

Setting up of Annealing 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, ROX &Cy5

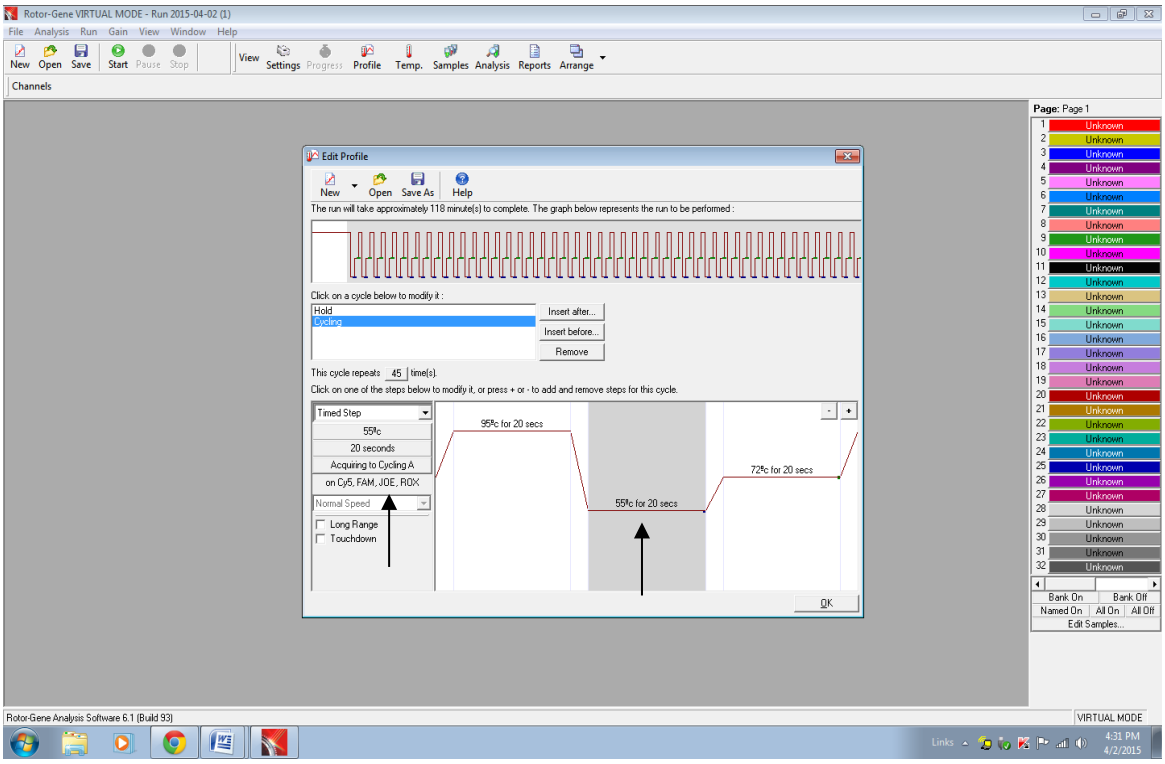

Fig. 10.

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

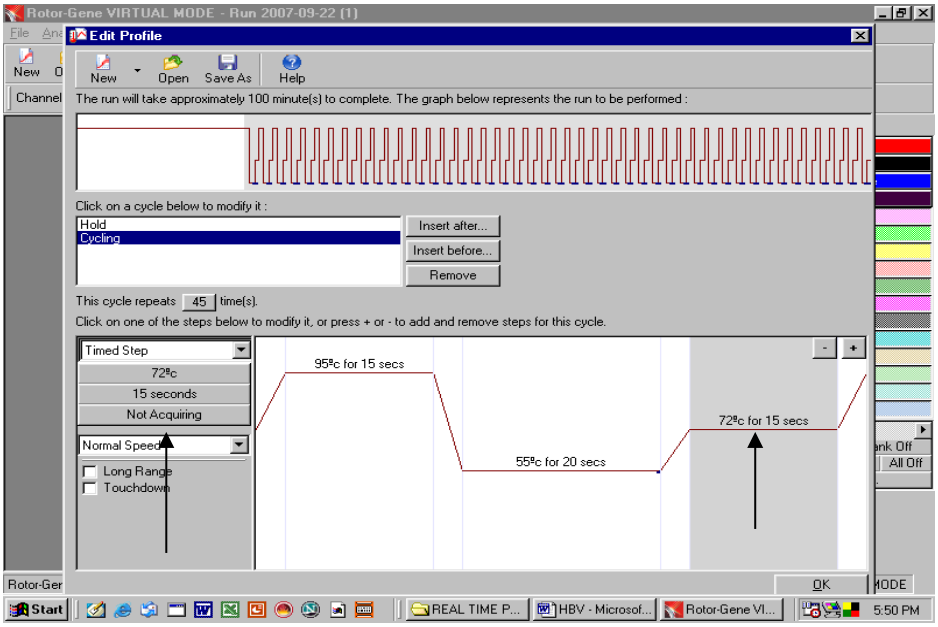

Fig. 11.

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

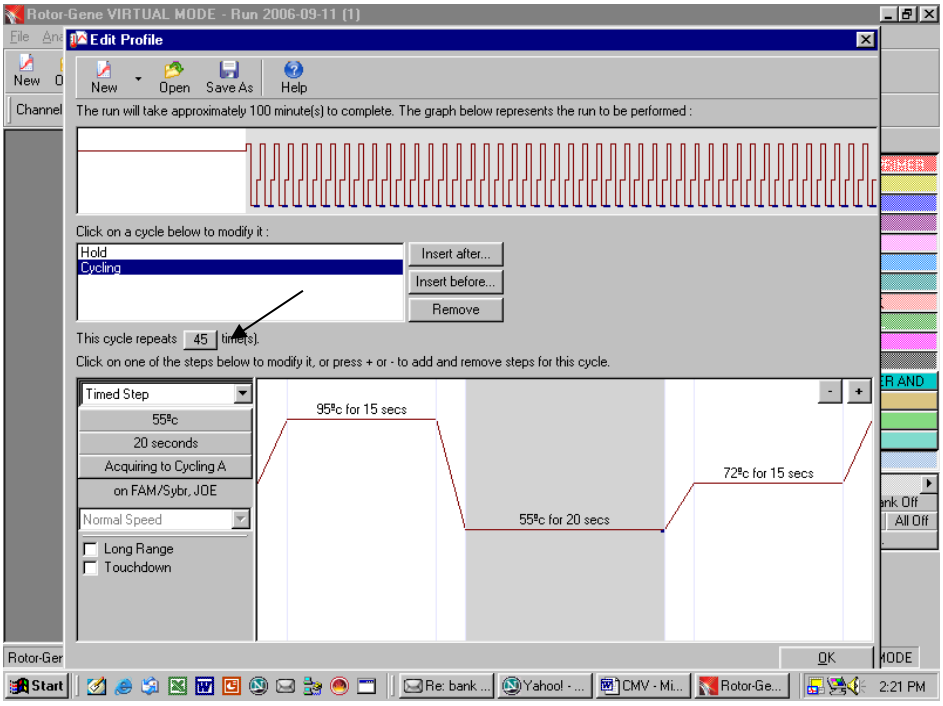

Fig. 12.

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

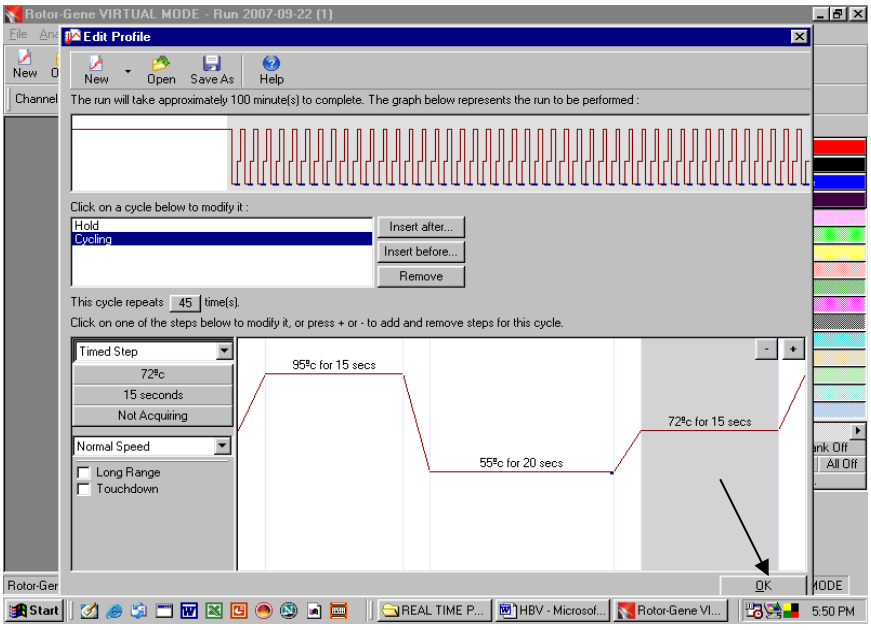

Fig. 13.

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

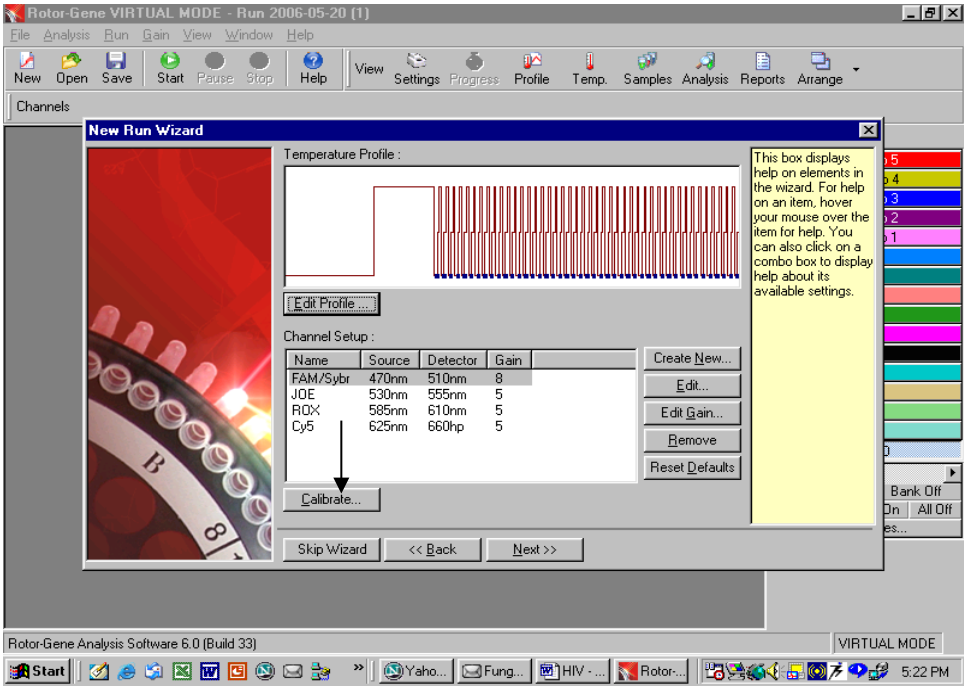

Fig.14.

The detection range of the fluorescence channels has to be determined according to the fluorescence intensities in the PCR tubes. This adjustment is done in the menu window *Auto Gain Calibration Setup* (activation in menu window *New Experiment Wizard* under *Calibrate*). Please set the calibration temperature to the annealing temperature of the amplification program (compare Fig. 16).

Adjustment of the fluorescence channel sensitivity as shown below.

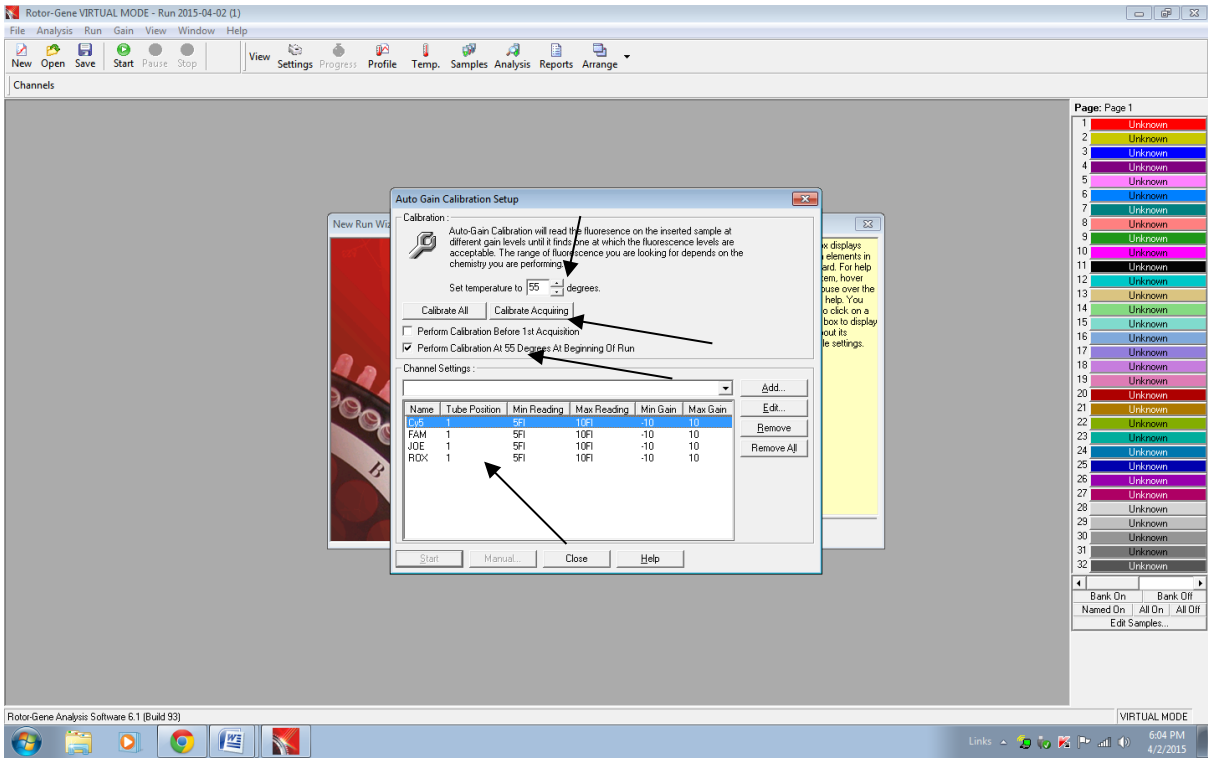

Fig. 15.

