# Geno-Sen's

# Factor V leiden (Rotor Gene)

# Real Time PCR Kit

**Qualitative**

for use with the

*Rotor Gene™ 2000/3000/6000*

(Corbett Research Australia)

IVD

**PACK INSERT**

Revised July 2016

CE


## Genome Diagnostics Pvt. Ltd.

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

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Table of Contents

***Factor V leiden 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 91116101 100 rxns | REF 91116102 50 rxns | REF 91116103 25 rxns |
|---|---|---|---|---|
| R1 Blue | Factor V leiden 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 |
| Fac V (PC) W Red | Factor V leiden *Positive Control (W)* | 1 Vial of 100µl | 1 Vial of 100µl | 1 Vial of 100µl |
| Fac V (PC) Mu Red | Factor V leiden *Positive Control (Mu)* | 1 Vial of 100µl | 1 Vial of 100µl | 1 Vial of 100µl |
| 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. FACTOR V LEIDEN Information

## Application

Factor V Leiden thrombophilia is characterized by a poor anticoagulant response to activated protein C (APC) and an increased risk of venous thromboembolism. The term "factor V Leiden" refers to the specific G-to-A substitution at nucleotide 1691 in the gene for factor V that predicts a single amino acid replacement (R506Q) at one of three APC cleavage sites in the factor Va molecule. Factor V Leiden is inactivated at a rate approximately ten times slower than normal factor V and persists longer in

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

the circulation, resulting in increased thrombin generation and a mild hypercoagulable state reflected by elevated levels of prothrombin fragment F1+2 and other activated coagulation markers. Individuals heterozygous for the factor V Leiden mutation have a slightly increased risk for venous thrombosis; homozygous individuals have a much greater thrombotic risk.

The diagnosis of factor V Leiden thrombophilia is suspected in individuals with a history of venous thrombosis or in families with a high incidence of venous thrombosis. The diagnosis of factor V Leiden thrombophilia is made either using a coagulation screening test or by DNA analysis of the *F5* gene, which encodes the factor V protein.

Although no clinical features are specific for factor V Leiden thrombophilia, the diagnosis is suspected in individuals with a history of venous thromboembolism (VTE) [deep vein thrombosis (DVT) or pulmonary embolism], especially in women with a history of VTE during pregnancy or in association with oral contraceptive use, and families with a high incidence of VTE.

There is growing consensus that testing for factor V Leiden should be performed in the following circumstances [ACMG Consensus Statement2001 , CAP Consensus Conference Statement 2002.

- A first VTE before 50 years of age
- A first unprovoked VTE at any age
- Recurrent VTE
- Venous thrombosis at unusual sites (such as cerebral, mesenteric, portal, and hepatic veins)
- VTE during pregnancy, the puerperium, or in association with oral contraceptive use or hormone replacement therapy
- A first VTE and a strong family history of VTE (No consensus or universally accepted definition of "strong family history" has been developed.)

Testing for factor V Leiden may also be considered for the following individuals:

- Asymptomatic adult family members of probands with a known factor V Leiden mutation, especially those with a strong family history of VTE at a young age
- Asymptomatic female family members of probands with known factor V Leiden who are pregnant or are considering oral contraceptive use or pregnancy
- Women with unexplained pregnancy loss during the second or third trimester
- Selected women with unexplained severe preeclampsia, placental abruption, or intrauterine growth retardation
- Women with a first VTE related to tamoxifen or other selective estrogen receptor modulators (SERM)

- Female smokers under 50 years of age with a myocardial infarction
- Individuals older than 50 years of age with a first provoked VTE in the absence of malignancy or of an intravascular device

Inherited thrombophilia predispose an individual to thrombotic events such as venous thrombosis, the third most common cardiovascular disease. Activated protein C (APC) resistance is regarded as the most prevalent coagulation abnormality associated with venous thrombosis. A point mutation at position 1691 of the Factor V gene, referred to as Factor V Leiden mutation, causes an arginine to glutamine substitution at position 506 in the Factor V protein and renders it partially resistant to inactivation by APC. Genetic analysis has demonstrated that this mutation, which has a relatively high prevalence in the general population (e.g., about 5% in Caucasians), accounts for 85% to 95% of APC resistance cases.

Samples which can be used: Whole Blood.

## 4. Precautions for PCR

The following aspects should always be taken care of:

- Store positive material (Specimens, Standards or amplicons) separately from all other reagents and add it to the reaction mix in a separate facility.
- Thaw all components thoroughly at room temperature before starting the assay.
- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice or in the Cooling Block.
- All the reagents including the NTC (except for standards & specimens) should be mixed & dispensed in pre-mix area.
- All the standards & specimens should be mixed & dispensed in extraction area.
- Use pipette tips with filters only.
- Always use disposable powder-free gloves

## 5. Additionally Required Materials and Devices

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

- Sterile pipette tips with aerosol barrier 2-20µl, 10-100µl, 100-1000µl,
- Disposable powder-free gloves
- Vortex mixer
- Centrifuge Desktop with rotor for 1.7 ml reaction tubes
- *Rotor Gene™ 2000/3000* or *Rotor Gene™ 6000*, Corbett Research (The Real time PCR Instrument)

## 6. Principle of Real-Time PCR

1. A 163 bp fragment of the Factor V gene is amplified from human genomic DNA using specific primers.
2. The amplicon is detected by fluorescence using a specific pair of Hybridization Probes. The Hybridization Probes consist of two different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of the PCR cycle. One probe is labeled at the 5´-end with Cy5 and to avoid extension, modified at the 3´-end by phosphorylation. The other probe is labeled at the 3´-end with fluorescein.
3. Only after hybridization to the template DNA, do the two probes come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, fluorescein, the donor fluorophore, is excited by the light source of the Instrument, and part of the excitation energy is transferred to Cy5 the acceptor fluorophore.
4. The emitted fluorescence of Cy5 is then measured by the Instrument.

Genotyping

The Hybridization Probes are also used to determine the genotype by performing a melting curve analysis after the amplification cycles are completed and the amplicon is present at increased concentration.

- The Cy5 labeled Hybridization Probe hybridizes to a part of the target sequence that is not mutated and functions as an anchor probe.
- The Fluorescein-labeled Hybridization Probe spans the mutation site (mutation probe).

During the melting curve analysis, increasing temperature causes the fluorescence to decrease because the shorter of the two probes (mutation probe) dissociates first and the two fluorescent dyes are no longer in close proximity. If the Factor V Leiden mutation is present, the mismatch of the mutation probe with the target destabilizes the hybrid so the decrease in fluorescence will occur at a lower temperature. With the wild-type genotype mismatches will not occur, and therefore the heteroduplex has a higher melting temperature (Tm). The heterozygous genotype exhibits a distinctive combination of properties.

## 7. Description of the Product.

The ***Geno-Sen's Factor V Leiden*** *PCR* Reagents constitute a ready to use system for detection of Factor V Leiden 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 Factor V Leiden and for subsequent melt detection of the specific amplicon. External Positive Controls (FV PC (W) & FV PC Mu) are also supplied.

## 8. Procedure

### 8.a DNA Extraction

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

| **Sample Material** | **Nucleic Acid Isolation Kit** | **Cat. Num. REF** |  |
|---|---|---|---|
| Whole Blood.etc. | ***Geno Sen's*** **® Genomic DNA Extraction Mini Kit — for DNA purification** | 98021 or 98022 | Genome Diagnostics Pvt. Ltd. India |
| | QIAamp Blood DNA Mini extraction Kit (50) | 51104 | QIAGEN |
| | Suitable Extraction kit which give a higher yield. | | |

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 *Factor V leiden Rotor Gene PCR Reagents* should not be used with phenol based isolation methods.

### 8.b *Inhibition Control:*

There is no Inhibition Control incorporated in the kit.

### 8.c Positive Controls

The Positive Controls provided in the kit (Fac V PC (W) & Fac V PC Mu) are treated in the same way as extracted samples and the same volume is used i.e. (2.5µl) instead of the sample. To define the Positive Controls in the *RotorGene™ 2000/3000/6000*, both Positive Controls should be used as defined in the menu window *Edit Samples* of the *RotorGene™* software. The same should also be defined as Positive Controls (see *Rotor Gene™* Manual).

## 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 Controls & at least one negative control (*Water, PCR grade*) are included per PCR run. Before each use, all reagents need to be thawed completely and mixed (by pipetting or by brief vortexing).

Please follow the pipeting scheme mentioned below for each sample depending upon the number of samples a mix can be prepared as follows. Each sample

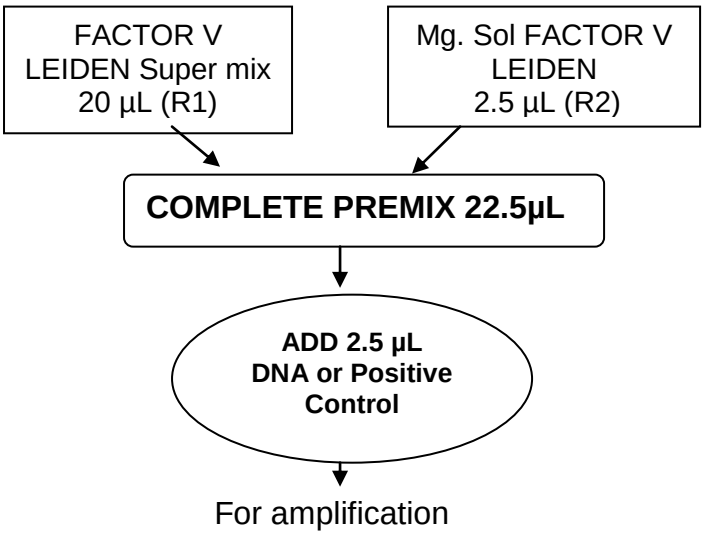


Fig. 4.

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

| FACTOR V LEIDEN MASTER MIX | 1 rxns. | 10 rxns. |
|---|---|---|
| FACTOR V LEIDEN Super Mix (R1) | 20 µL | 200 µL |
| FACTOR V LEIDEN Mg Sol. (R2) | 2.5 µL | 25 µL |
| Total | 22.5µL | 225µL |

Fig. 5.

Pipette 22.5 µl of the Master Mix into each labelled PCR tube. Then add 2.5 µl of the earlier extracted DNA to each sample tube and mix well by pipeting up and down. Correspondingly, 2.5 µl of the Positive Controls i.e. (Fac V PC (W) & Fac V PC Mu) must be used as a positive control and 2.5 µl of water (*Water, PCR grade*) as a negative control. Close the PCR tubes and transfer the FACTOR V LEIDEN tubes into the rotor of the *RotorGene*™ instrument. The *RotorGene*™ software versions 5.0.53 and higher require a Locking Ring (accessory of the *RotorGene*™, Corbett Research) to be placed on top of the rotor to prevent accidental opening of the tubes during the run.

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

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

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

Program the *RotorGene*™ *2000/3000* for these 5 steps according to the parameters shown in Fig. 6-24. All specifications refer to the *RotorGene*™ software version 6.0.33. Please find further information on programming the *RotorGene*™ in the *RotorGene*™ *2000/3000 Operator's Manual*,. In the illustrations these settings are shown by arrows

## Setting of general assay parameters & Reaction volume.

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

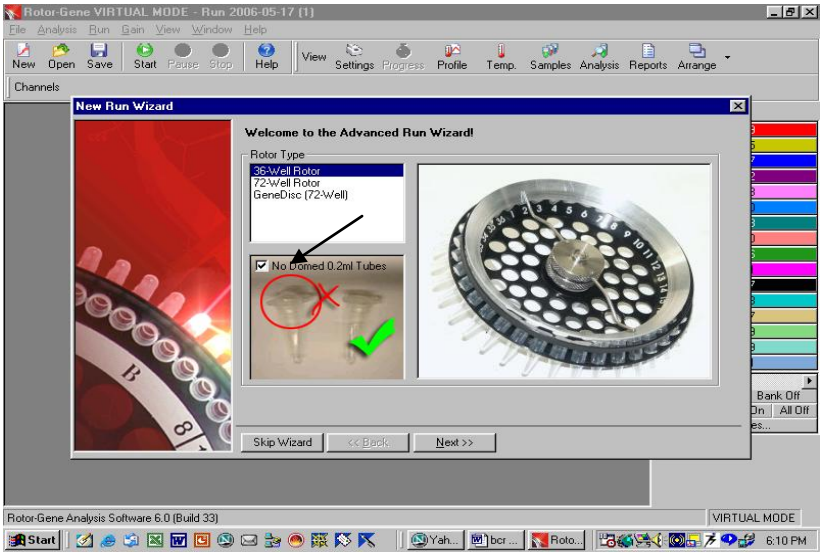


Fig. 6.