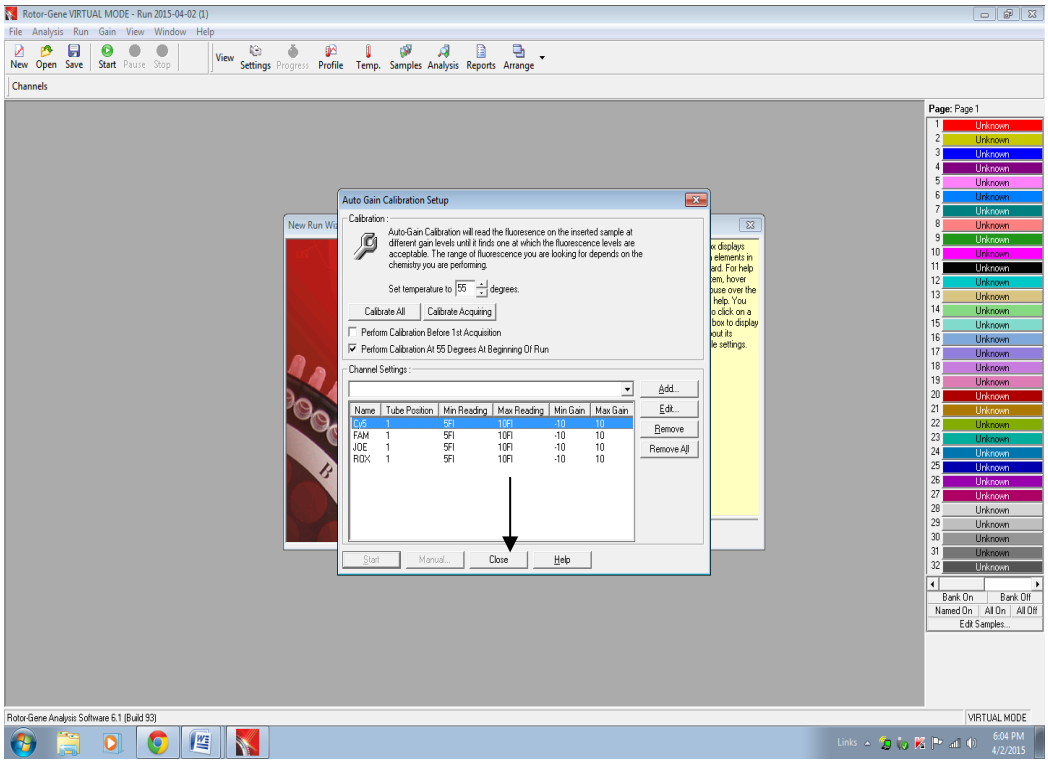

Fig. 16.

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

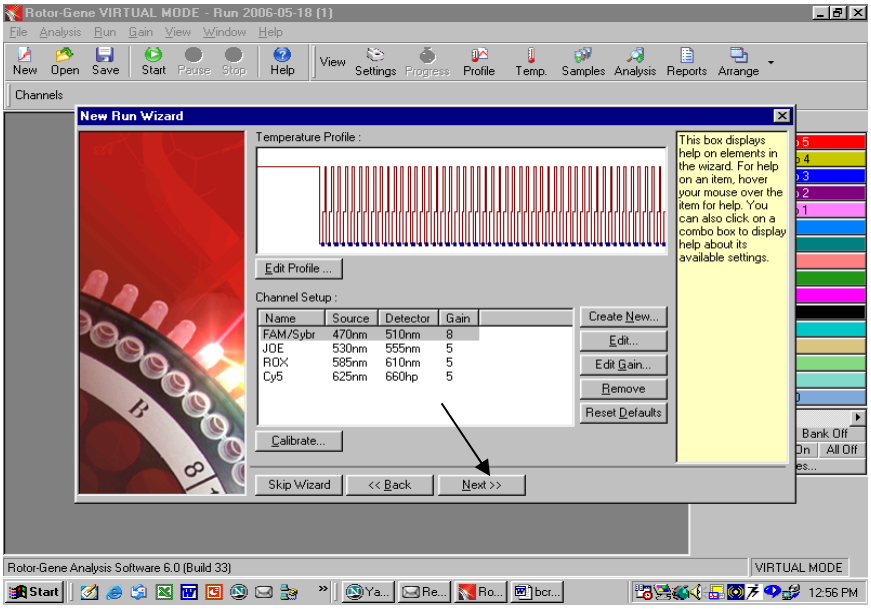

Fig. 17.

Starting of the *Rotor Gene*™ run.

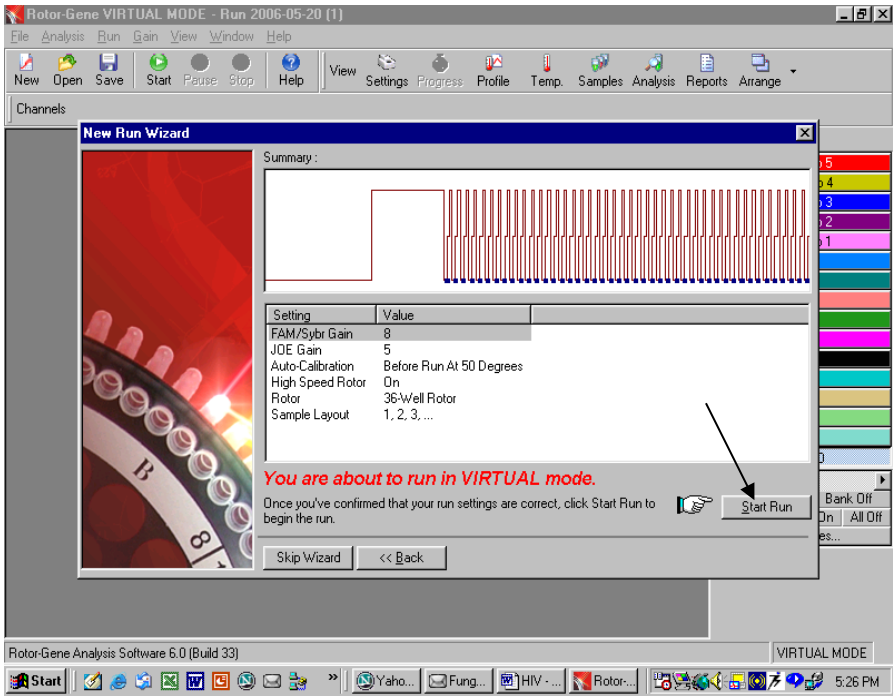

Fig. 18:
Press Start Run Button.

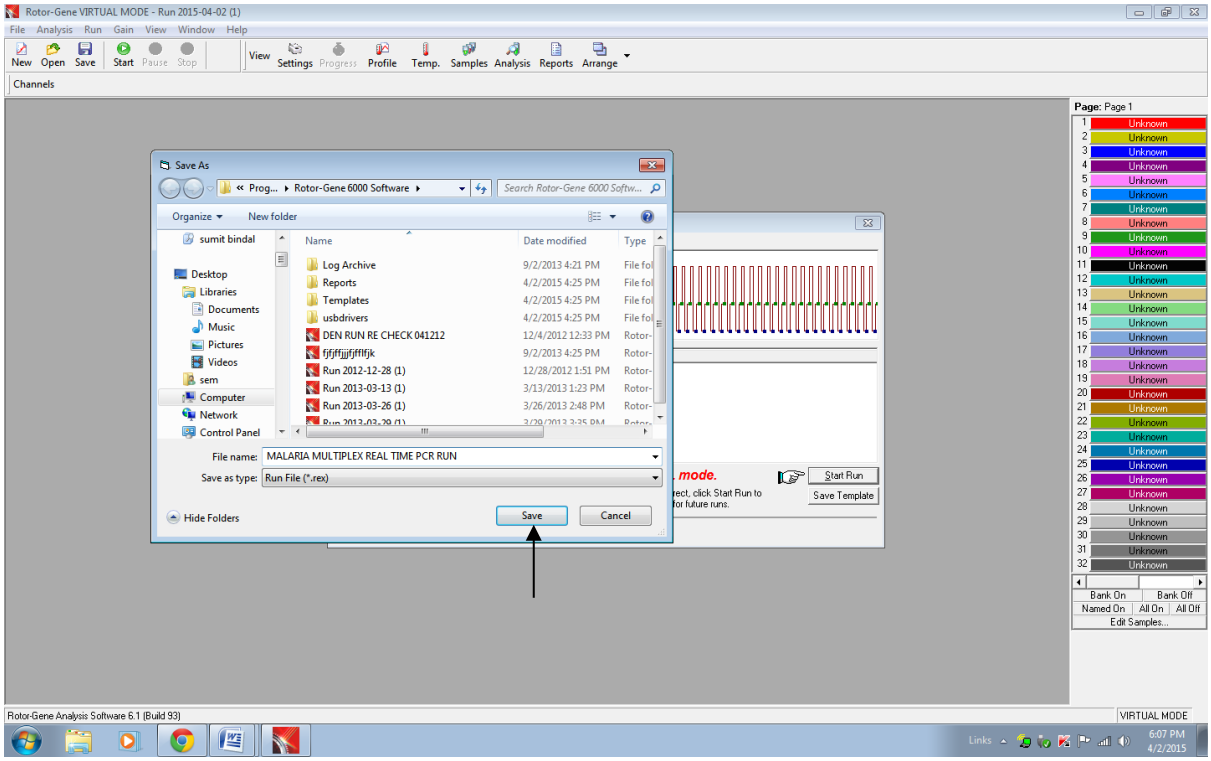

Fig.19.

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 *Rotor Gene*™ 6000

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

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

Program the *RotorGene*™ 6000 for these 5 steps according to the parameters shown in Figures 20-35 below All specifications refer to the *Rotor Gene*™ 6000 software version 1.7 Please find further information on programming the *Rotor Gene*™ in the *Rotor Gene*™ 6000 *Operator's Manual*,. In the illustrations these settings are shown by arrows

g) Setting of general assay parameters & Reaction volume.

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

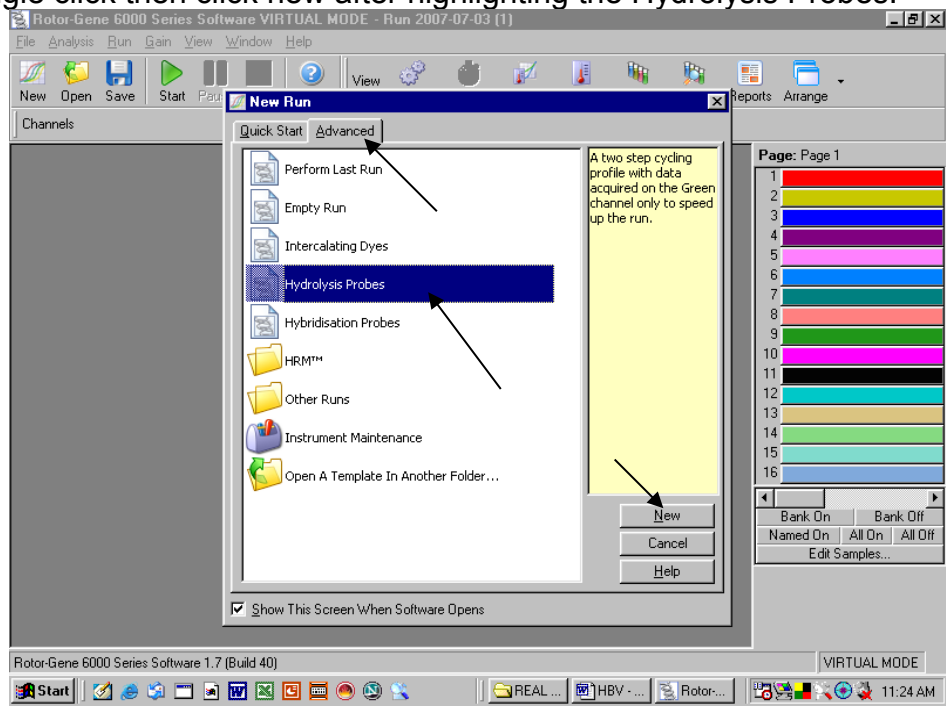

Fig. 20.

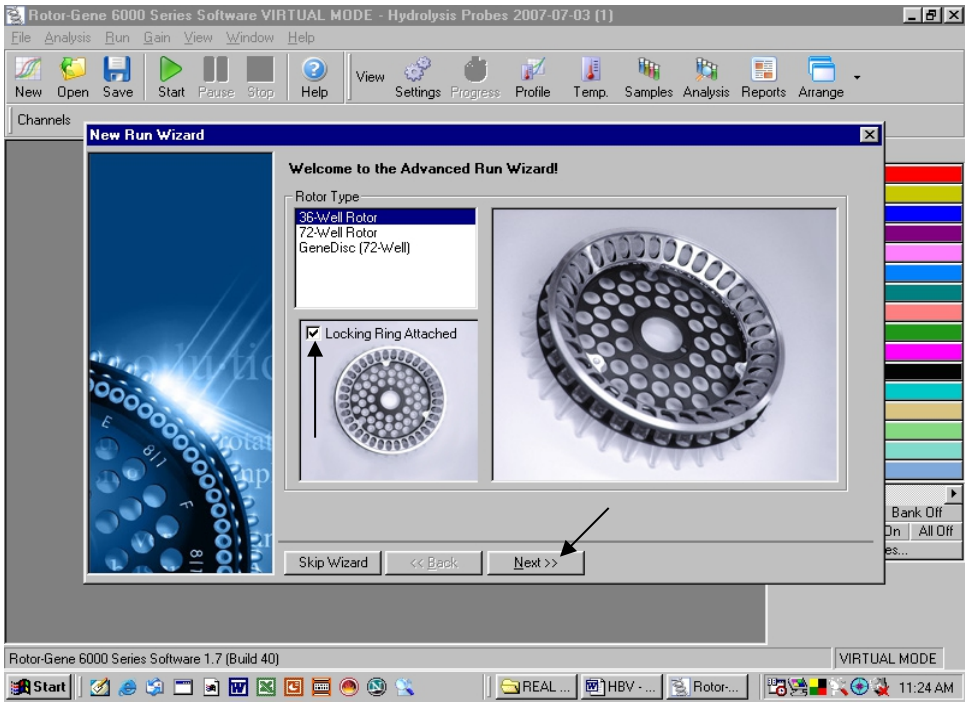

Fig. 21.