## Confirmation of Reaction Volume as follows.

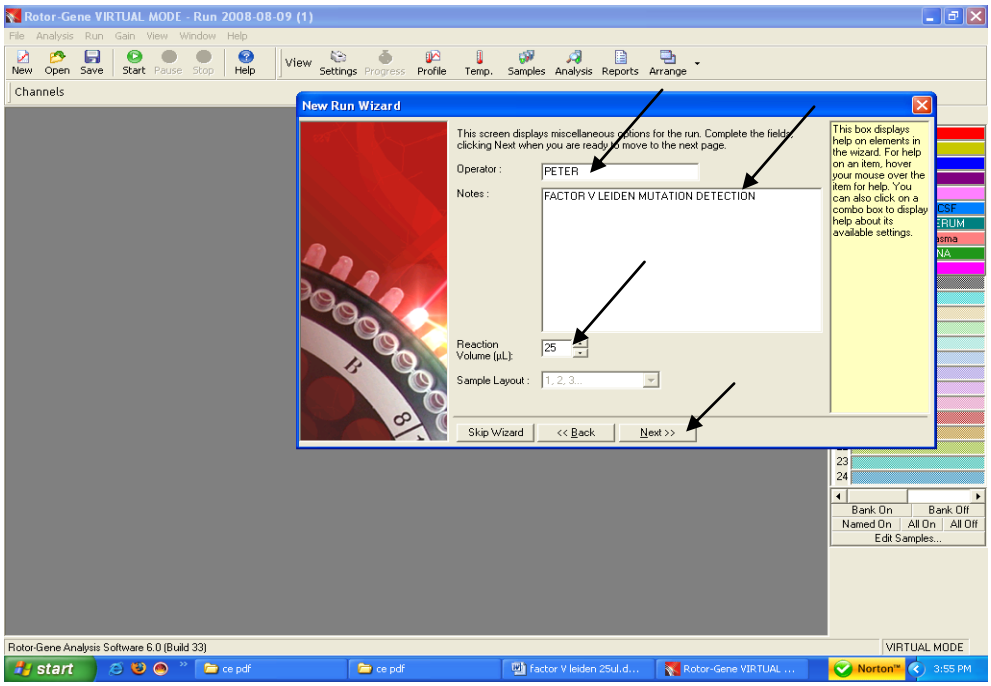


Fig. 7. Setting of general assay parameters.

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

## THERMAL PROFILE & CALIBRATION:

**Here the thermal profile for the assay will be defined.**

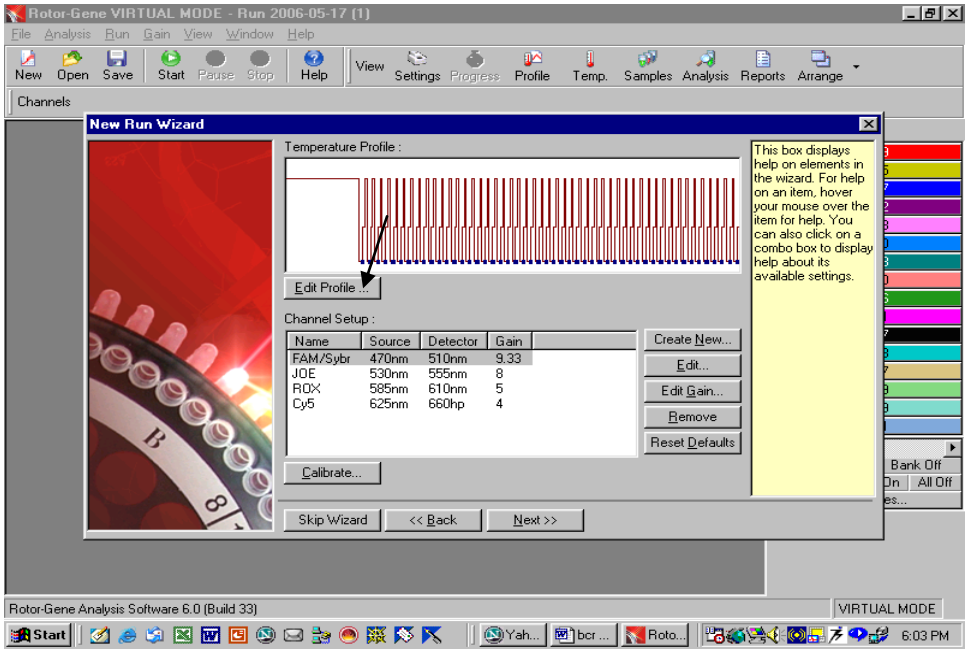


Fig. 8.

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

**CYCLING PROFILE: Hold** 95°C for 10 minutes as below

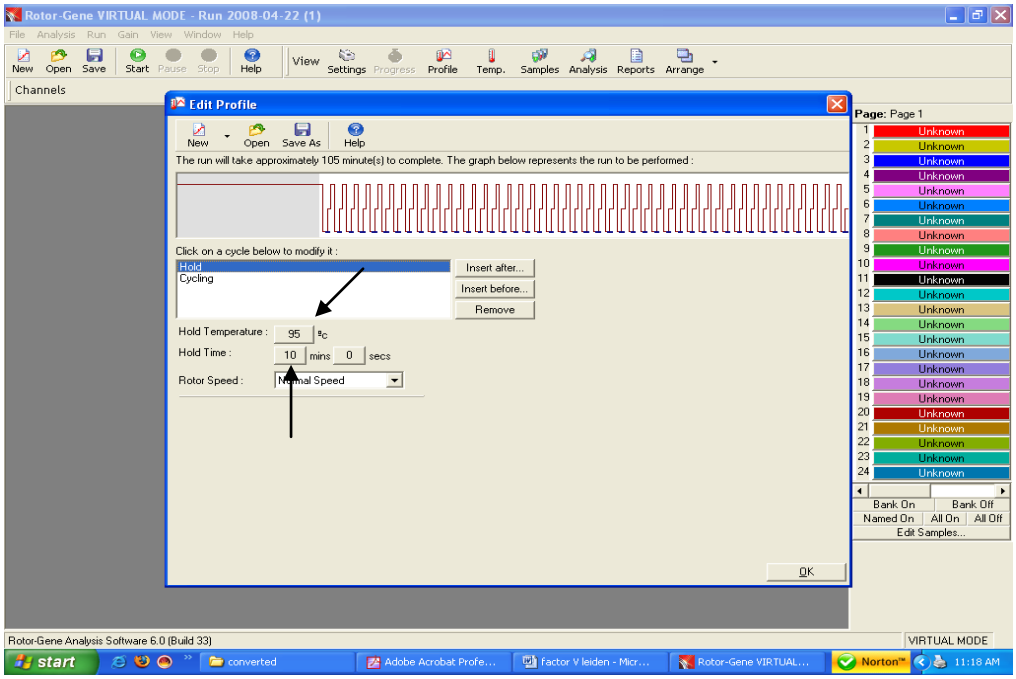


Fig. 9. Initial activation of the Hot Start enzyme.

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

95°C for 15 seconds.

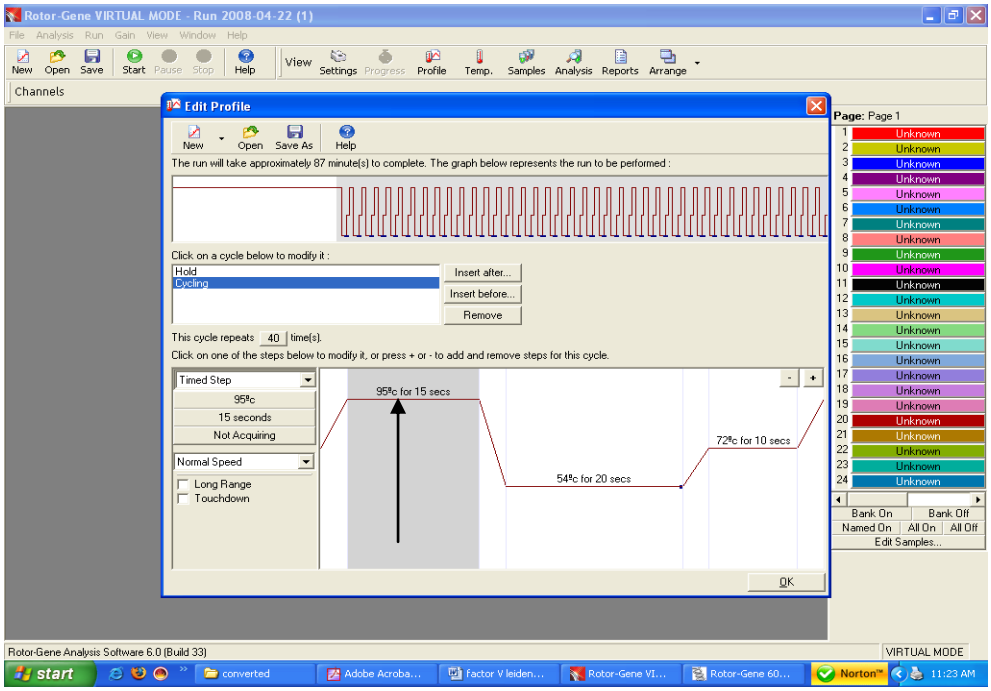


Fig. 10.

**Setting up of Anneling step in the cycling profile as depicted below i.e. 54**°C for 20 **Seconds and defining the Data acquiring channel i.e FAM & Cy5**

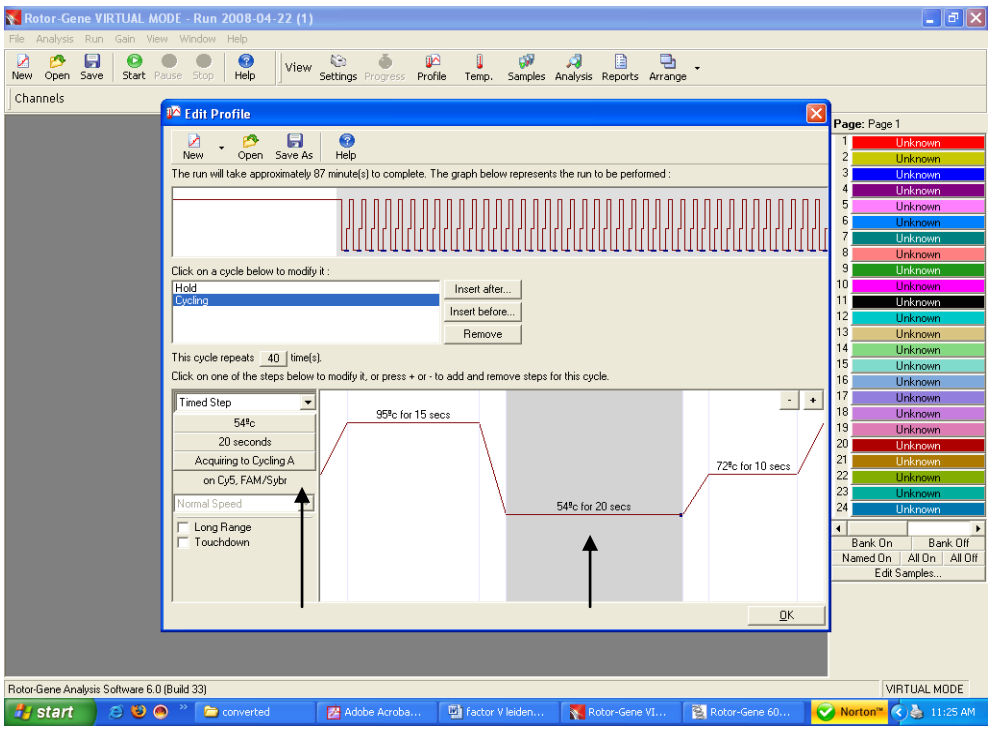


Fig. 11.

**Setting up of Extension step in the cycling profile as depicted below i.e. 72**°C for 10 **Seconds**

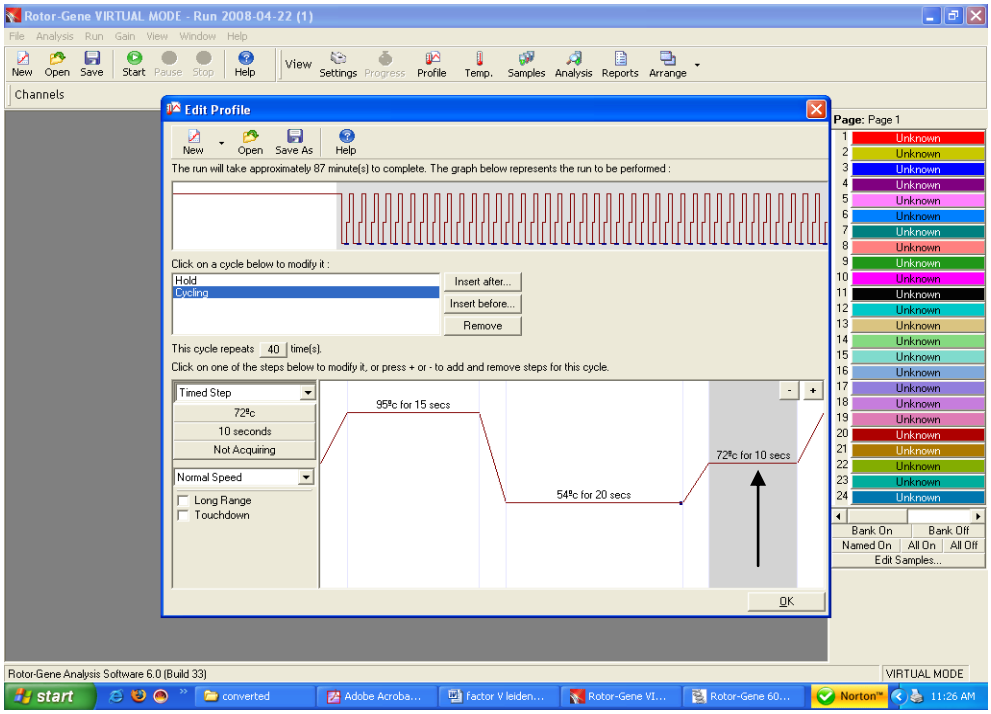


Fig. 12.

**Setting up of Number of Cycles to 40 cycles in the cycling profile as depicted below.**

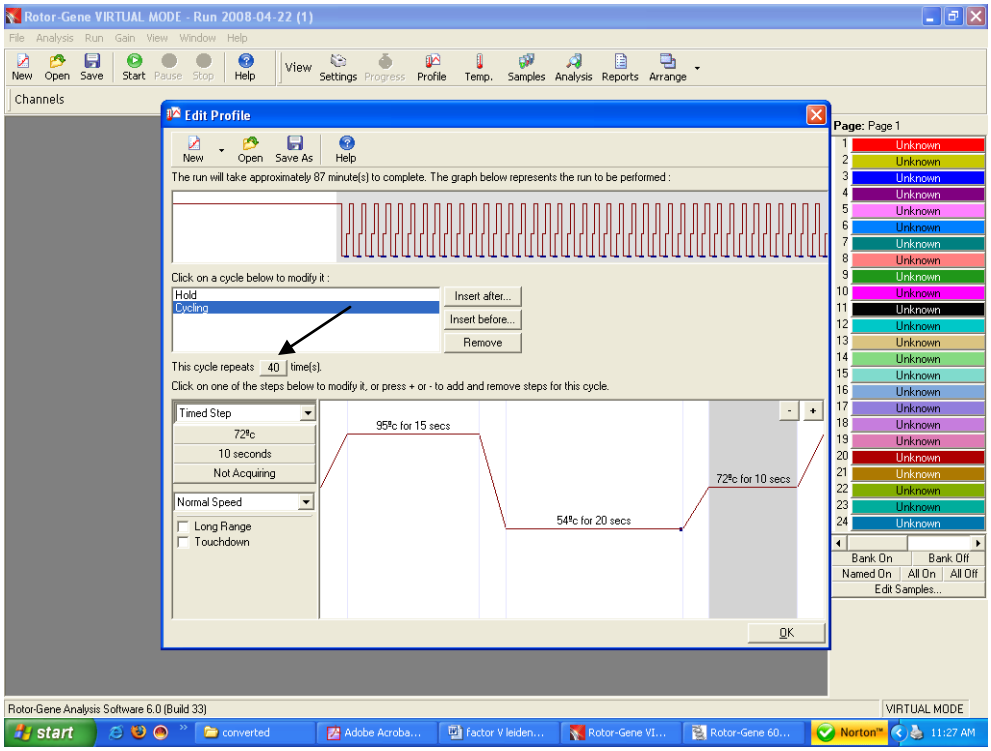


Fig. 13.

## Setting up of Melt:

Hold-1,,, 95°C for 15 seconds.

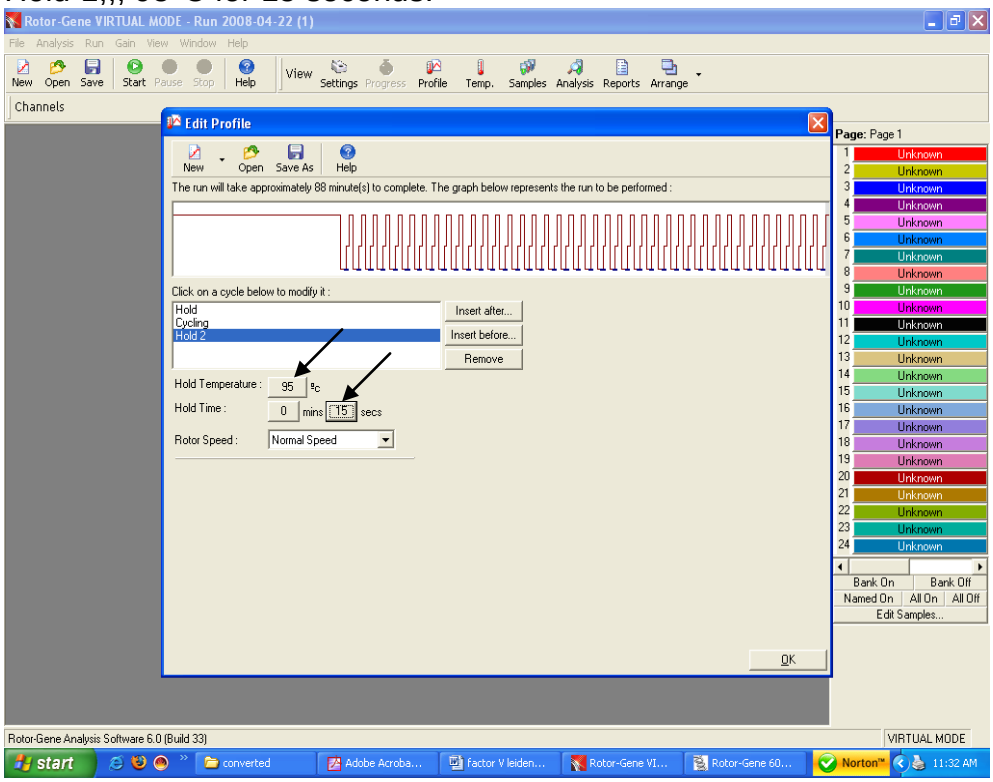


Fig. 14.

***Hold-2,, 40°C for 1 minute***

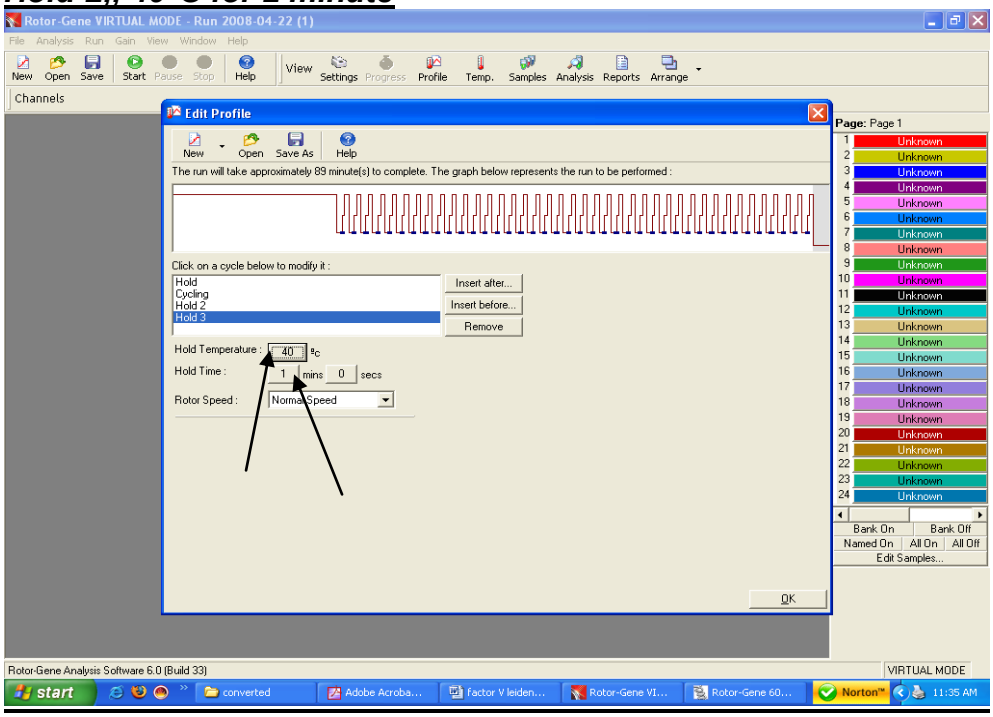


Fig. 15.

***HOLD-3,,, 50°C FOR one minute***

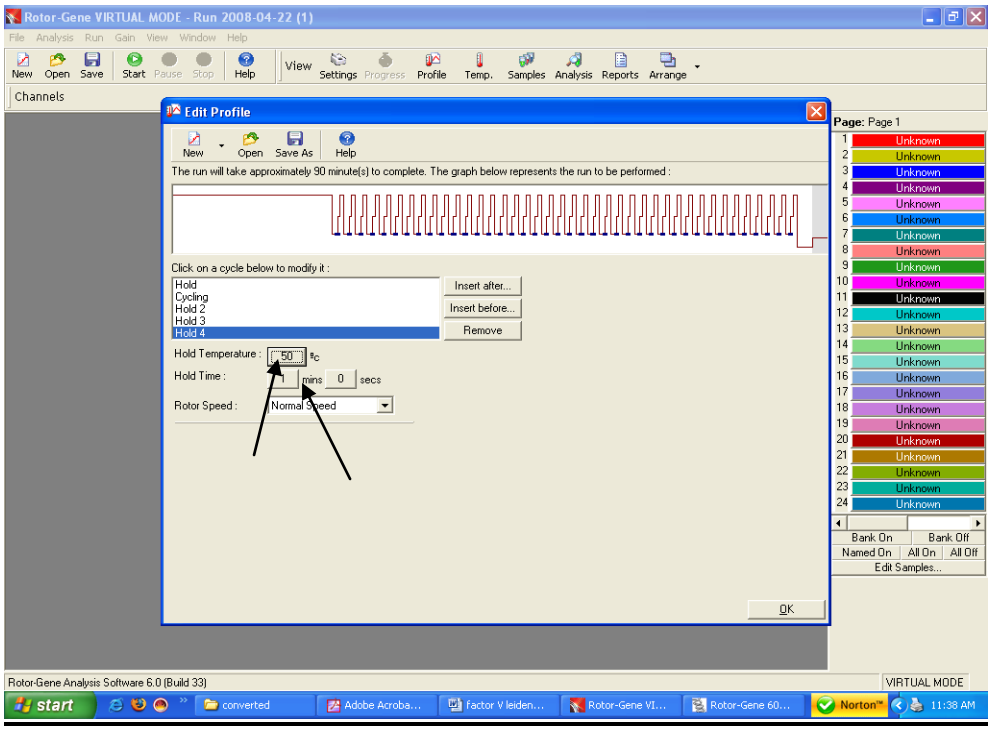


Fig. 16.

***DEFINING FINAL MELT CYCLING: The conditions are defined below:***

***The temperature range is 50°C - 80°C***

***Rising by 1°C***

***Wait for 60 second's on 1st, step***

***Wait for 5 seconds for each step afterwards.***

***Acquire on FAM.***

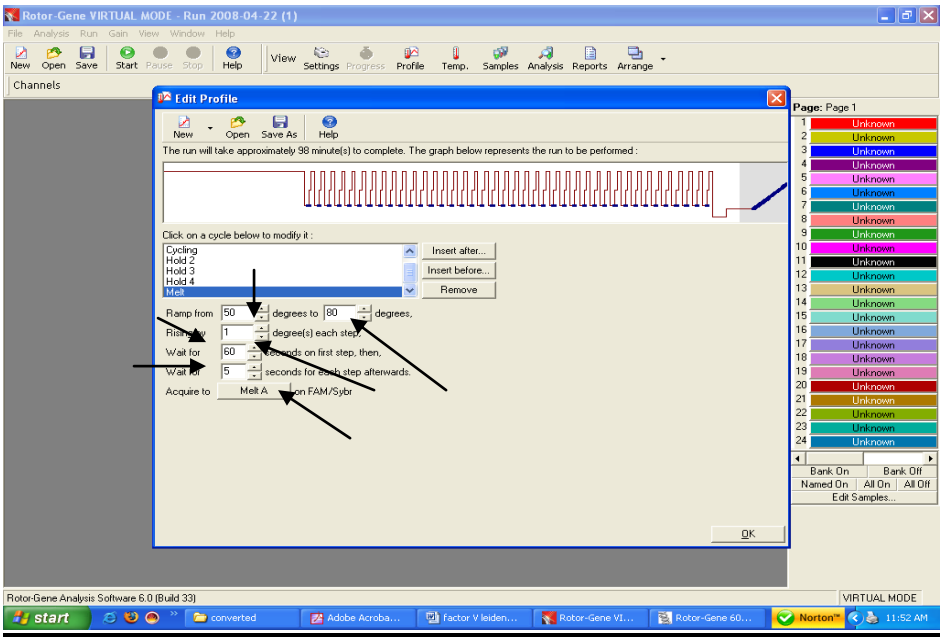


Fig. 17.