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.

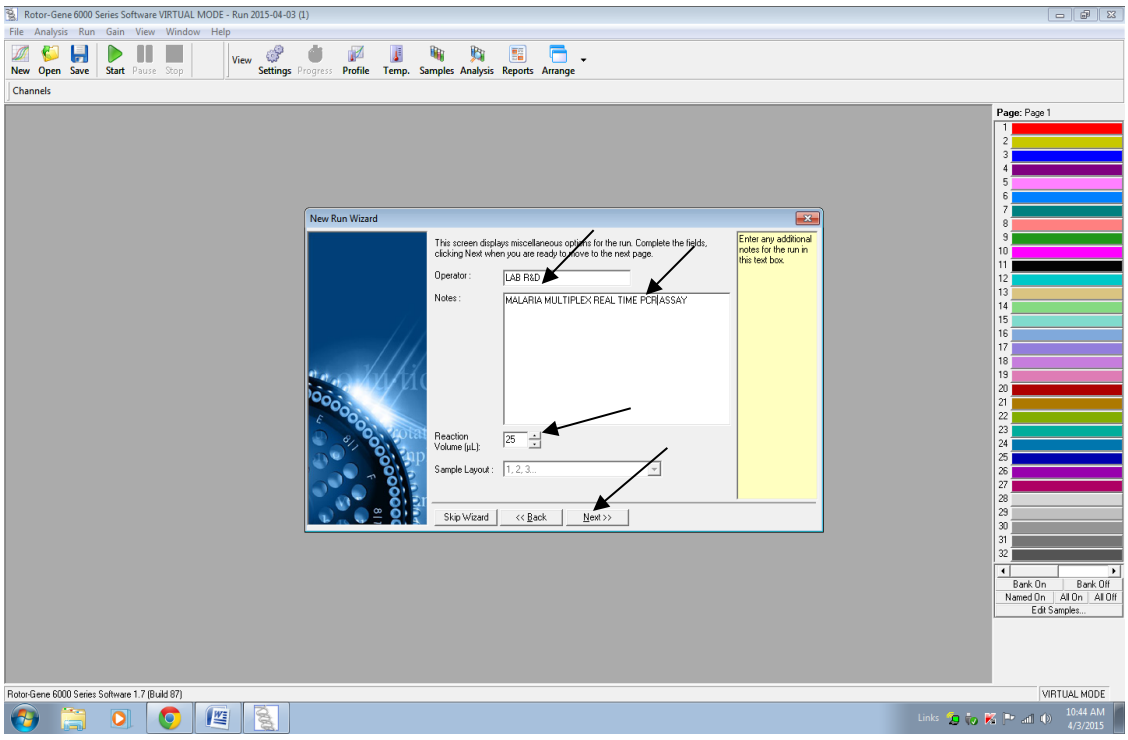

Fig. 22.

- 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.

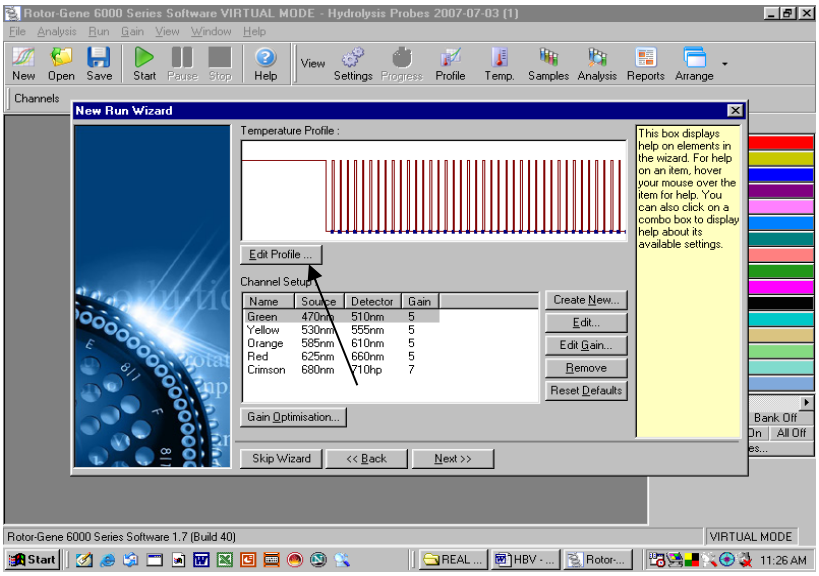

Fig. 23.

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

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

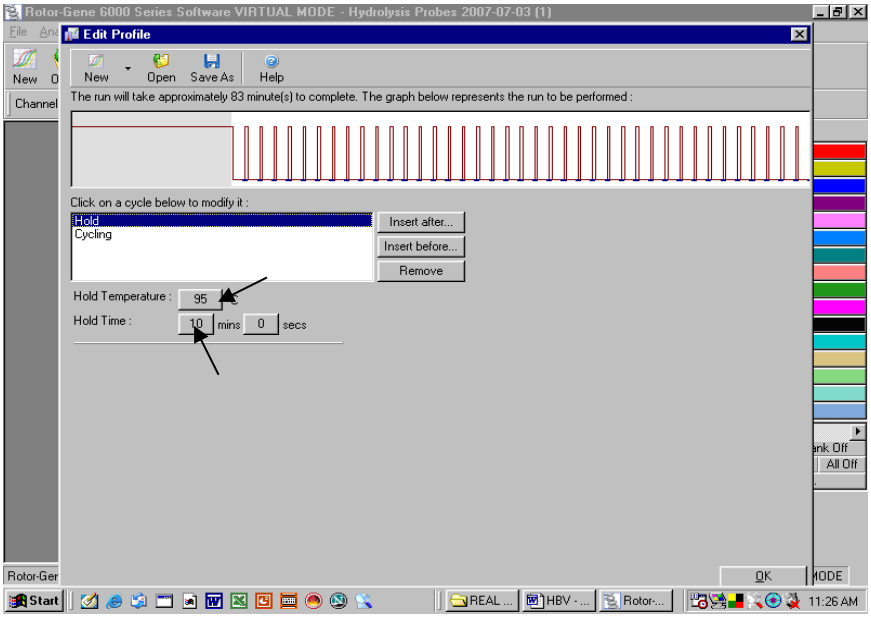

Fig. 24. Initial activation of the Hot Start enzyme. Generally the window will open with the Hold Temp as 95°C and the Hold Time as 10 minutes. In case it is different then set the same to as shown above.

j) Setting the Cycling and acquisition.

When clicked on Cycling the window will open as below.

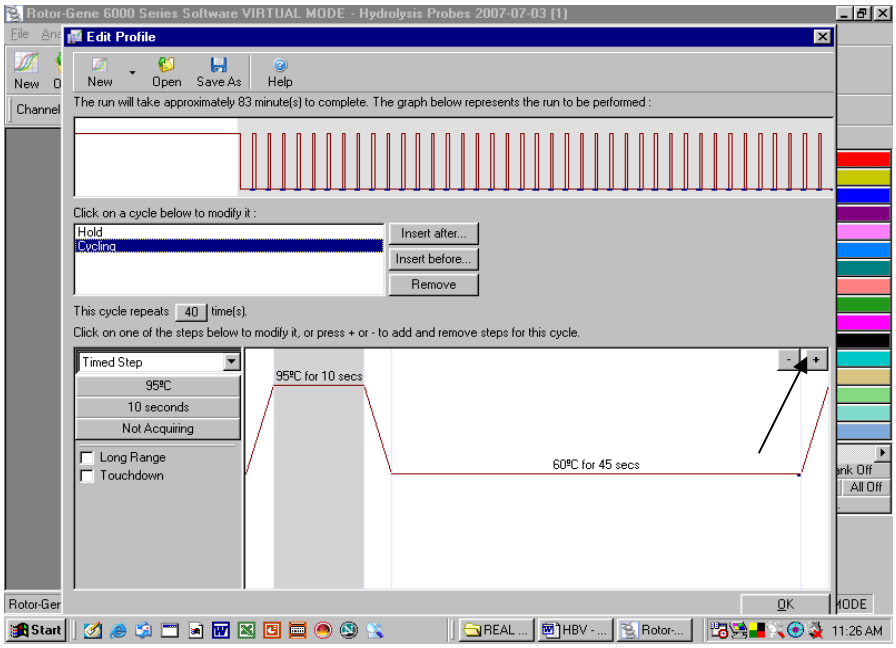

Fig. 25.

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.

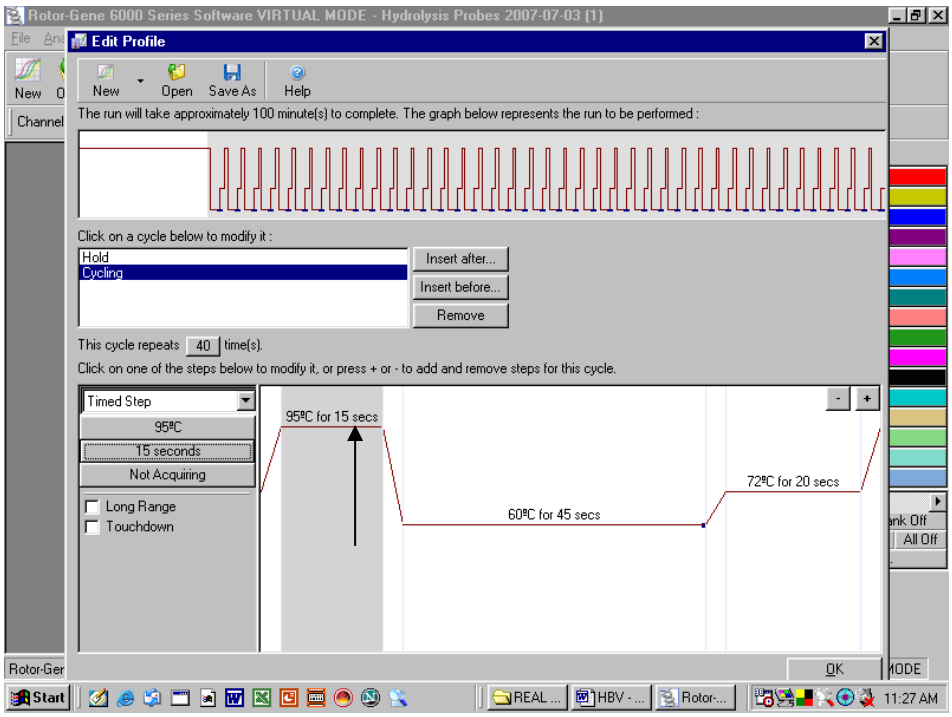

Fig. 26.

Setting up of Annealing step in the cycling profile as depicted below i.e. 55°C for 20 Seconds **and defining the Data acquiring channel i.e. GREEN,RED ORANGE & YELLOW.**

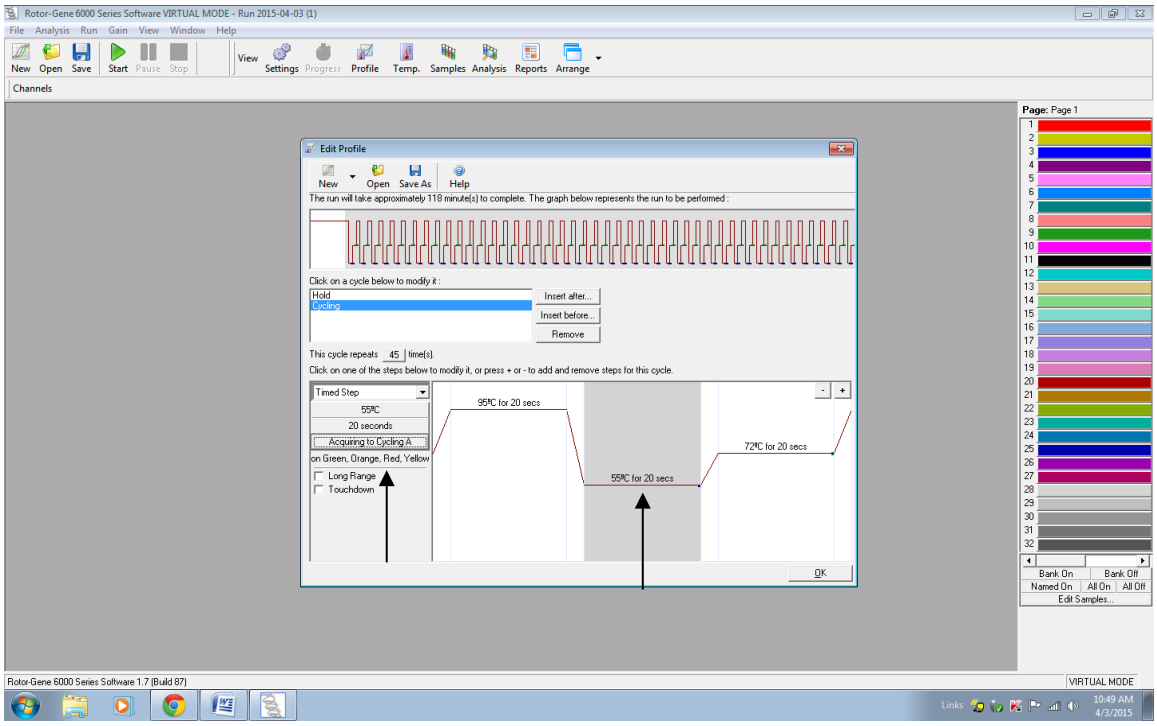

Fig. 27.

After setting the Anneling temperature and the time for annealing 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, Red, Orange & Yellow

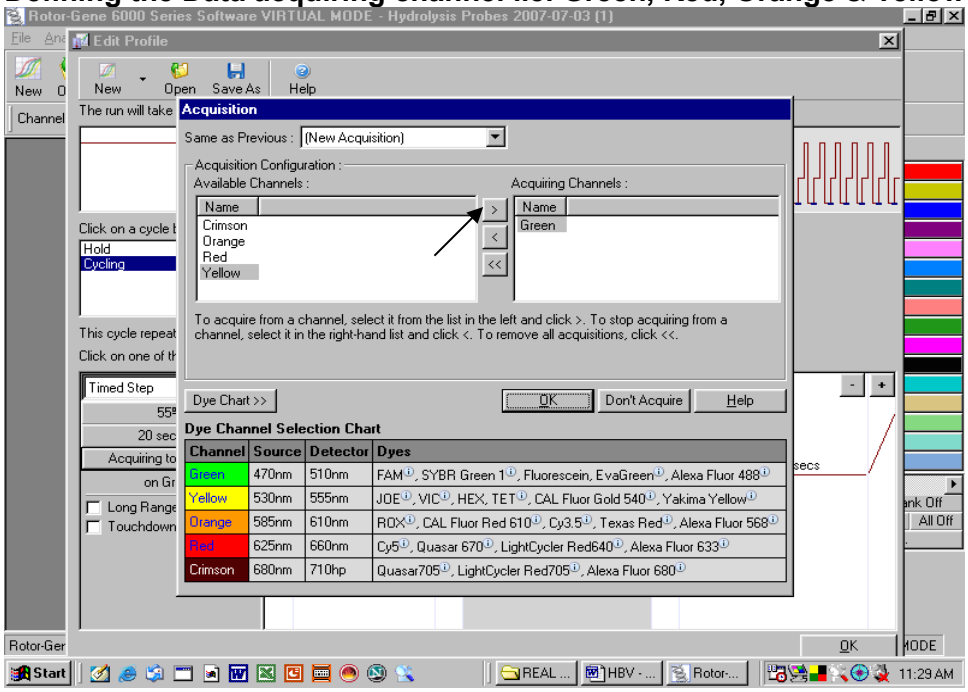

Fig. 28.

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, Red and Orange. In case any other Channel appears besides Green, Red & Orange on the right then the same be shifted to the left.

Confirmation of Channels as shown below.

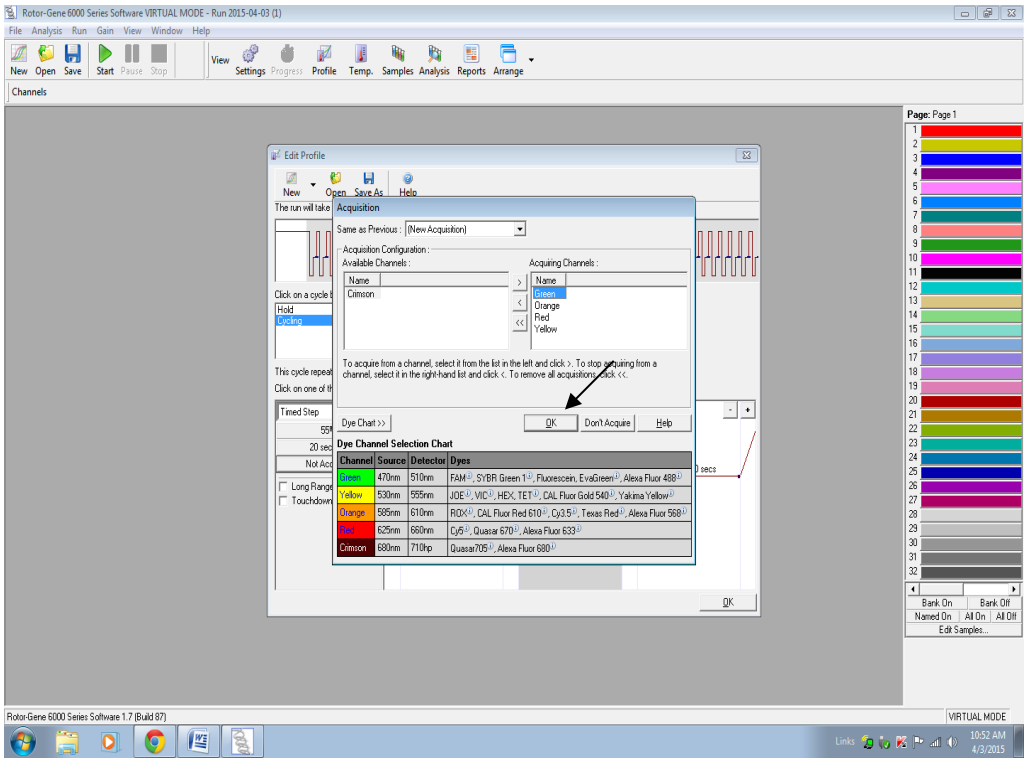

Fig. 29.

Once the Red, Orange, 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

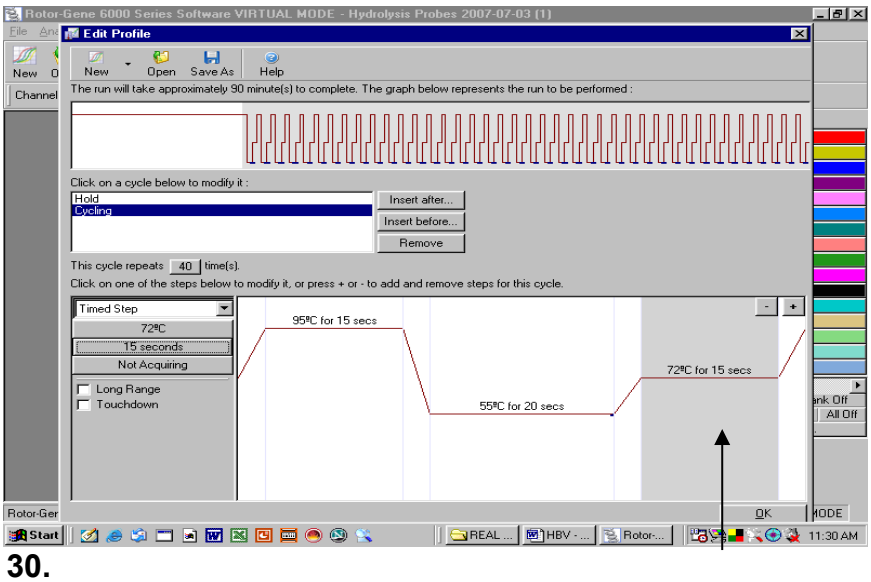

Fig. 30.

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

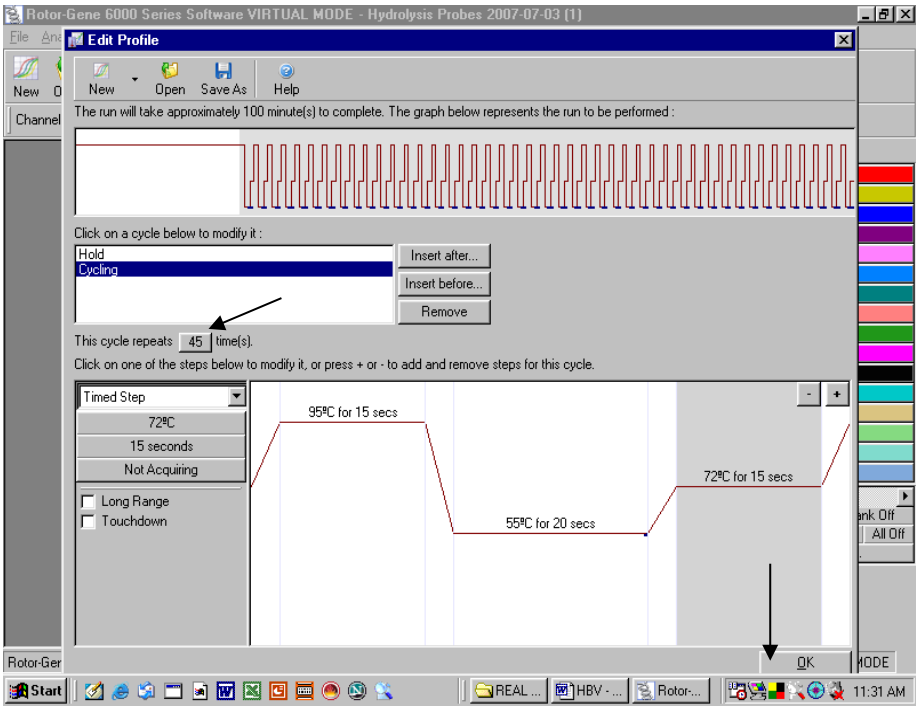

Fig. 31.

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.

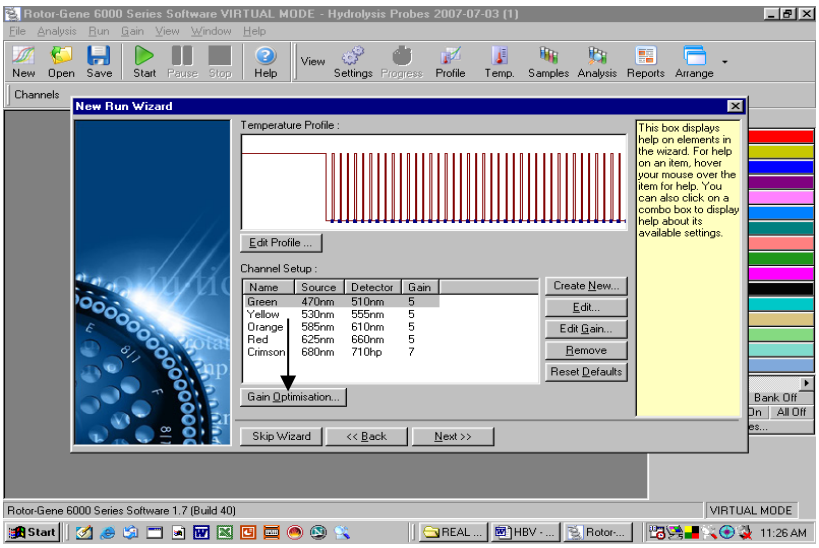

Fig. 32.

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.33.)

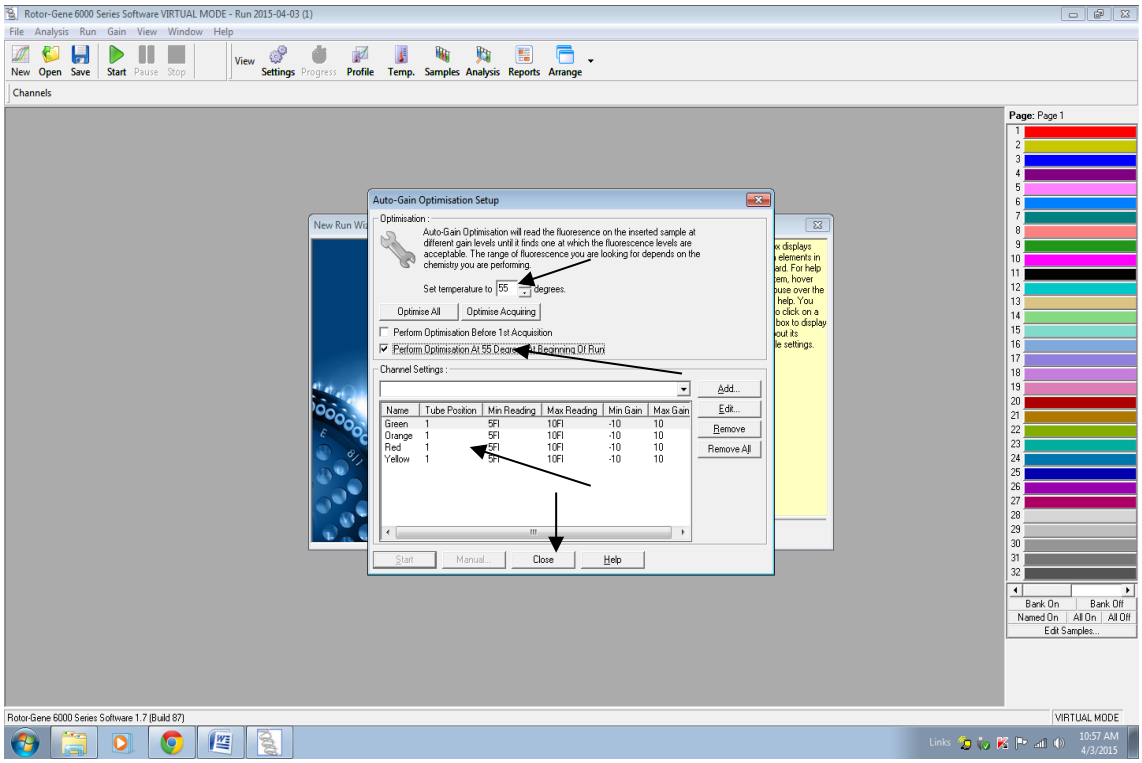

Fig. 33.

The following needs to be done.

- Click on the Optimise Acquiring
- Click on Set temperature To Adjust the Temperature to 55°C.
- Click on the Box Perform Optimisation at 55 degree at beginning of Run.
- Just see that below the channel settings there appear only two channels i.e. Green and Yellow. In case it is different then the channels have not been set properly. Close the window and reset the channels.
- Then Press Close.

L) PRESS Start RUN

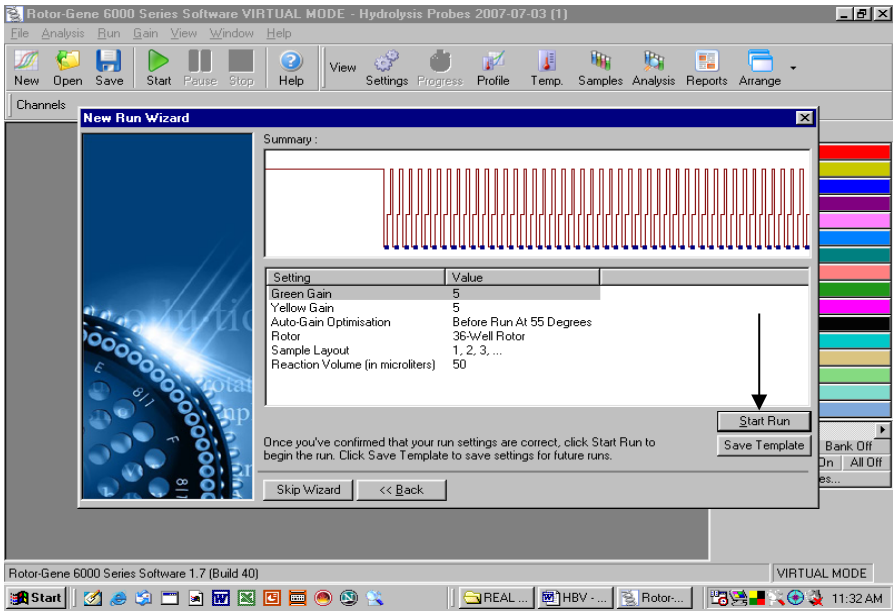

Fig. 34.