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

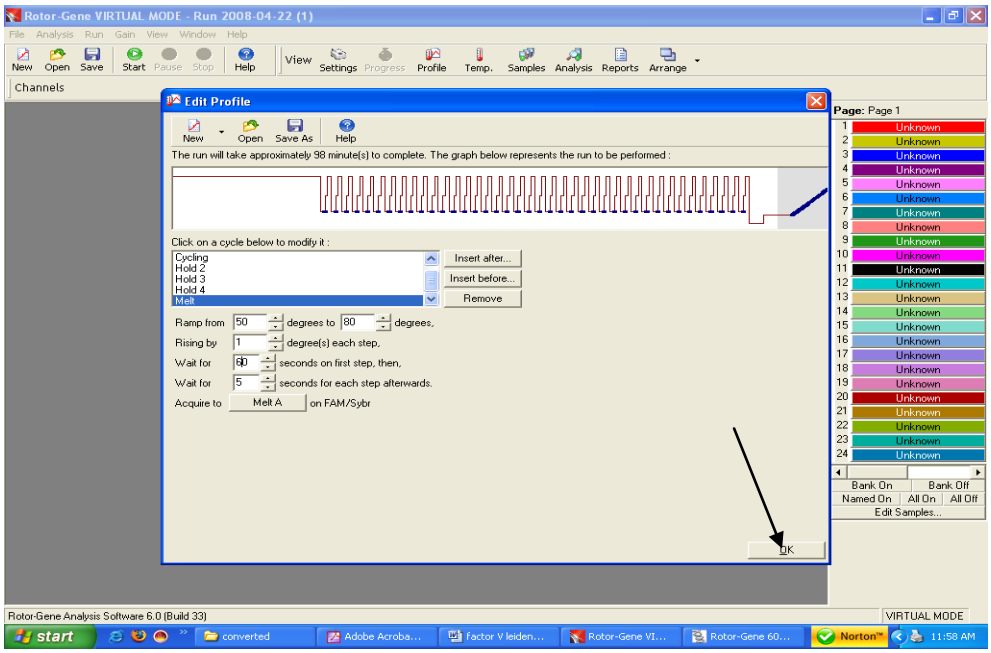


Fig. 18.

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

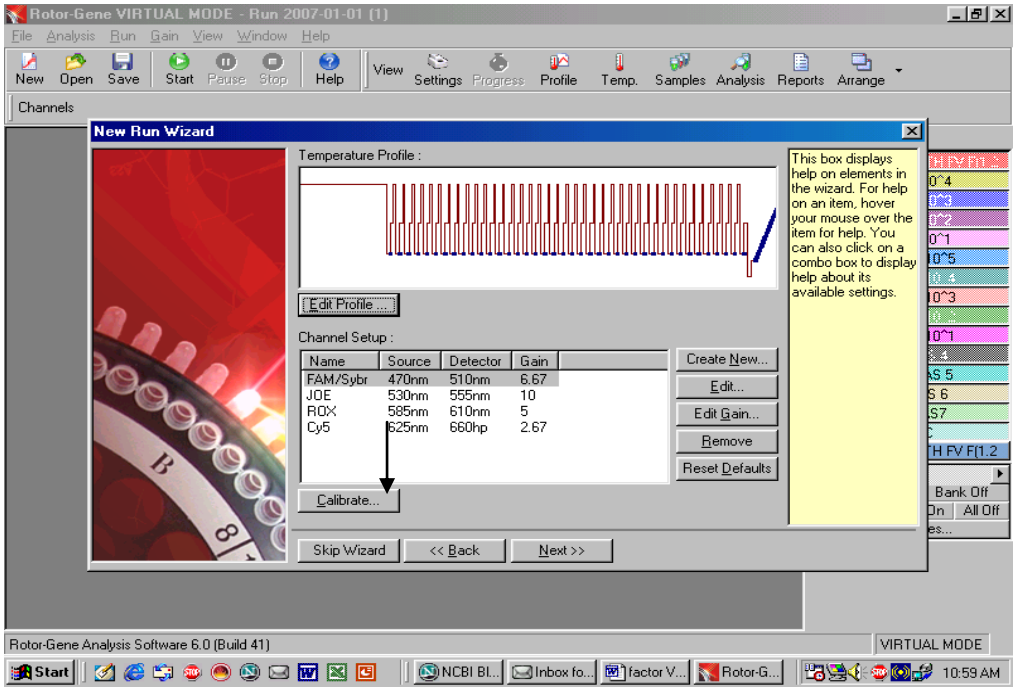


Fig. 19.

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. 20).

**Adjustment of the fluorescence channel sensitivity as shown below.**

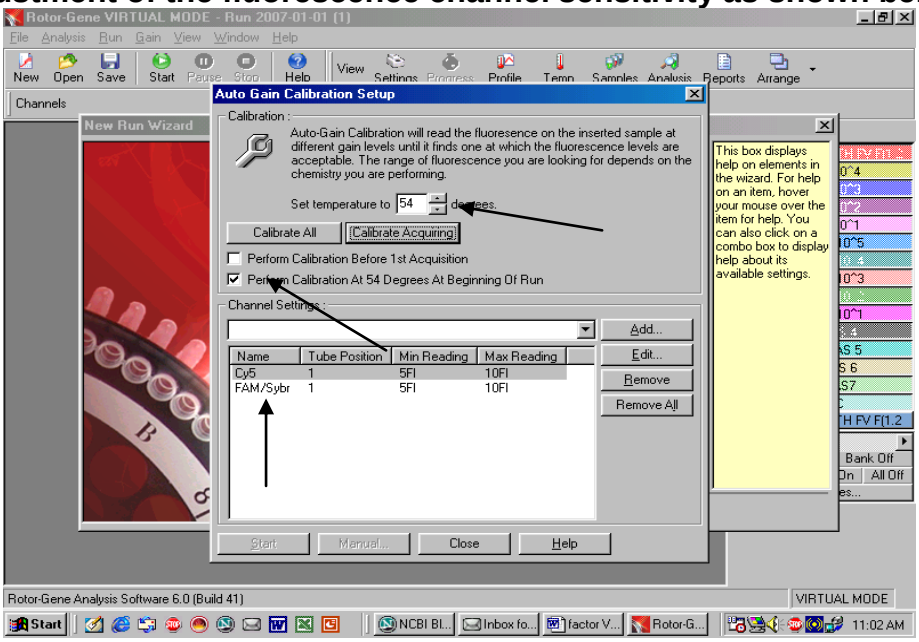


Fig. 20.

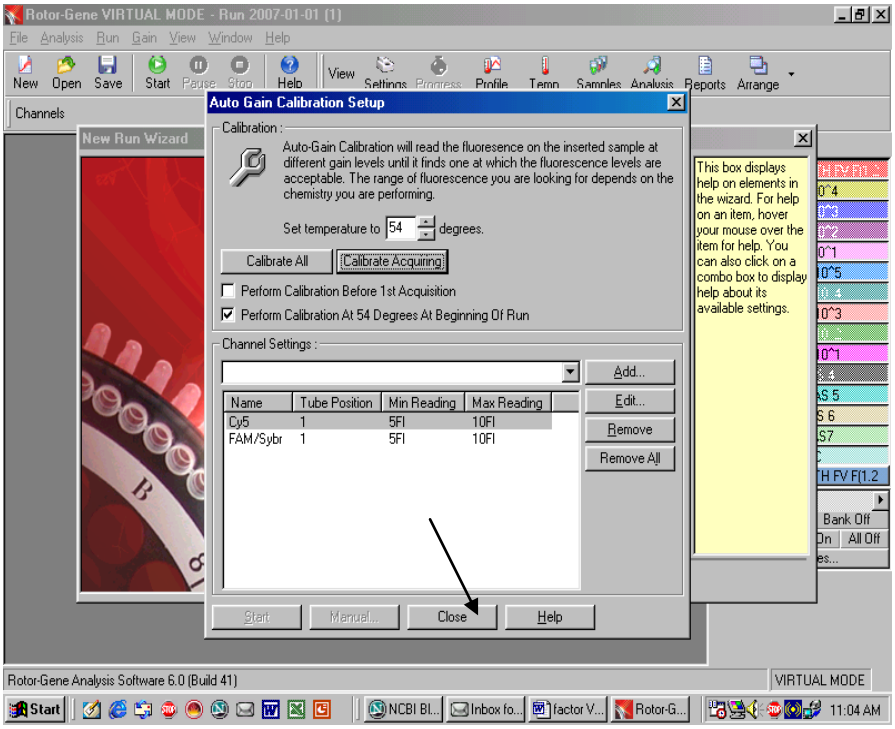


Fig. 21.

Please do not forget to click on the box against " Perform calibration at 54°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**

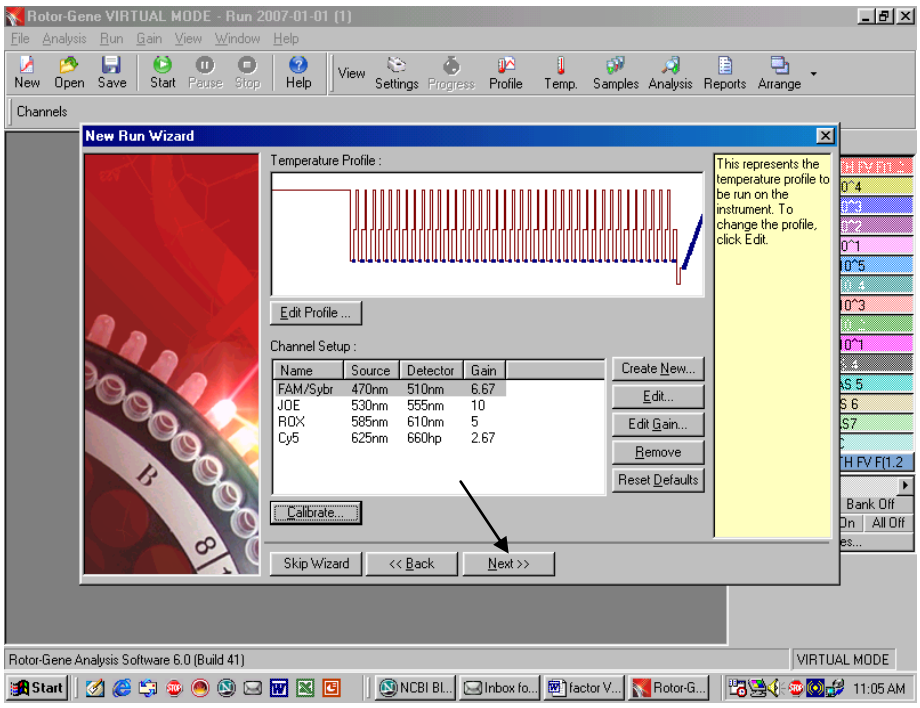


Fig. 22.