Saving the RUN File

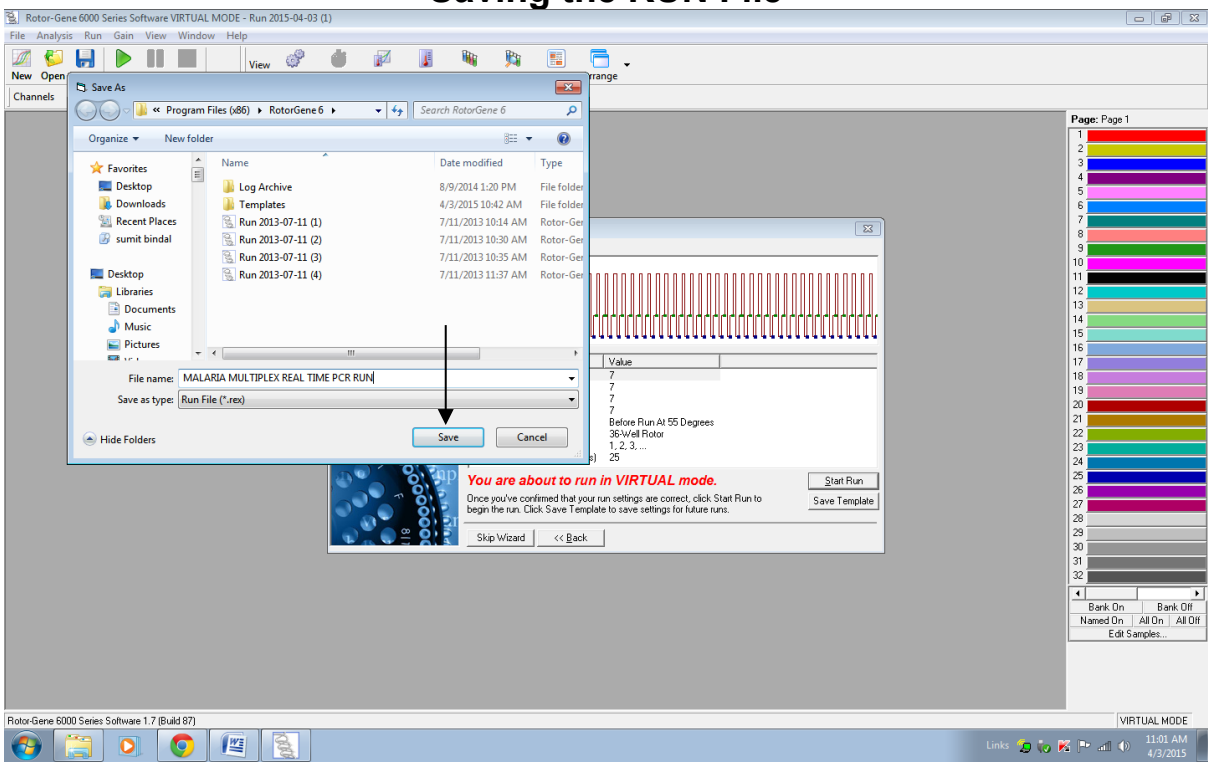

Fig. 35.

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

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

9. a) Generated Data Interpretation & Analysis for Rotor Gene 2000/3000

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

The following results are possible:

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

The result of the analysis is positive: The sample contains **P.falciparum DNA**.

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

No P.falciparum DNA detectable. It can be considered negative.

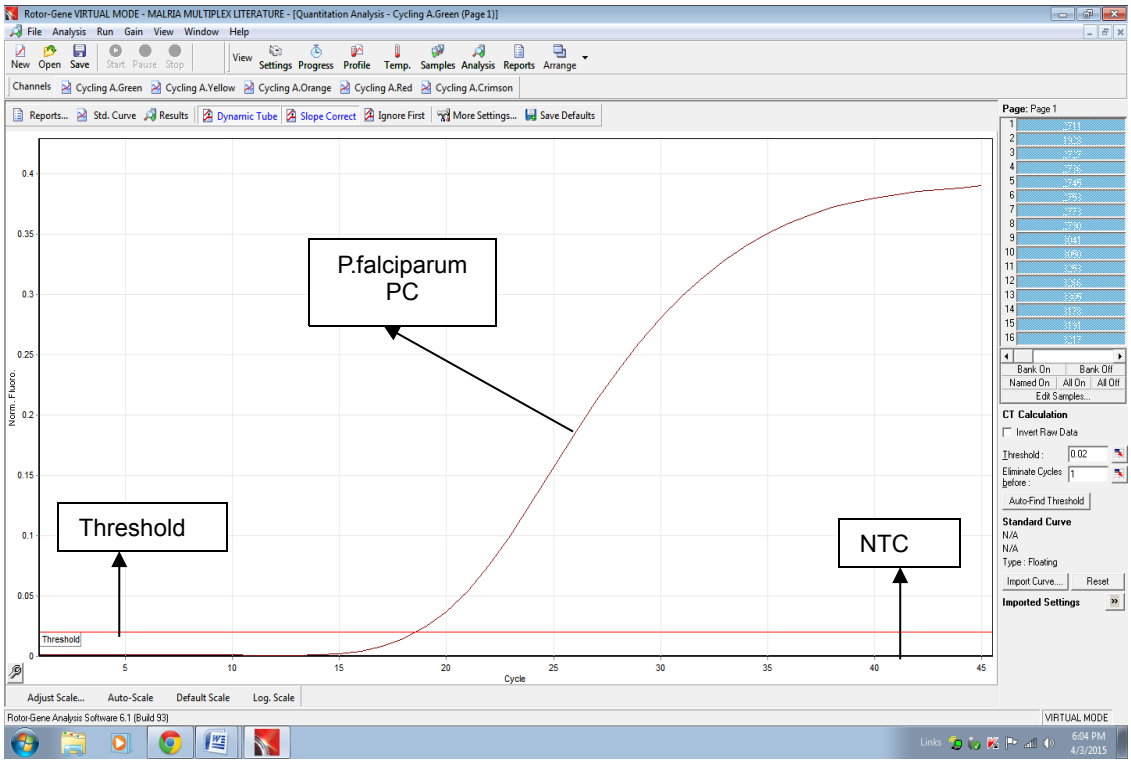

Fig.36: Detection of the **Malaria P.falciparum PC** in fluorescence channel Cycling A.FAM. NTC: non-template control

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

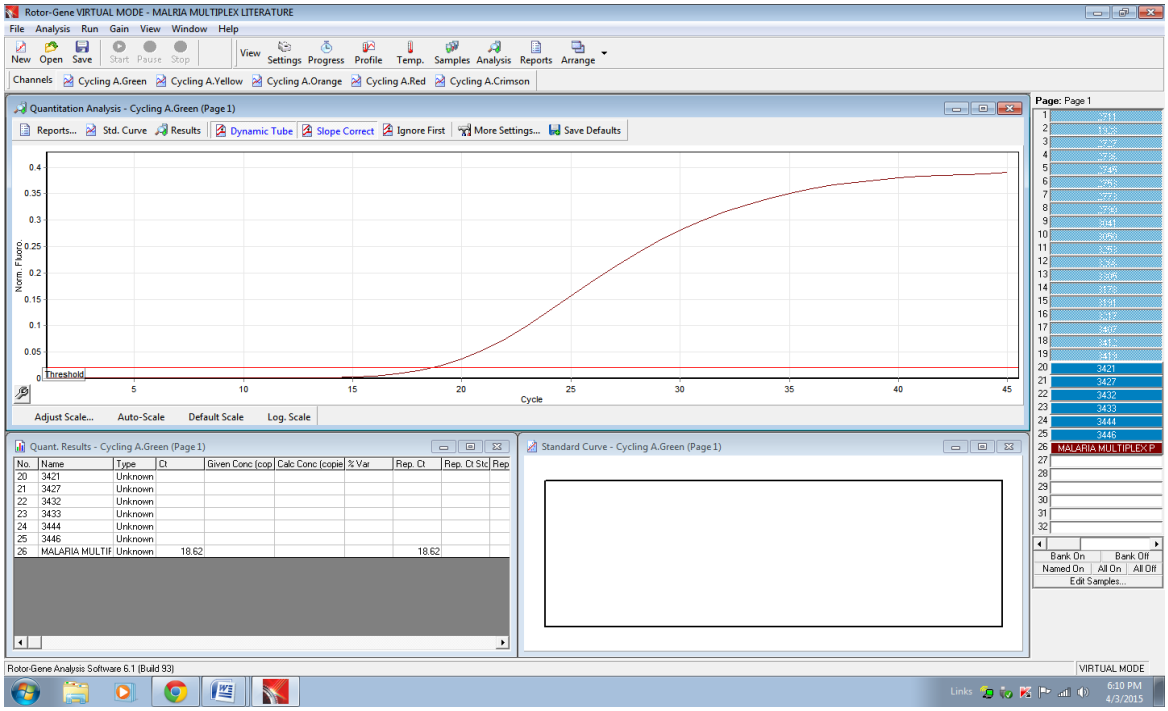

Fig. 37.

Example of analysed data for P.falciparum where amplification curve can be seen in FAM channel.

3. A signal is detected in fluorescence channel Cycling A.Cy5.

The result of the analysis is positive: The sample contains **P.VIVAX** DNA.

4. In fluorescence channel Cycling A.Cy5 no signal is detected.

No P.vivax DNA detectable. It can be considered negative.

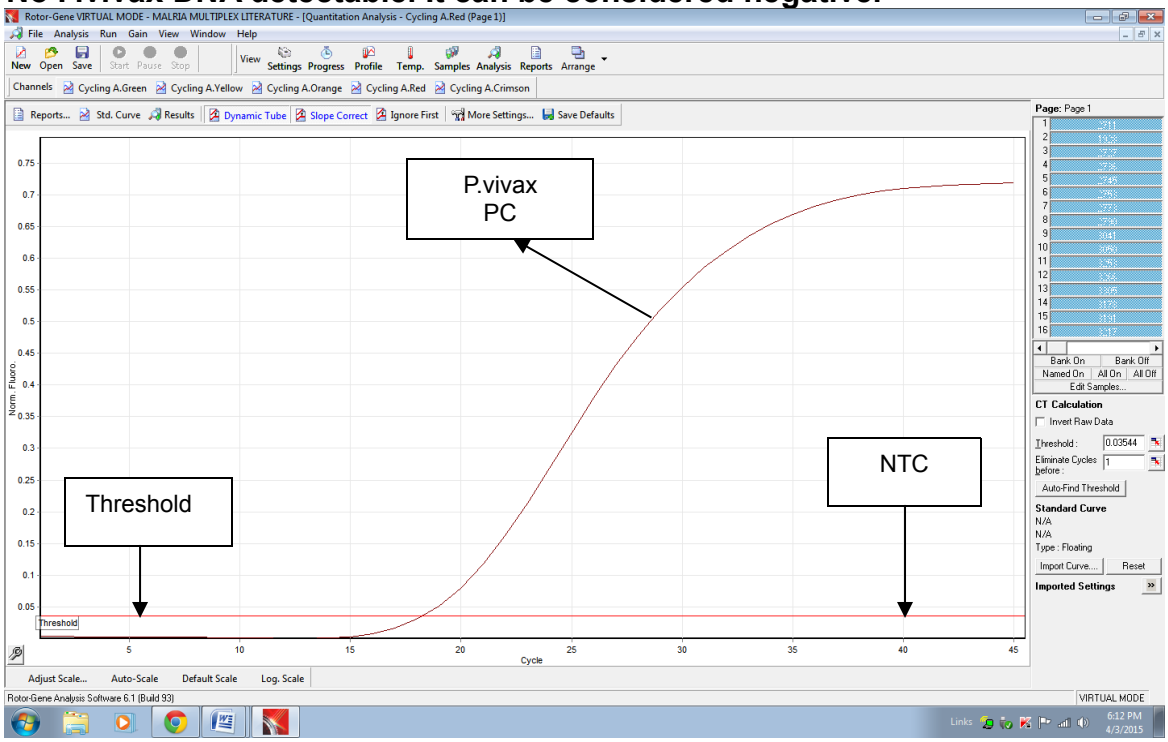

Fig.38: Detection of the **Malaria P. vivax** in fluorescence channel Cycling **A.Cy5**. NTC: non-template control

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

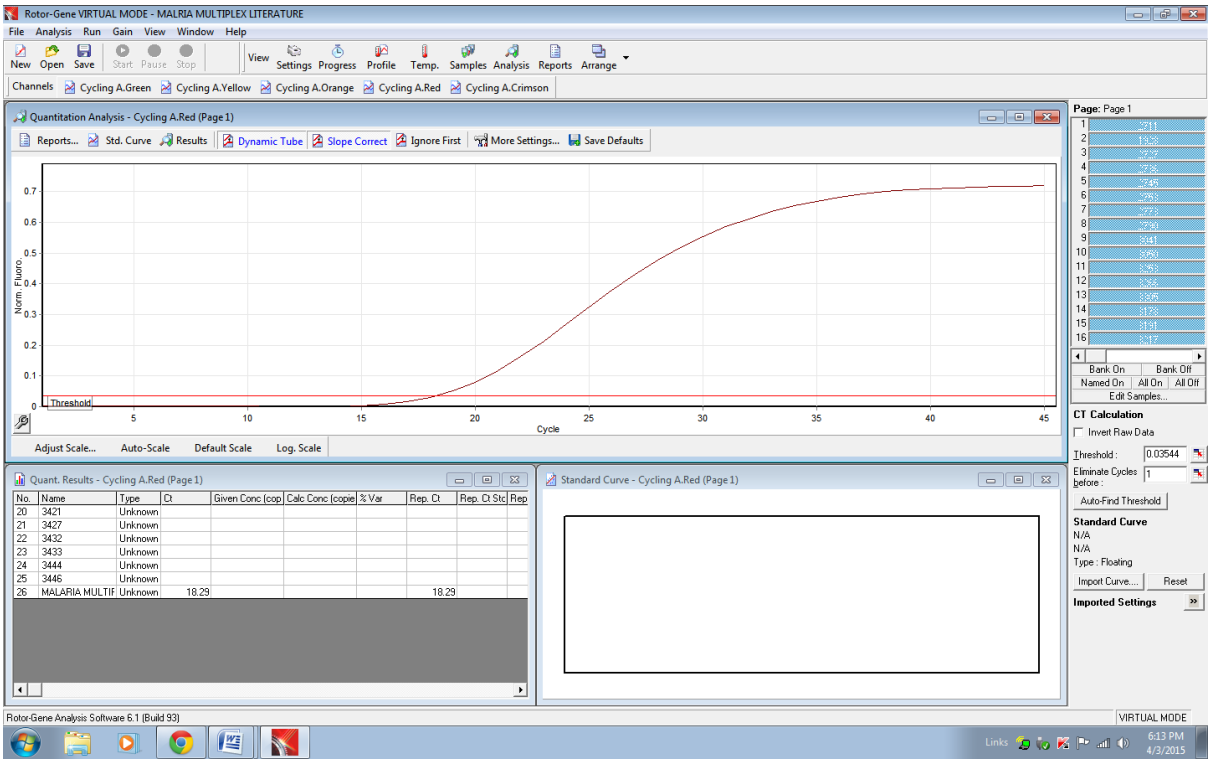

Fig. 39.