Starting of the *Rotor Gene*™ run. **Press Start Run Button.**

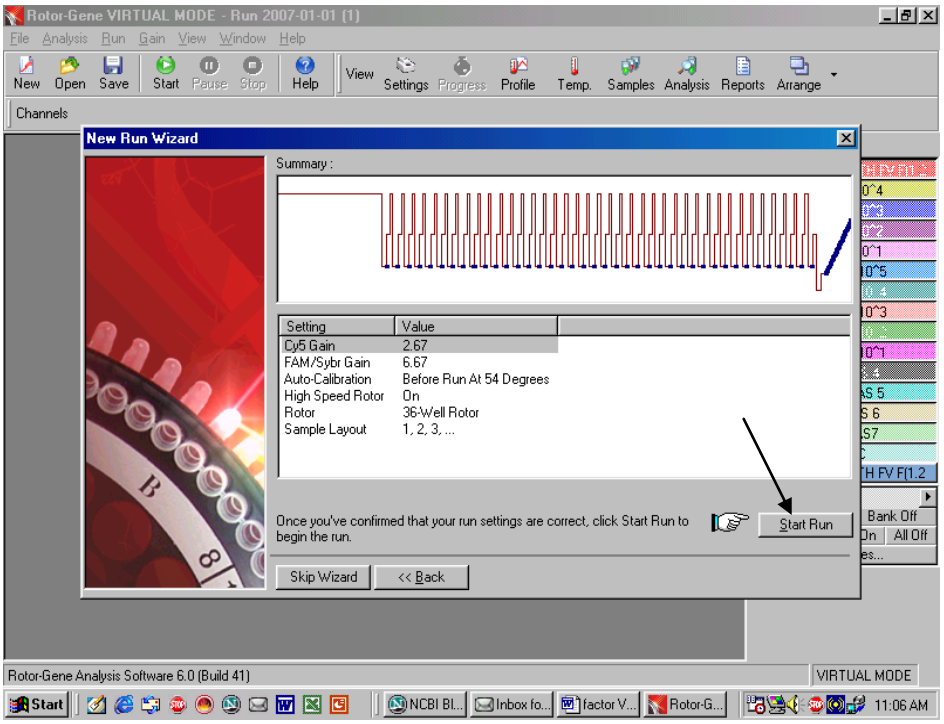


**Fig. 23:**

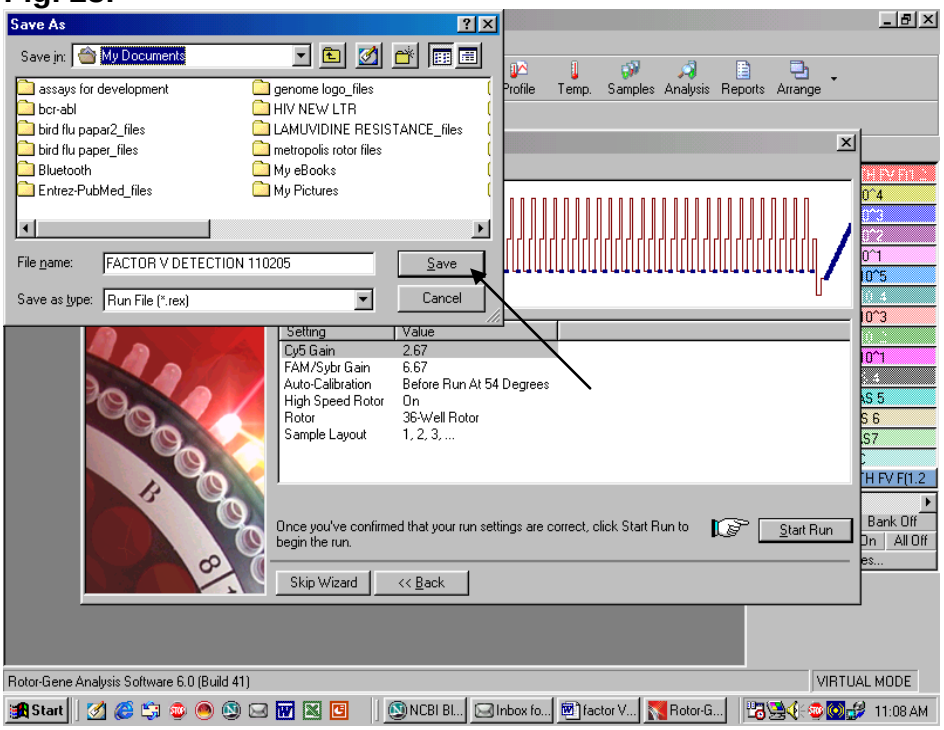


Fig. 24.

**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 Factor V leiden 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*™ 6*000* for these 5 steps according to the parameters shown in Fig. 25-45. All specifications refer to the *RotorGene*™ software version 1.7.40. Please find further information on programming the *RotorGene*™ in the *RotorGene*™ 6*000 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.

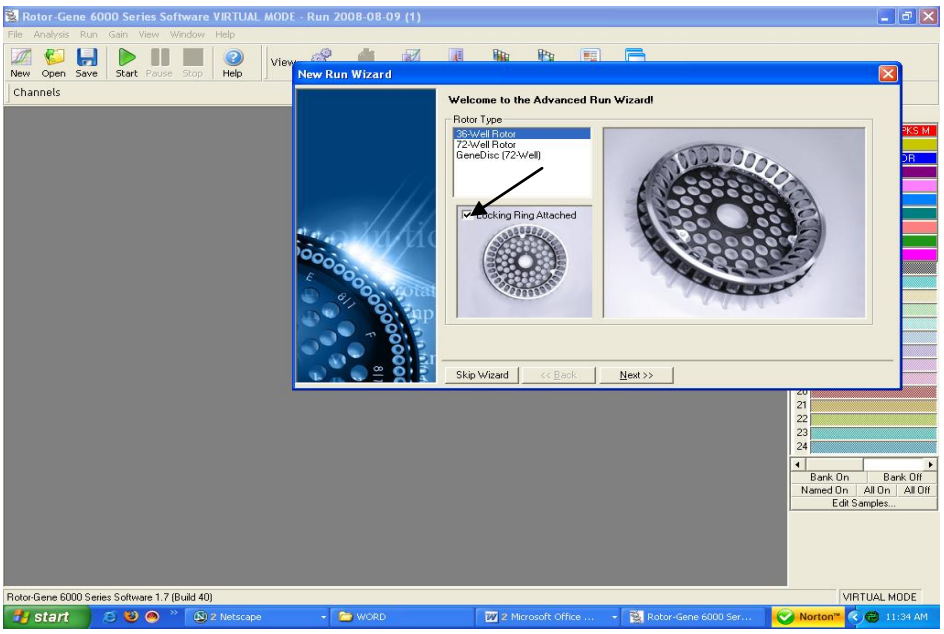


Fig. 25.

**Confirmation of Reaction Volume as follows.**

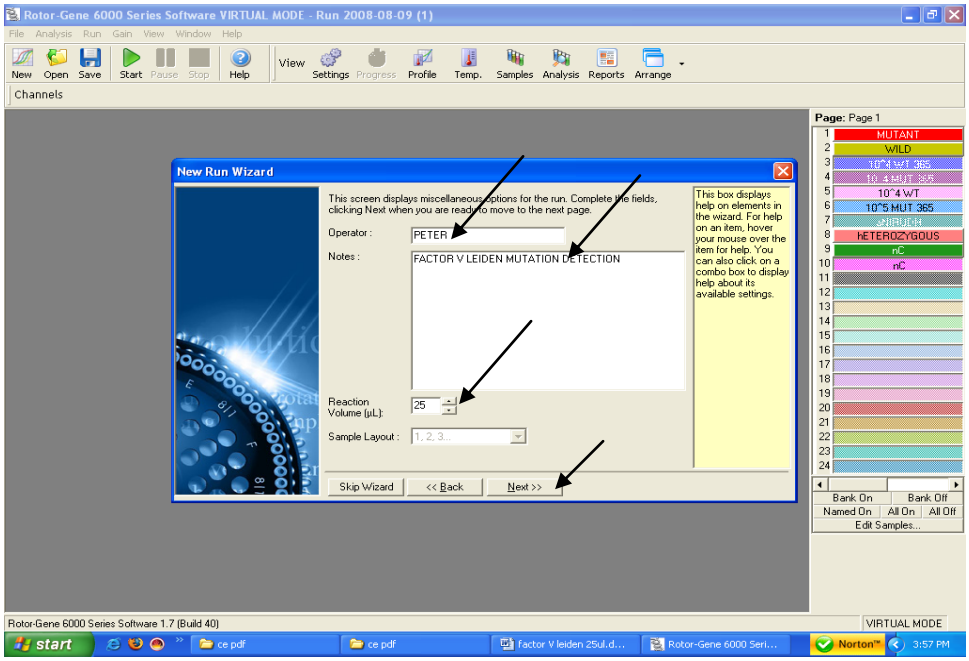


Fig. 26. Setting of general assay parameters.

- 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.**

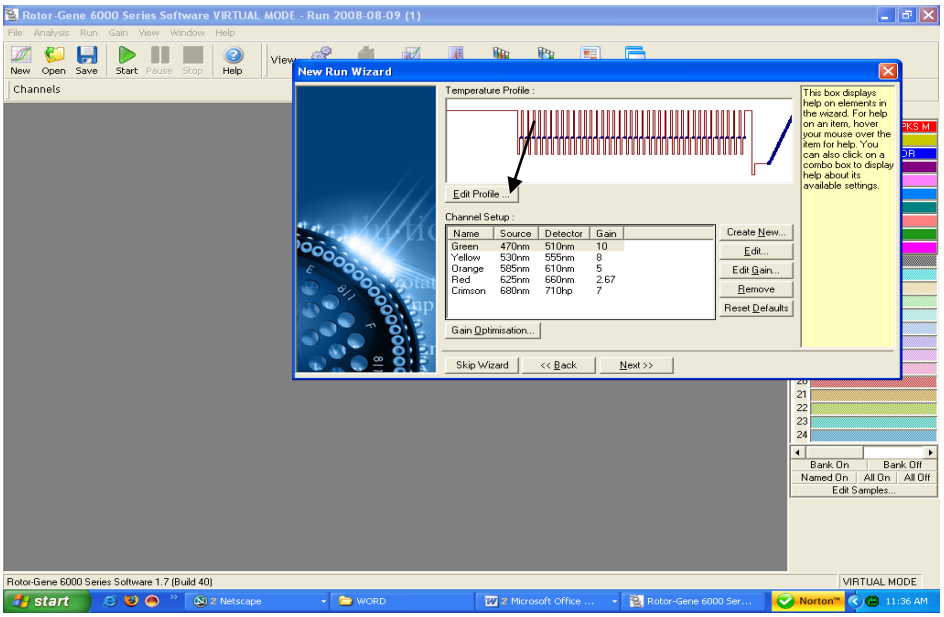


Fig. 27.

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

**CYCLING PROFILE: Hold** 95°C for 10 minutes as below

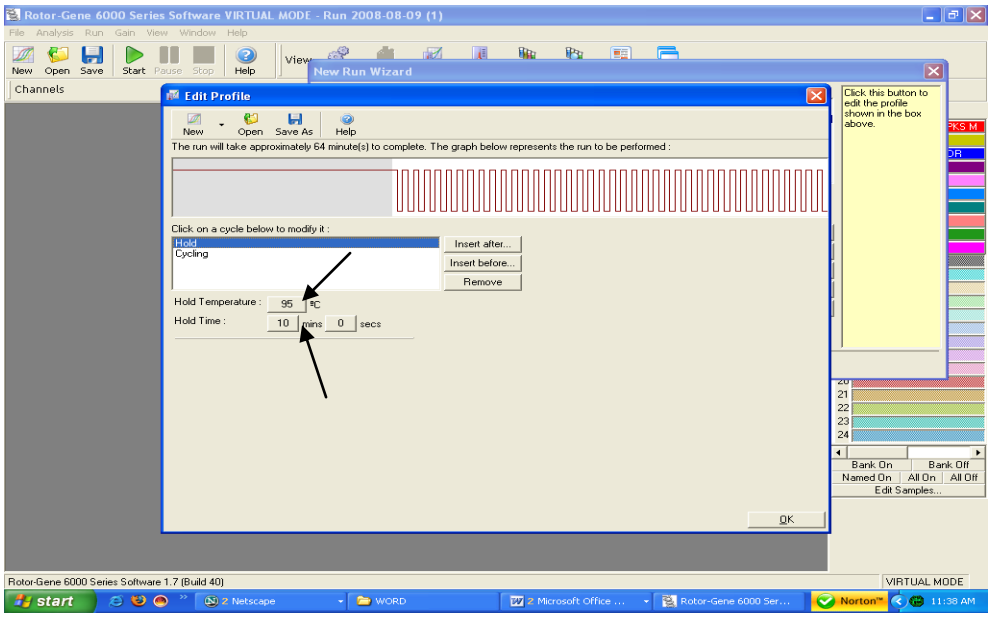


Fig. 28. Initial activation of the Hot Start enzyme.

# Setting the Cycling and acquisition.

When clicked on Cycling the window will open as below.

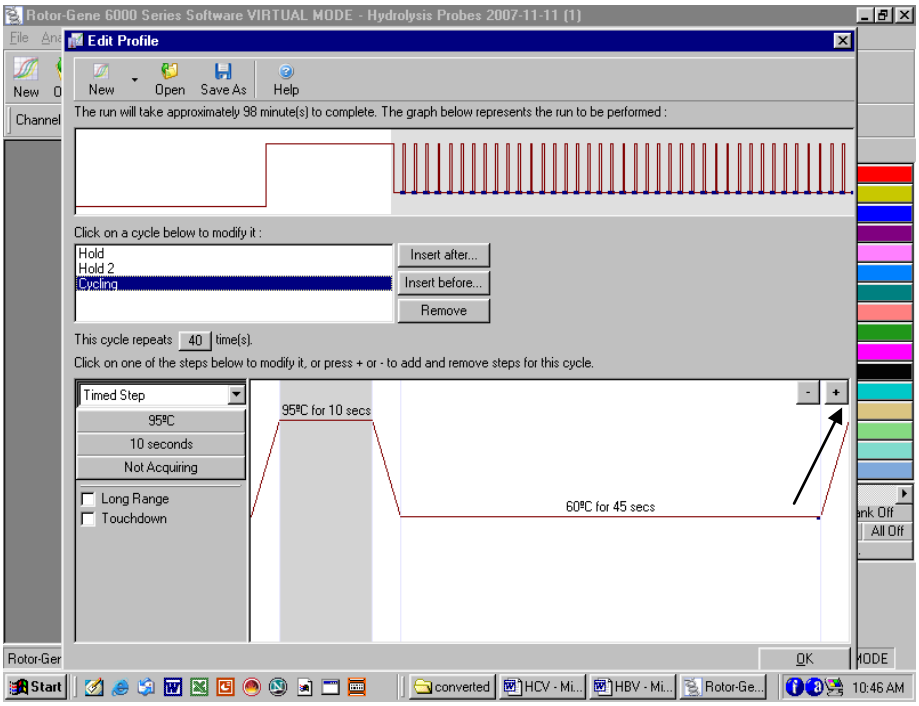


**Fig. 29.**

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.