Example of analysed data for P.VIVAX where all the amplification curves can be seen in Cy5.

5. A signal is detected in fluorescence channel Cycling A.ROX.

The result of the analysis is positive: The sample contains **P.OVALE DNA**.

6. In fluorescence channel Cycling **A.ROX** no signal is detected.

No P.OVALE DNA detectable. It can be considered negative.

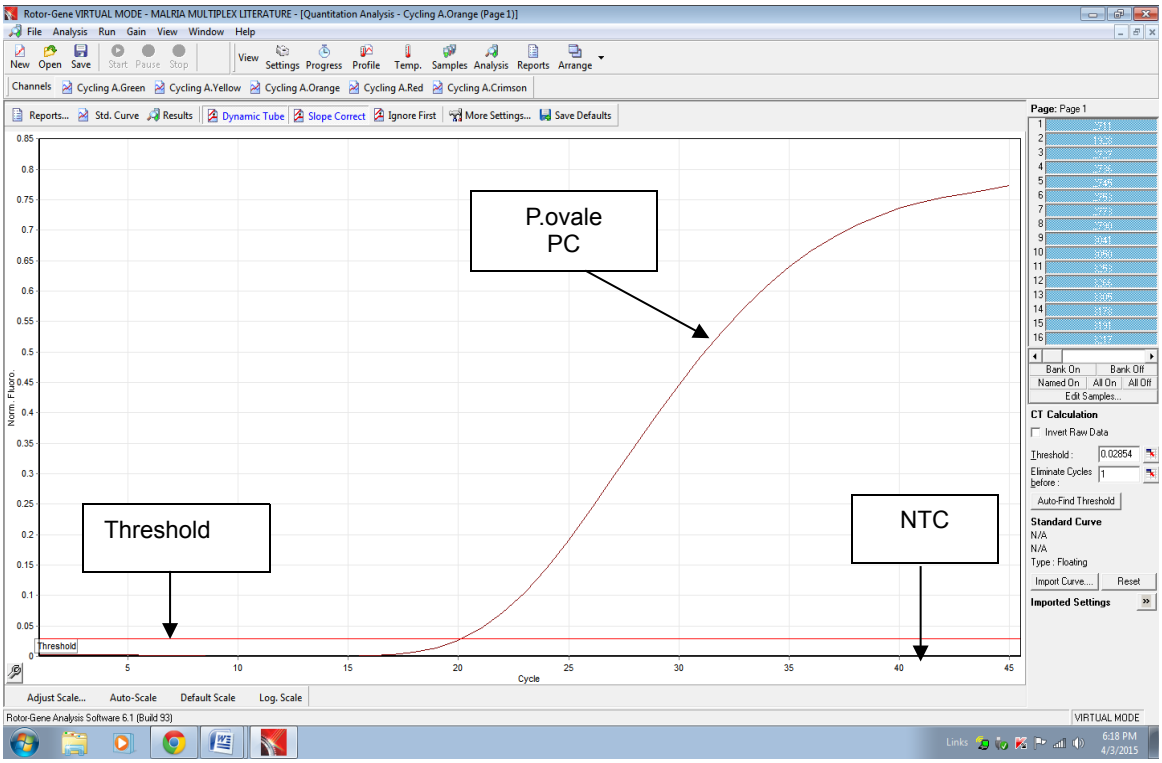

Fig.40: Detection of the Malaria P.ovale PC in fluorescence channel Cycling **A.ROX**.

NTC: non-template control

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

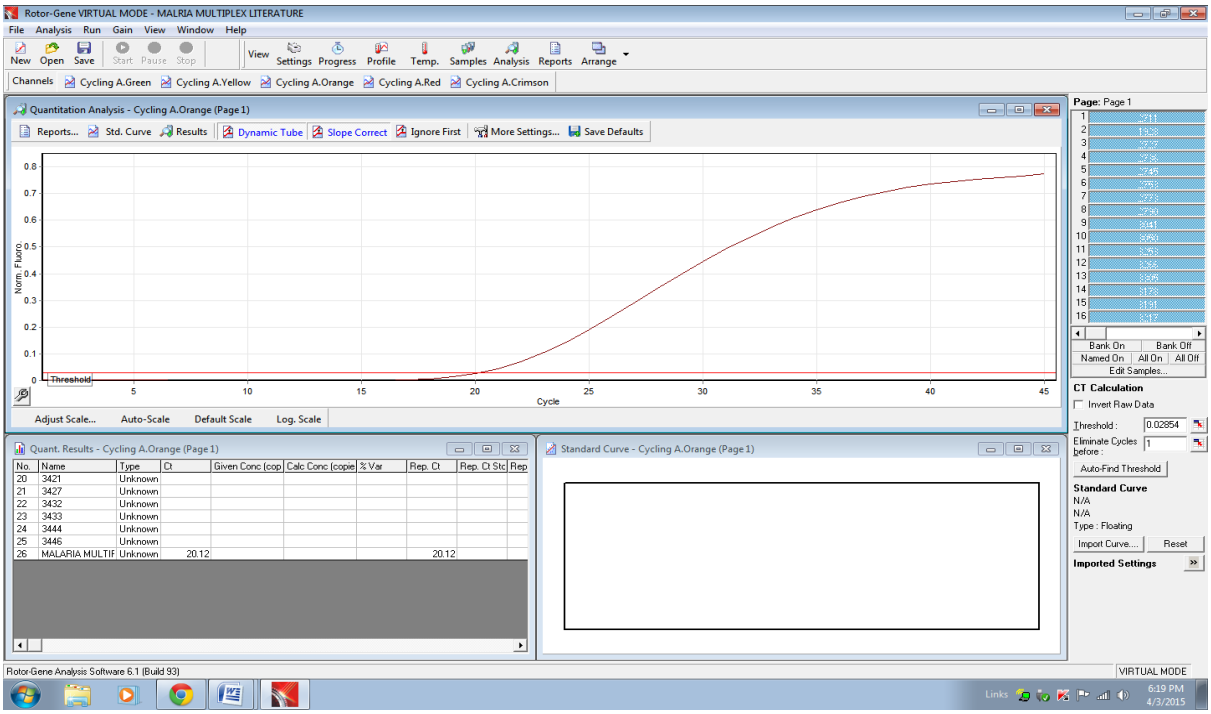

Fig. 41.

Example of analysed data for P.OVALE where all the amplification curves can be seen in ROX.

7. A signal is detected in fluorescence channel Cycling A.JOE.

The result of the analysis is positive: The sample contains **P.MALARIAE** DNA.

8. In fluorescence channel Cycling A.JOE no signal is detected.

No P.MALARIAE DNA detectable. It can be considered negative.

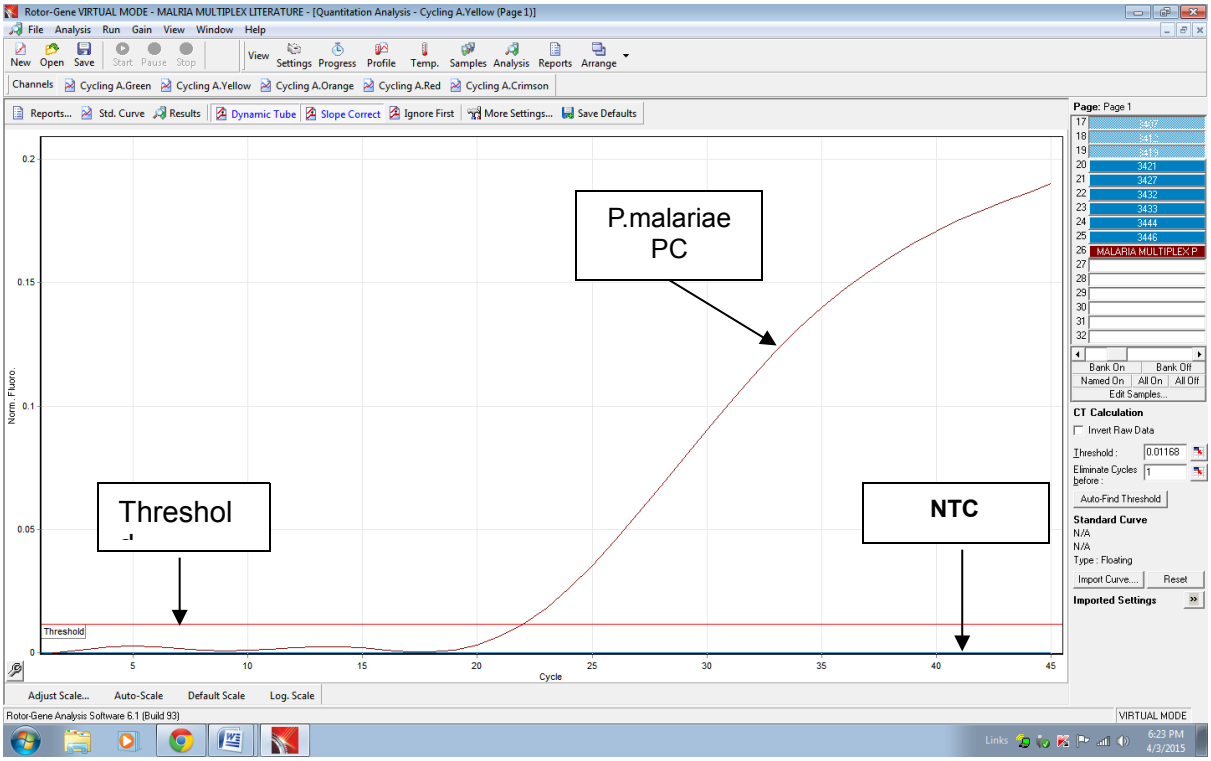

Fig.42: Detection of the **Malaria P. malariae** PC in fluorescence channel Cycling A.JOE. NTC: non-template control.

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

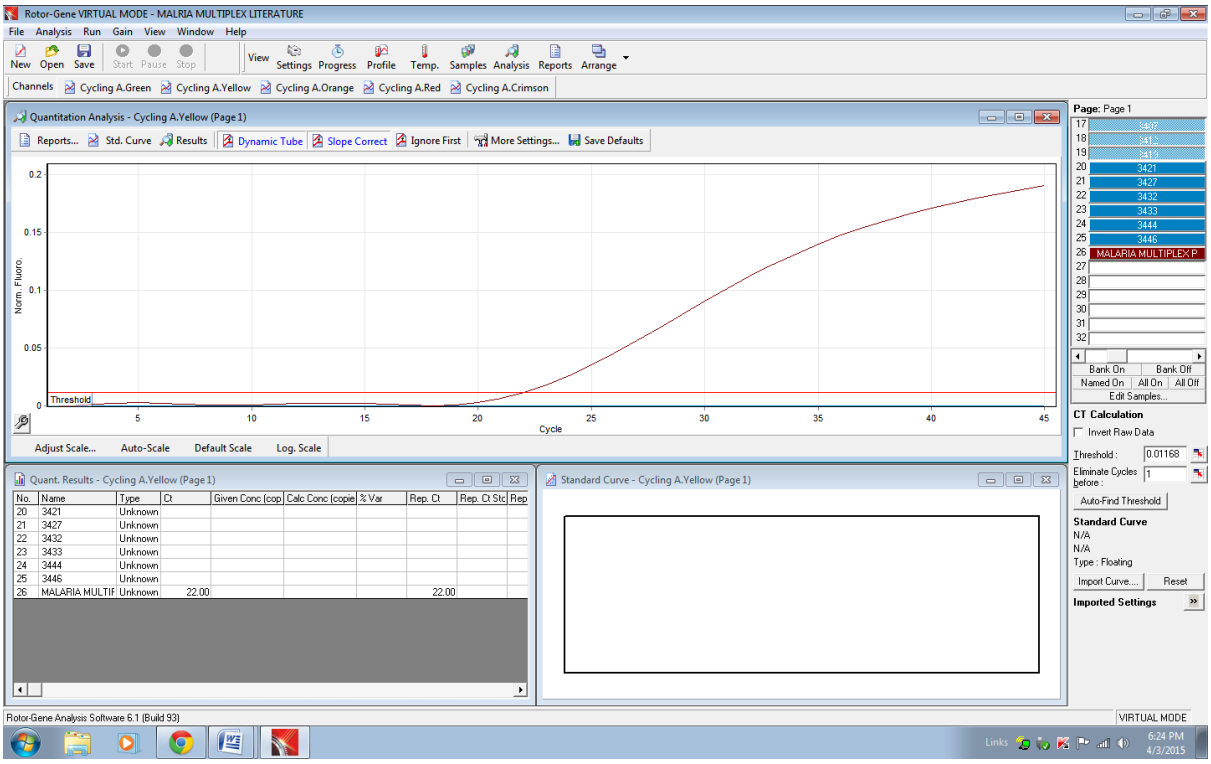

Fig. 43.
Example of analysed data for P.MALARIAE where all the amplification curves can be seen in JOE CHANNEL.

9. b) Generated Data Interpretation & Analysis for Rotor Gene 6000

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

The following results are possible:

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

The result of the analysis is positive: The sample contains **P.falciparum** DNA.

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

No P.falciparum DNA detectable. It can be considered negative.

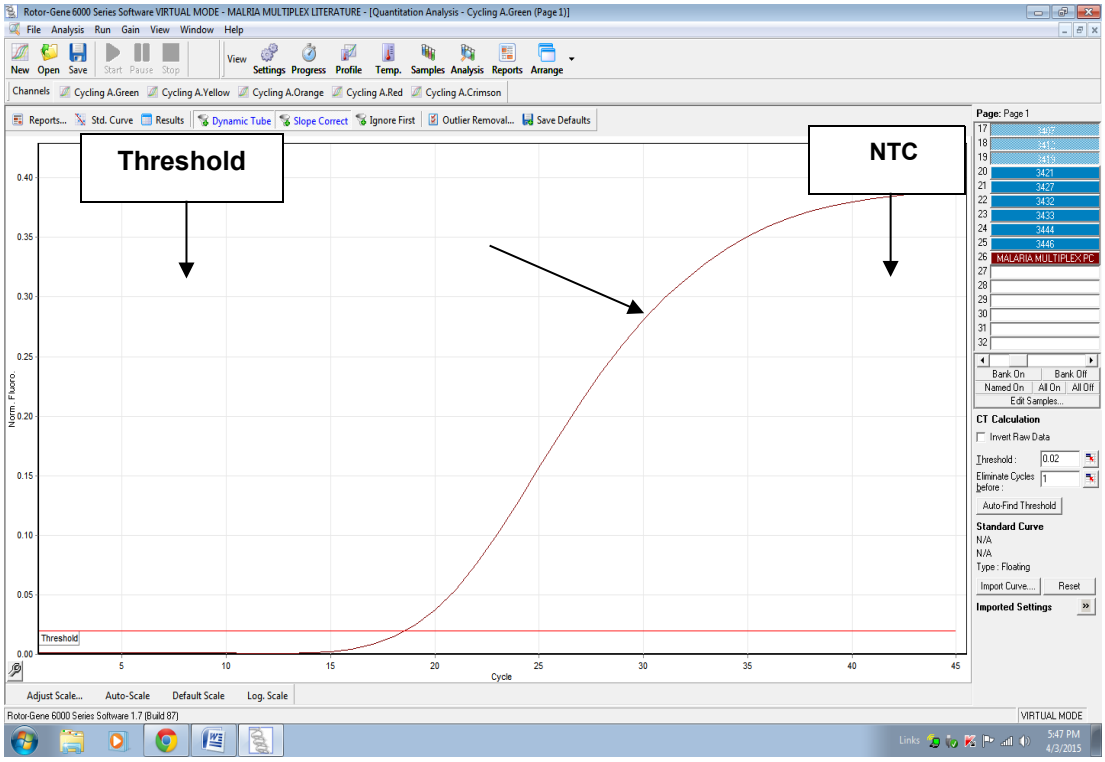

Fig.44: Detection of the Malaria P.falciparum PC in fluorescence channel Cycling A.GREEN. NTC: non-template control

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

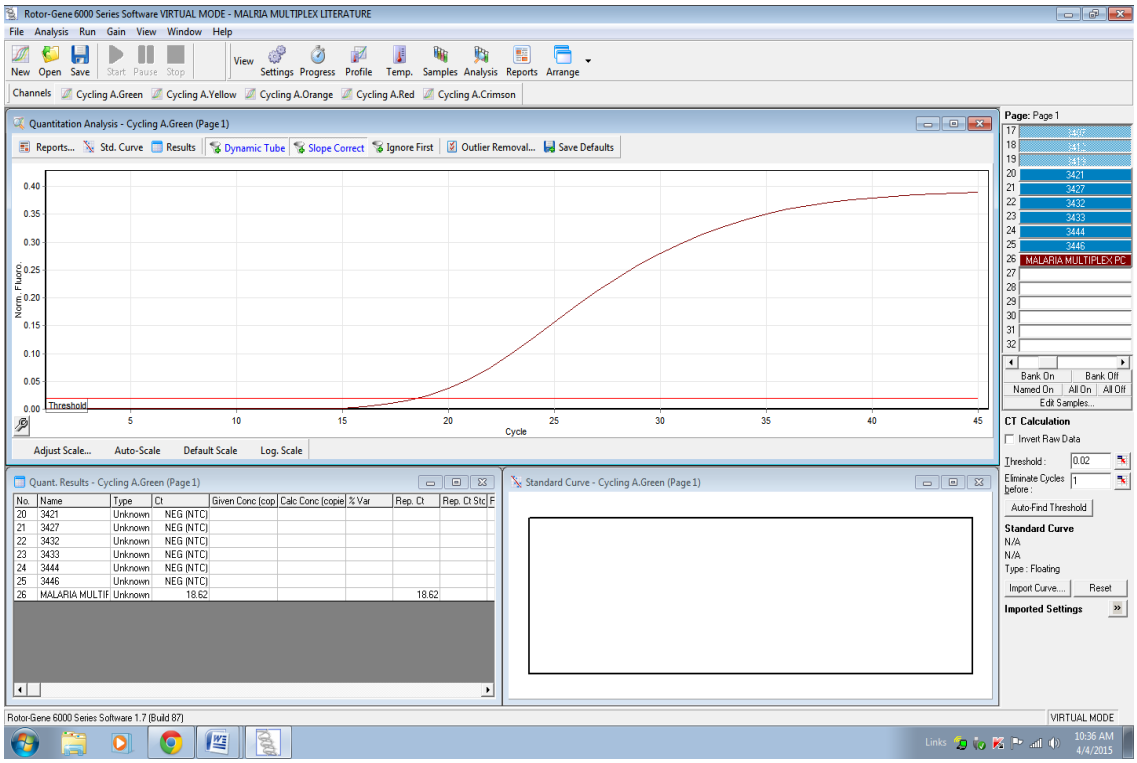

FIG.45.Example of analysed data for P.falciparum where amplification curve can be seen in GREEN channel.

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

The result of the analysis is positive: The sample contains **P.VIVAX** DNA.

4. In fluorescence channel Cycling **A.RED** no signal is detected.

No P.vivax DNA detectable. It can be considered negative.

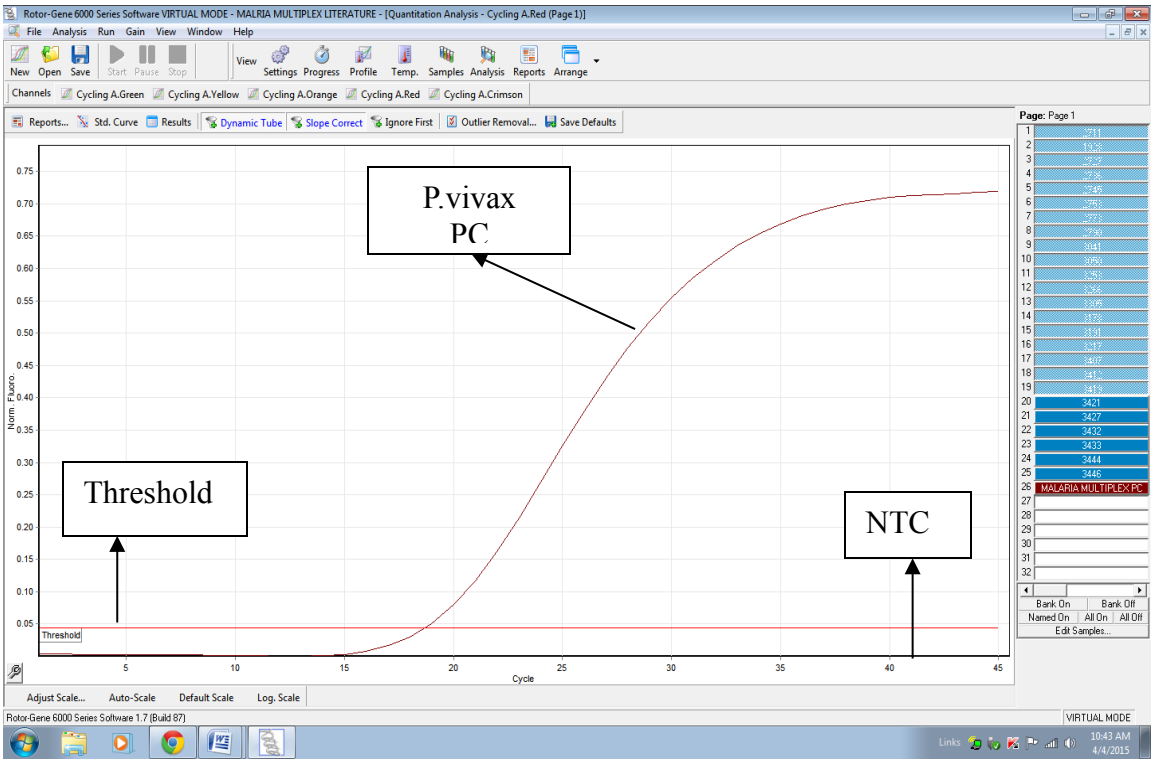

Fig.46: Detection of the **Malaria P.vivax PC** *in* fluorescence channel Cycling A.RED. NTC: non-template control

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

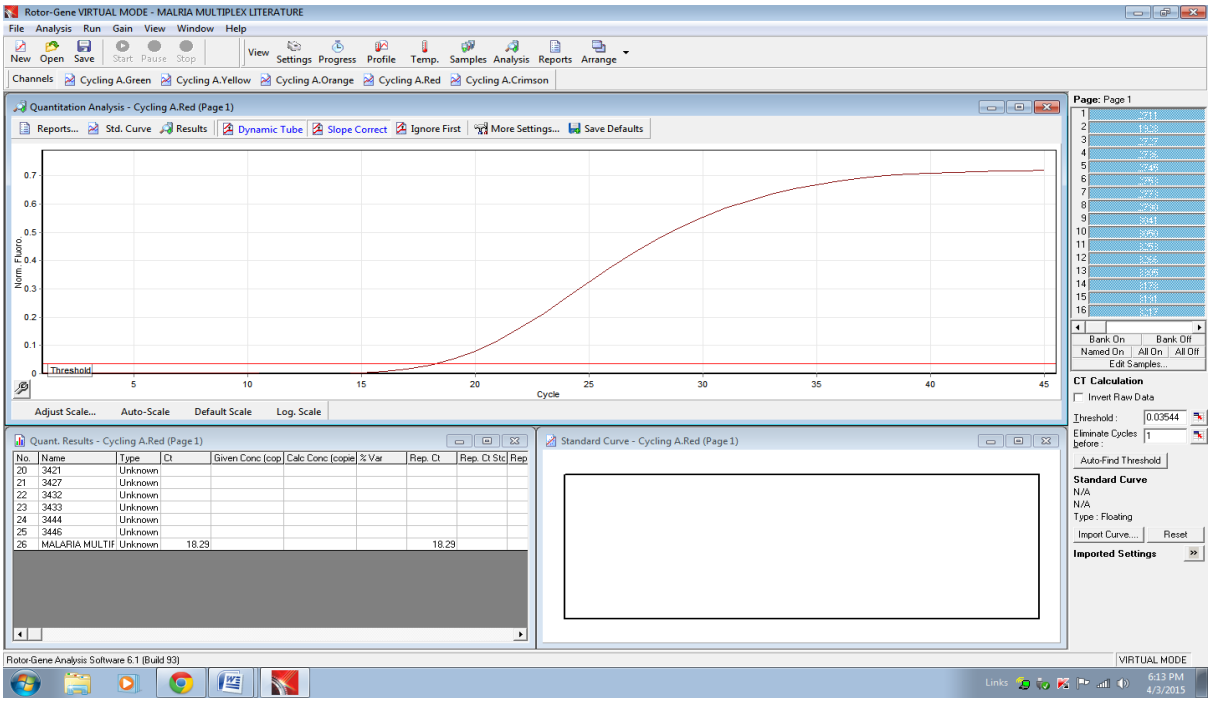

FIG.47.Example of analysed data for P.vivax where amplification curve can be seen in RED channel.

5. A signal is detected in fluorescence channel Cycling A.ORANGE.

The result of the analysis is positive: The sample contains **P.OVALE** DNA.

6. In fluorescence channel Cycling **A.ORANGE** no signal is detected.

No P.OVALE DNA detectable. It can be considered negative.

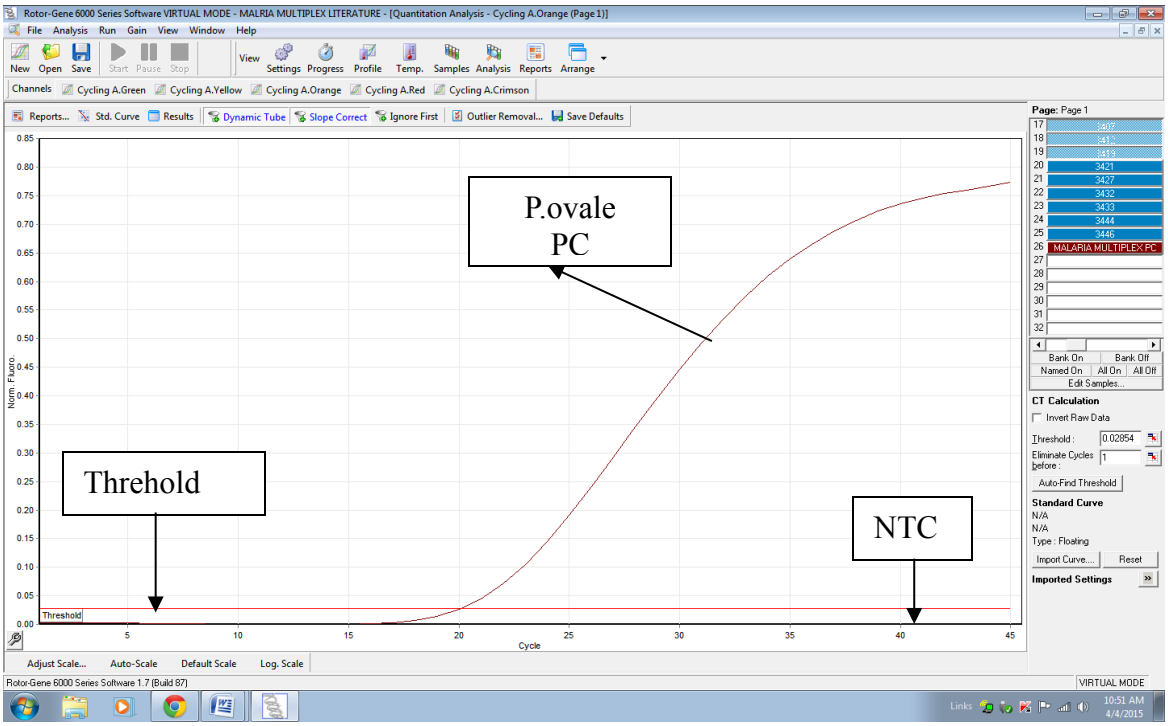

Fig.49: Detection of the **Malaria P.ovale PC** in fluorescence channel Cycling A.ORANGE. NTC: non-template control