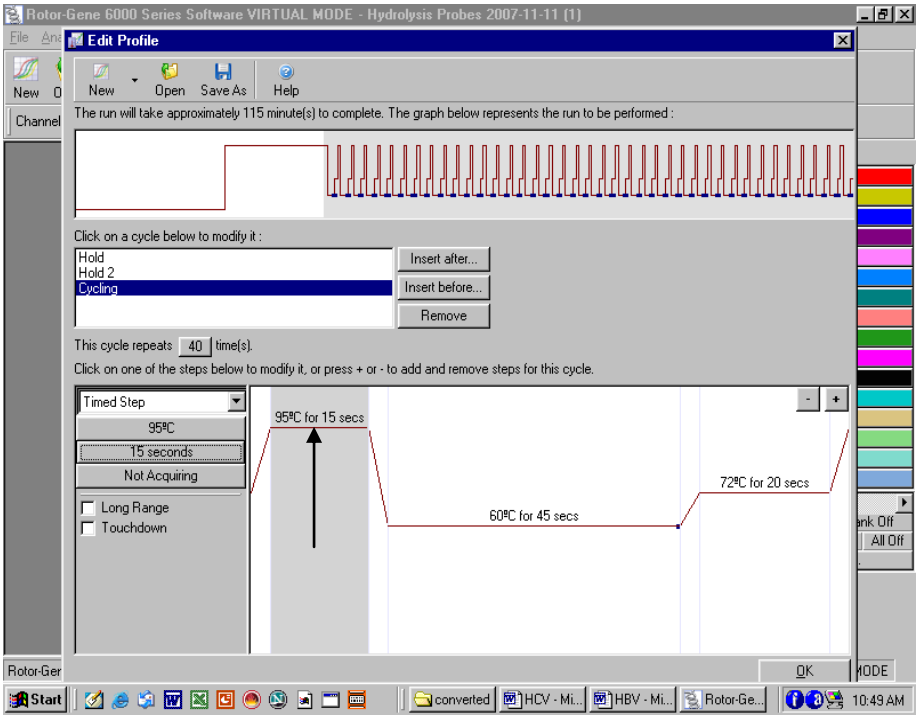


**Fig. 30.**

Setting up of Anneling step in the cycling profile as depicted below i.e. 54°C for 20 Seconds

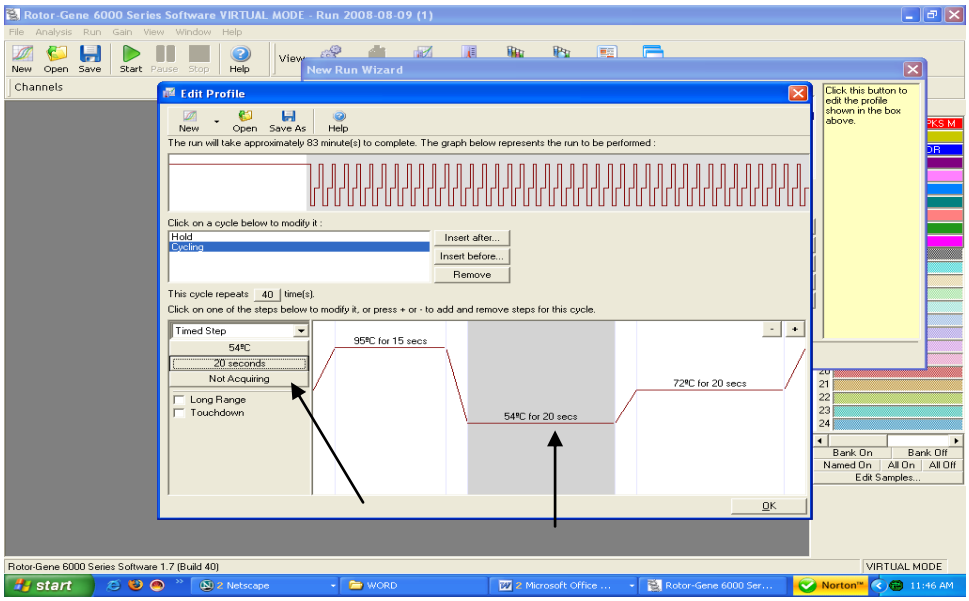


**Fig. 31.**

**After setting the Anneling temperature and the time for anneling click on the "Not acquiring " as shown by arrow. A New window will open as shown below.**

**Defining the Data acquiring channel i.e Green (FAM) & RED (CY5)**

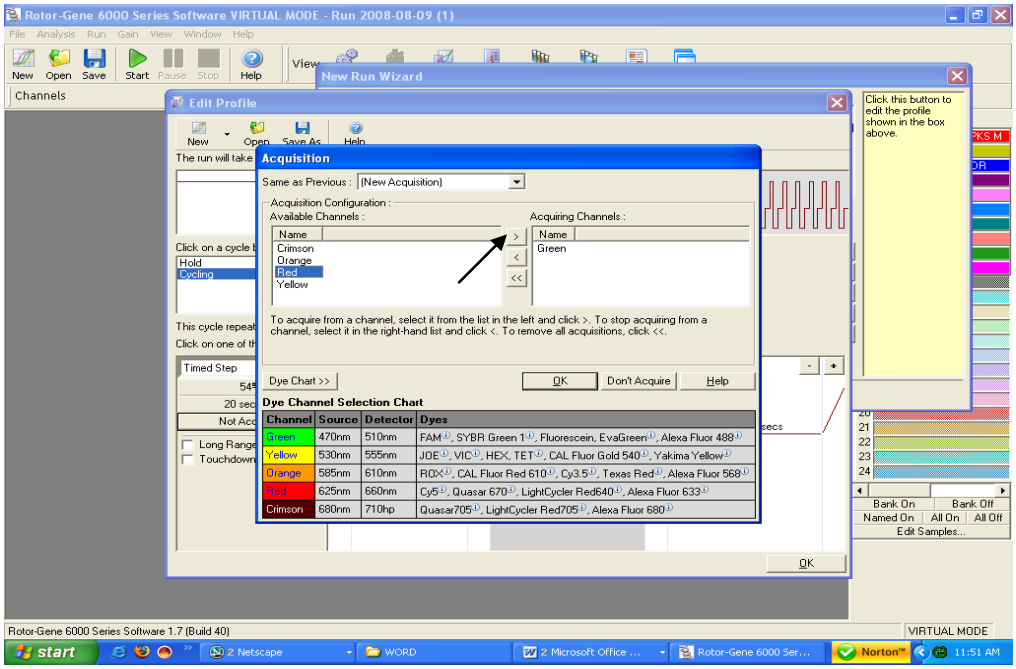


**Fig. 32.**

Highlight the Red and then press the right arrow. Just see before shifting the Red 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.

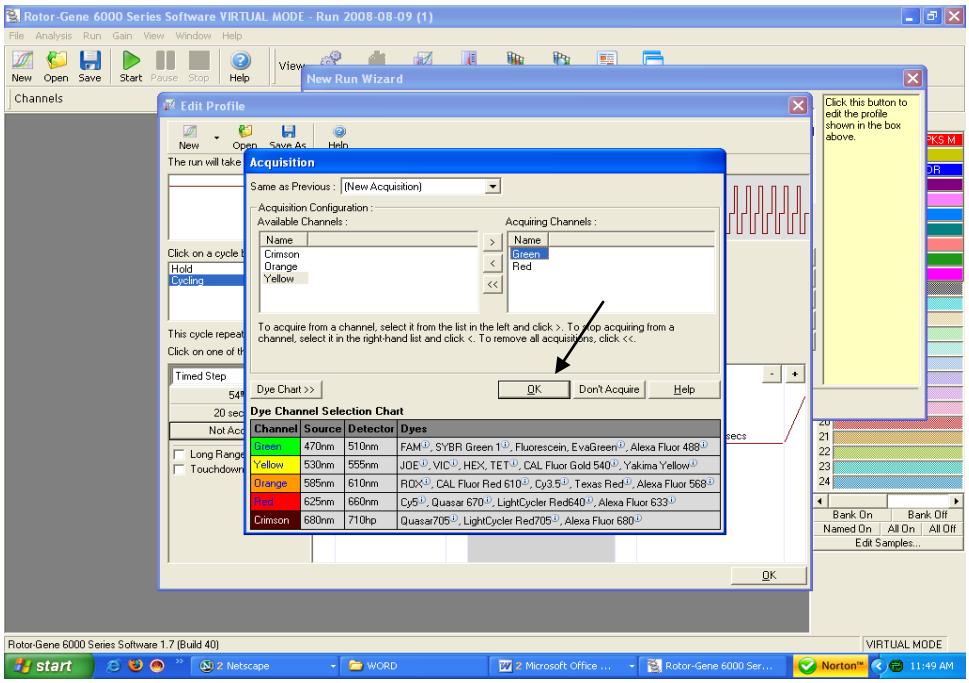


**Fig. 33.**

Once the Green & Red 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 10 Seconds

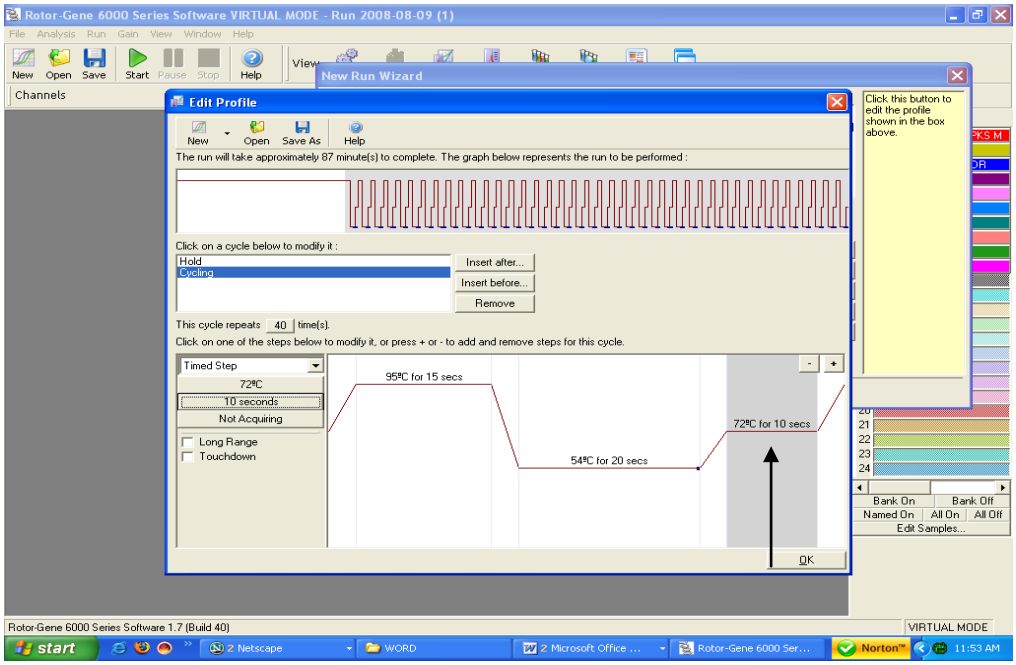


**Fig. 34.**

**Setting up of Number of Cycles to 40 cycles in the cycling profile as depicted below.**

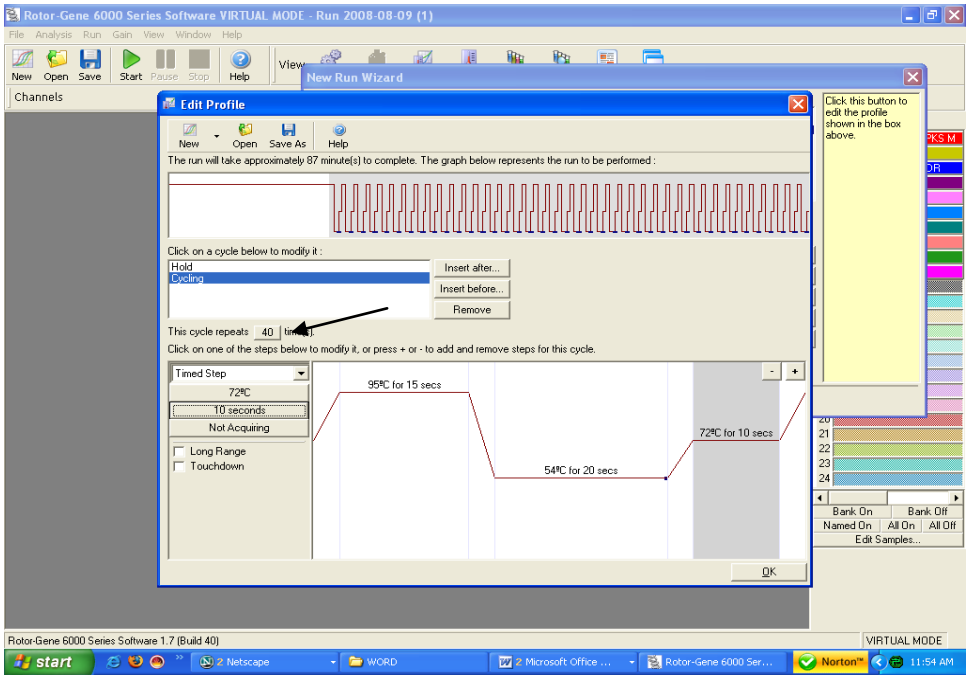


Fig. 35.