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

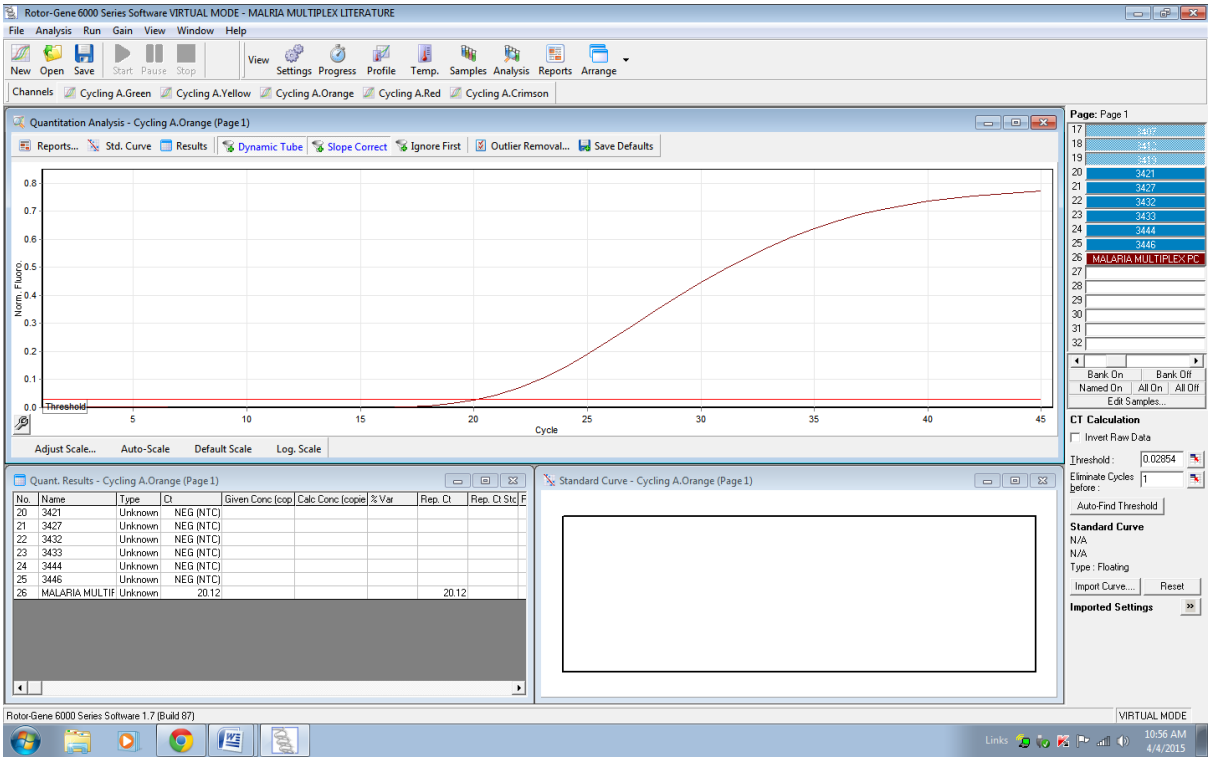

FIG.50. Example of analysed data for P.OVALE where amplification curve can be seen in ORANGE channel.

7. A signal is detected in fluorescence channel Cycling A.YELLOW.

The result of the analysis is positive: The sample contains **P.MALARIAE** DNA.

8. In fluorescence channel Cycling **A.YELLOW** no signal is detected.

No P.MALARIAE DNA detectable. It can be considered negative.

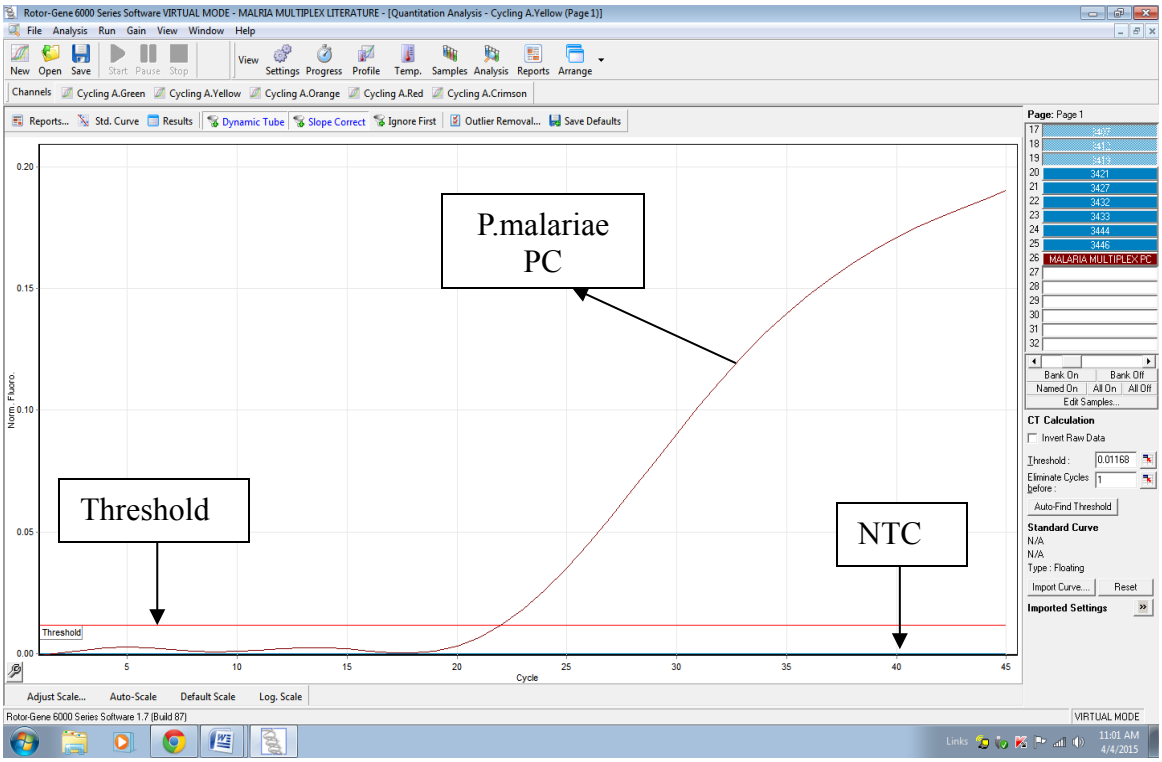

Fig.51: Detection of the Malaria P.malariae PC in fluorescence channel Cycling **A.YELLOW**. NTC: non-template control

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

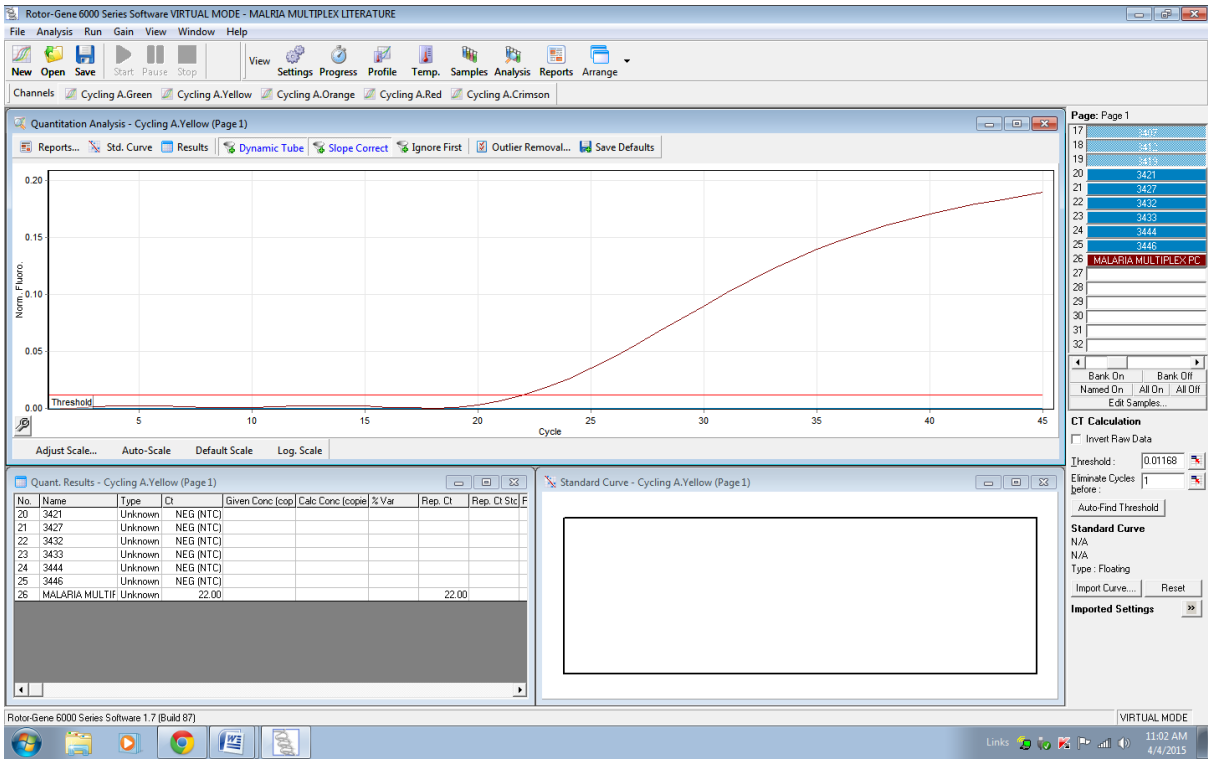

FIG.52. Example of analysed data for P.MALARIAE where amplification curve can be seen in YELLOW channel.

10. A) Troubleshooting for 2000/3000

1. No signal with POSITIVE CONTROL (P.falciparum & P. Vivax.P.OVALE & P.MALARIAE) in fluorescence channel Cycling A.FAM/CY5/ROX/JOE.

- 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/CY5/ROX/JOE:

- The PCR conditions do not comply with the protocol.

  ➔ Repeat the PCR with corrected settings.

- The **MALARIA MULTIPLEX Super Mix *R1*** has been thawed and frozen too often.
- The **MALARIA MULTIPLEX 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 **MALARIA MULTIPLEX Super Mix *R1***.

- **The PCR was inhibited.**

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

10. B) Troubleshooting for 6000

1. No signal with POSITIVE CONTROL (P.falciparum & P. Vivax.P.OVALE & P.MALARIAE) in fluorescence channel Cycling A. Green/Red/Orange/Yellow.

- 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/Red/Orange/Yellow:

- The PCR conditions do not comply with the protocol.

➔ Repeat the PCR with corrected settings.

- The **MALARIA MULTIPLEX Super Mix *R1*** has been thawed and frozen too often.
- The **MALARIA MULTIPLEX 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 **MALARIA MULTIPLEX Super Mix *R1***).

- **The PCR was inhibited.**

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

11. Specifications

11.a Sensitivity and Reproducibility

In order to determine the sensitivity of the ***Geno-Sen's*** **MALARIA MULTIPLEX** *Real Time PCR Kit*, a dilution series has been set up from 10^6 down to 10^0 Copies/µl of **MALARIA MULTIPLEX** DNA and analyzed with the ***Geno-Sen's*** **MALARIA MULTIPLEX** *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*** **MALARIA MULTIPLEX** *Real Time PCR Kit* is consistently 100 Copies/ml. This means that there is 95% probability that 100 Copies/ml will be detected.

11.b. Specificity

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

Vericella Zoster Virus	TTV	N. Meningitis
Human Herpes Virus 1 & 2	Hepatitis C Virus	S. Pneumonia
Epstein barr Virus	Hepatitis E Virus	ENTEROVIRUS
Cytomagalovirus	HIV-1	Mycobacterium tuberculosis
Chlamydia pneumonia	HIV-2	Hepatitis A
Parvovirus B 19	WEST NILE VIRUS	Staphylococcus aureus
Dengue Virus 1-4	H. Influenza	Salmonella enteritidis
Leprosy	Hepatitis B Virus	Scrub typhus
B.pseudomallie	JEV	Leptospira interrogans.

Further studies are underway on this aspect.

12. Warranty:

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

Diagnostics Pvt. Ltd. option, replacement of the purchasing price. Any complaint on damaged goods during transport must be directed to the handling or transport agent.

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

13. Limitations of product use:

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

If you have any further questions or problems, please contact our technical support at dharam@vsnl.com OR pbpl@vsnl.net.

14. EXPLANATION OF SYMBOLS USED:

Symbol	Explanation
	**Catalog Number**
	**Contains Sufficient for <n> reactions.**
	**Caution, Consult accompanying Documents**
	**Batch Number**
	***In Vitro* Diagnostic Medical Device**
	**Manufacturing Date**
	**Expiry date (Use before)**
	**Manufacturer.**
	**European Representative.**
	**Temperature limitation.**

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

S.NO.	PRODUCT
1	HIV-1 RG quantitative Real time PCR kit.

2	HBV RG quantitative Real time PCR kit.
3	HCV RG quantitative Real time PCR kit.
4	HCV Genotyping 1/2/3/4 RG qualitative Real time PCR kit.
5	HEV RG quantitative Real time PCR kit.
6	HAV RG quantitative Real time PCR kit.
7	JEV RG quantitative Real time PCR kit.
8	ENTEROVIRUS RG quantitative Real time PCR kit.
9	DENGUE RG quantitative Real time PCR KIT
10	HSV 1 & 2 RG quantitative Real time PCR kit.
11	CMV RG quantitative Real time PCR kit.
12	Hanta Virus RG quantitative Real time PCR kit.
13	Measles Virus RG quantitative Real time PCR kit.
14	West Nile Virus RG quantitative Real time PCR kit.
15	H5 N1 (Bird Flu) RG quantitative Real time PCR kit.
16	Chikungunya RG quantitative Real time PCR kit.
17	TTV RG quantitative Real time PCR kit.
18	SARS RG quantitative Real time PCR kit.
19	JC/BK Virus RG quantitative Real time PCR kit.
20	MTb Complex RG quantitative Real time PCR kit.
21	MTb Complex /MOTT RG quanlitative Real time PCR kit.
22	Chlamydia pneumonia RG quantitative Real time PCR kit.
23	Streptococcous pneumonia RG quantitative Real time PCR kit.
24	N. Meningitis RG quantitative Real time PCR kit.
25	H. Influenza RG quantitative Real time PCR kit.

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

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