# Setting up of Melt:

Hold-1,,, 95°C for 15 seconds.

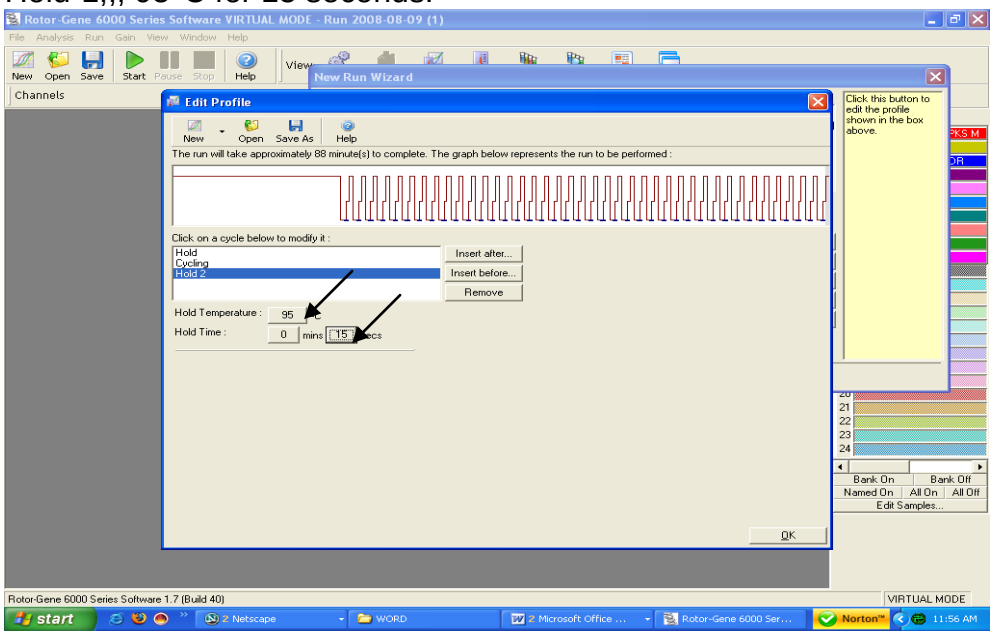


Fig. 36.

***Hold-2,,, 40°C for 1 minute***

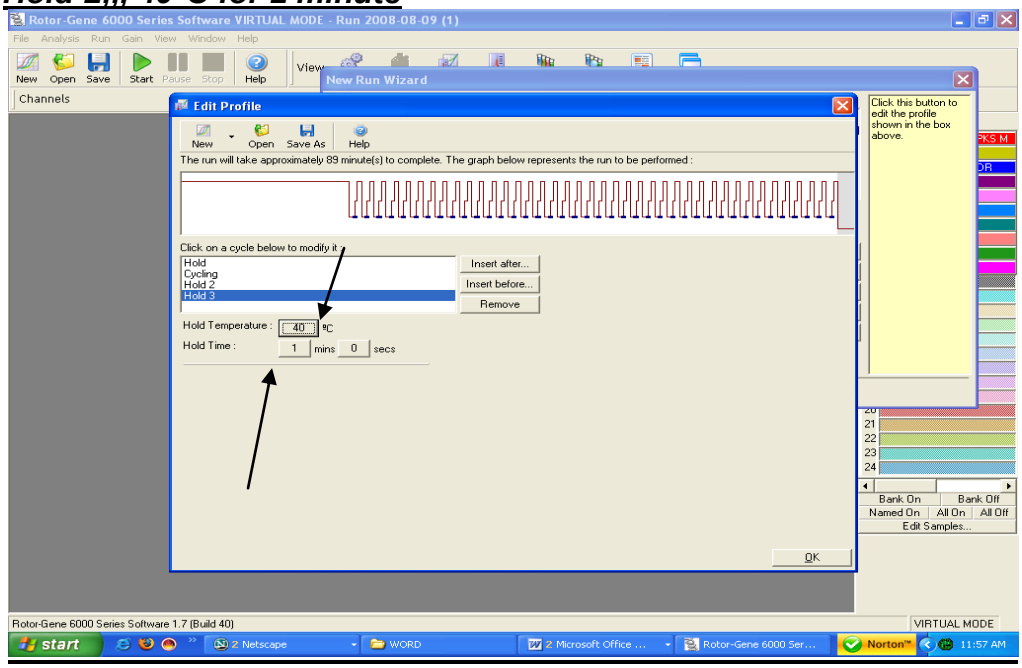


Fig. 37.

***HOLD-3,,, 50°C FOR one minute***

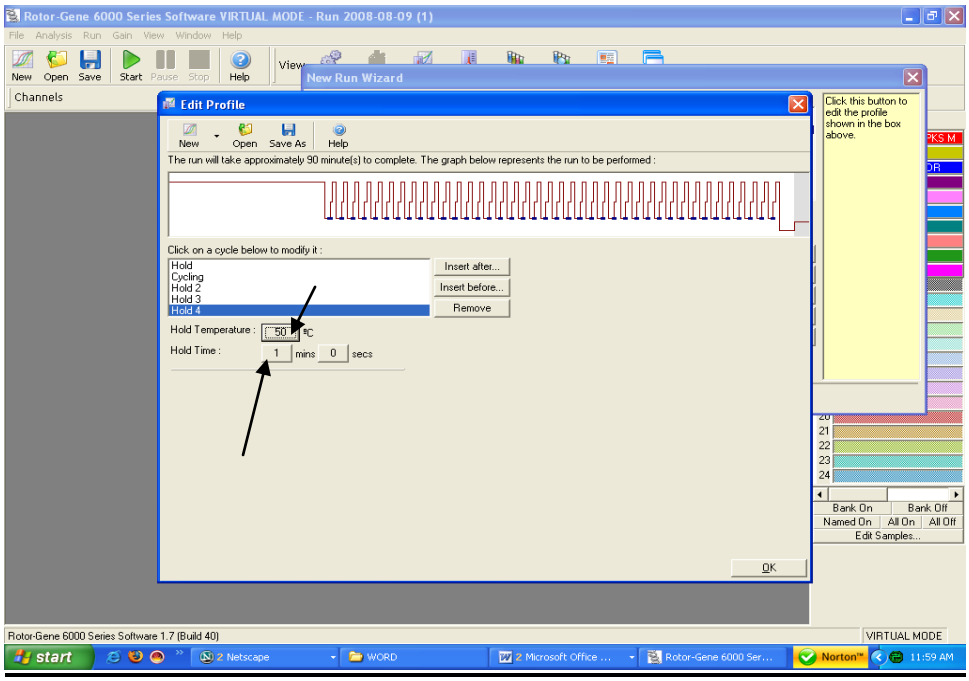


Fig. 38.

***DEFINING FINAL MELT CYCLING: The conditions are defined below:***

***The temperature range is 50°C - 80°C***

***Rising by 1°C***

***Wait for 90 second's on 1st, step***

***Wait for 5 seconds for each step afterwards.***

***Acquire on Green.***

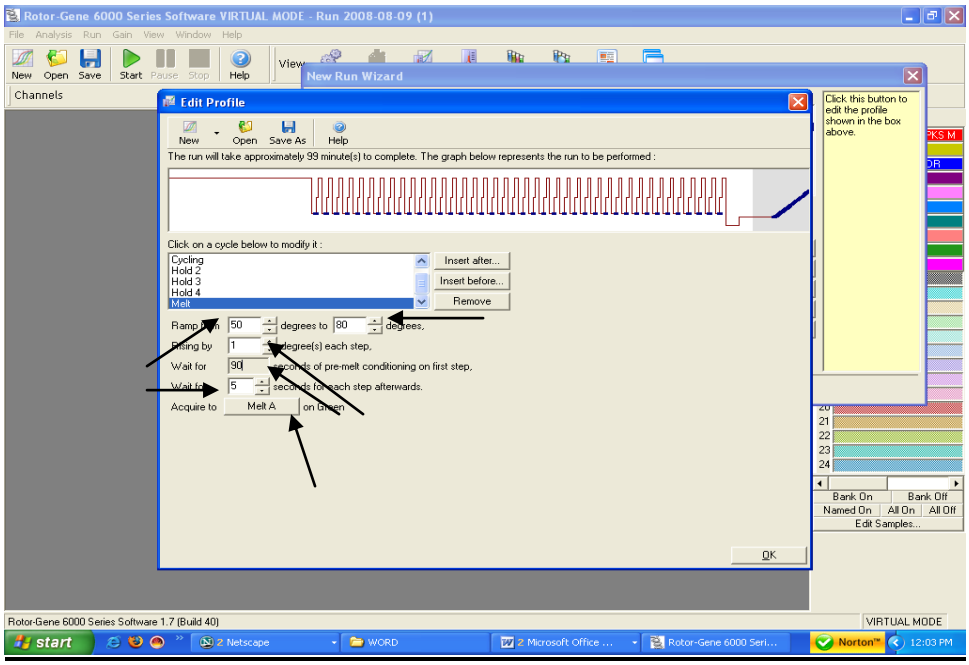


Fig. 39.

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

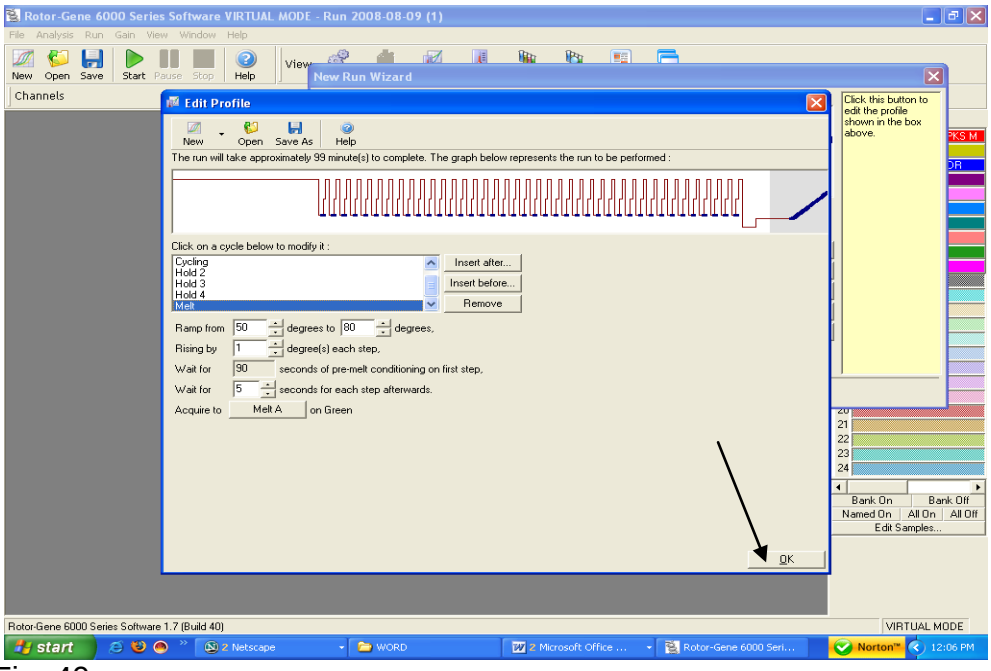


Fig. 40.

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

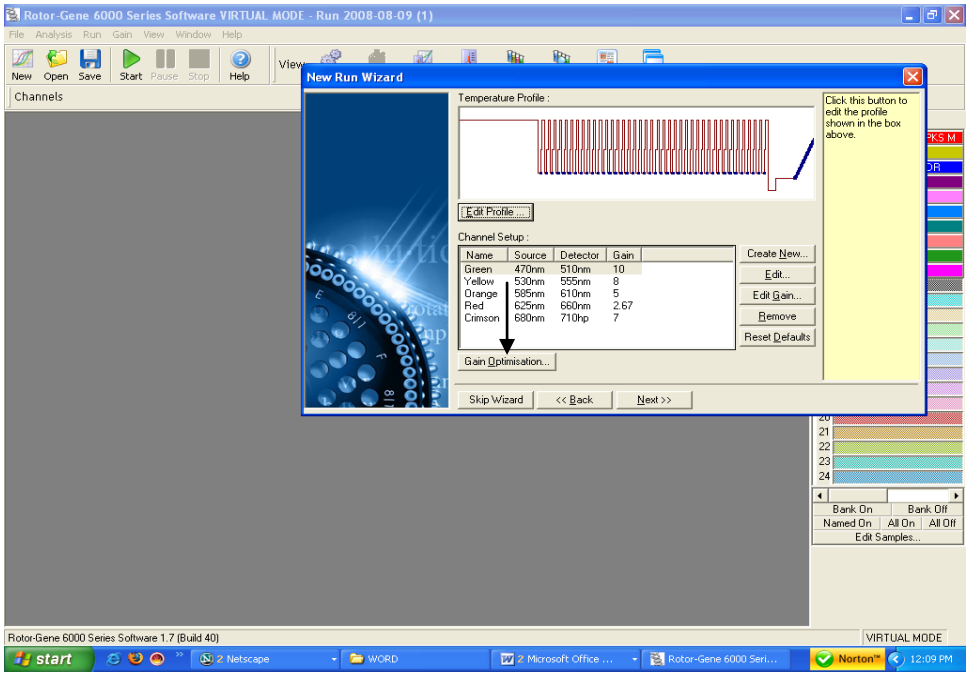


Fig. 41.

**Adjustment of the fluorescence channel sensitivity as shown below.**

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 *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.42 .)

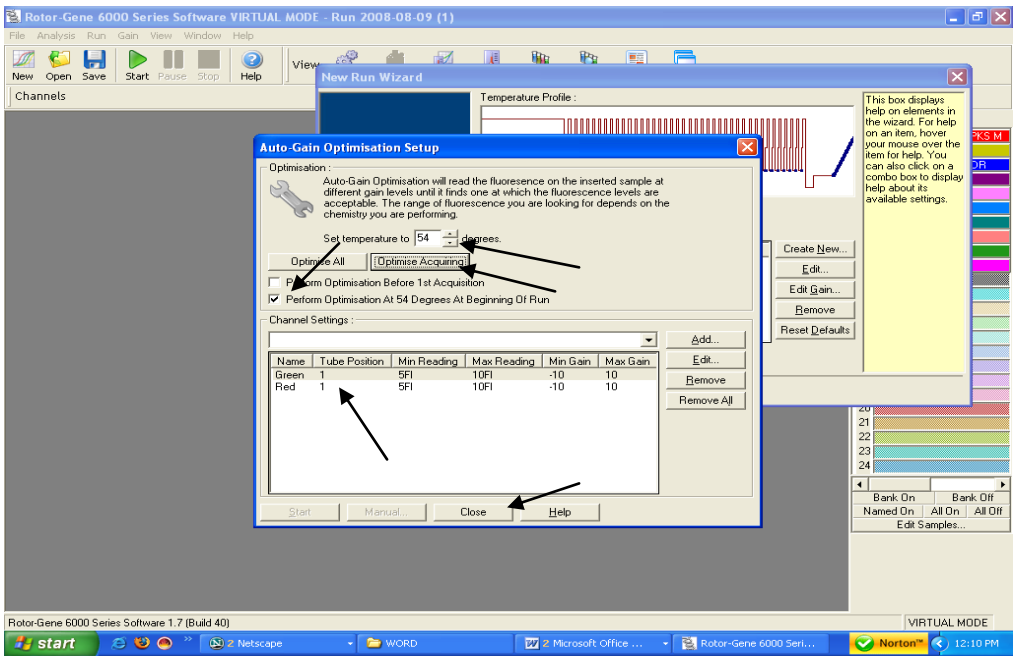


**Fig. 42.**

**The following needs to be done.**

- Click on the Optimise Acquiring
- Click on Set temperature To Adjust the Temperature to 54°C.
- Click on the Box Perform Optimisation at 54°C at the beginning of the Run.
- Just see that below the channel settings there appear only two channels i.e. Green and Red. 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.

Please do not forget to click on the box against " Perform calibration at 54°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**

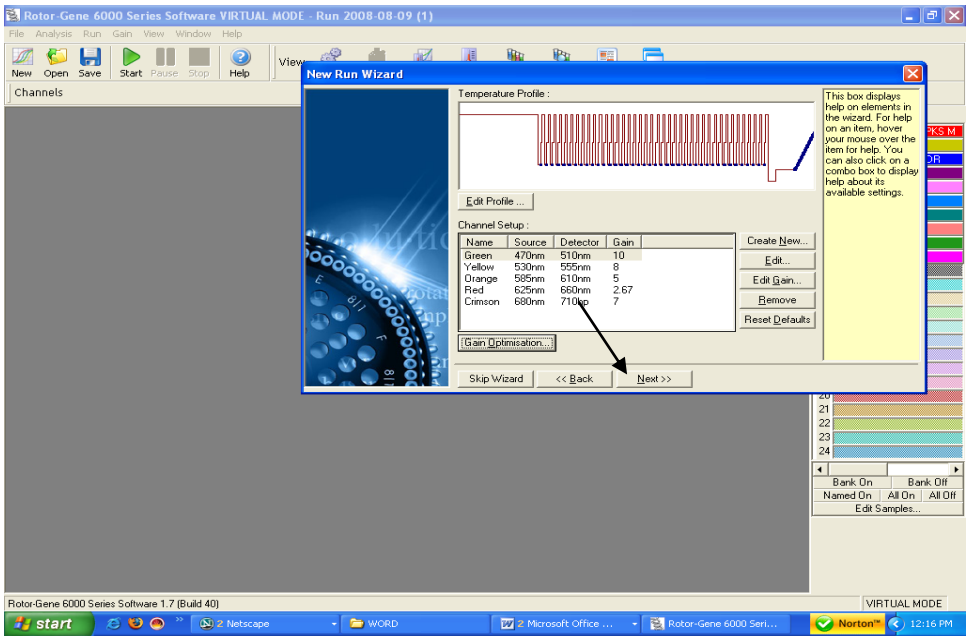


Fig. 43.

Starting of the *Rotor Gene*™ run. **Press Start Run Button.**

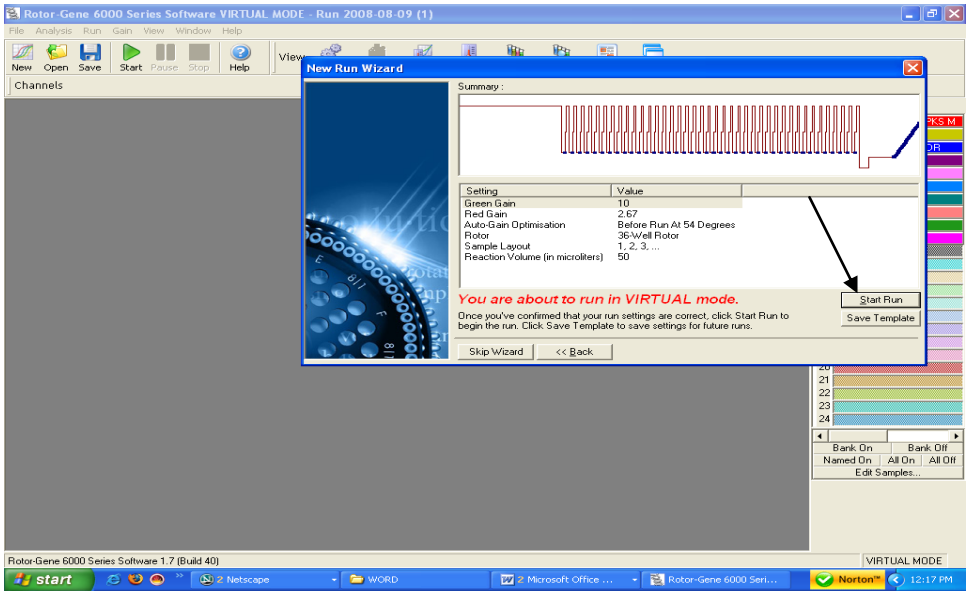


**Fig. 44:**

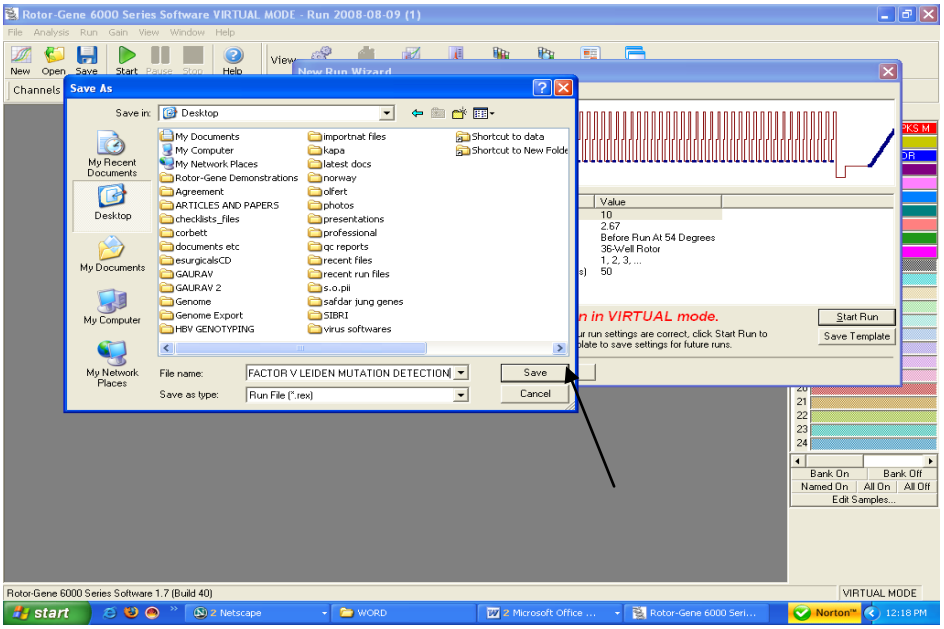


Fig. 45.

## 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.

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

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

However for convenience the procedure is detailed below.

1. Go to the Analysis part.

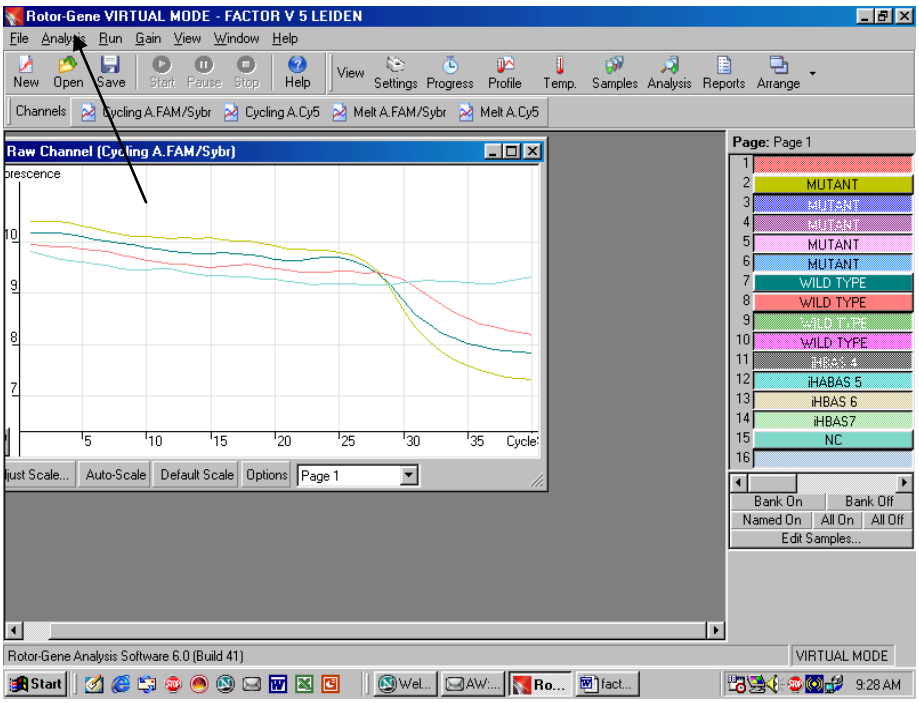


Fig. 46.

After the window is opened click on the following as shown in figure below.

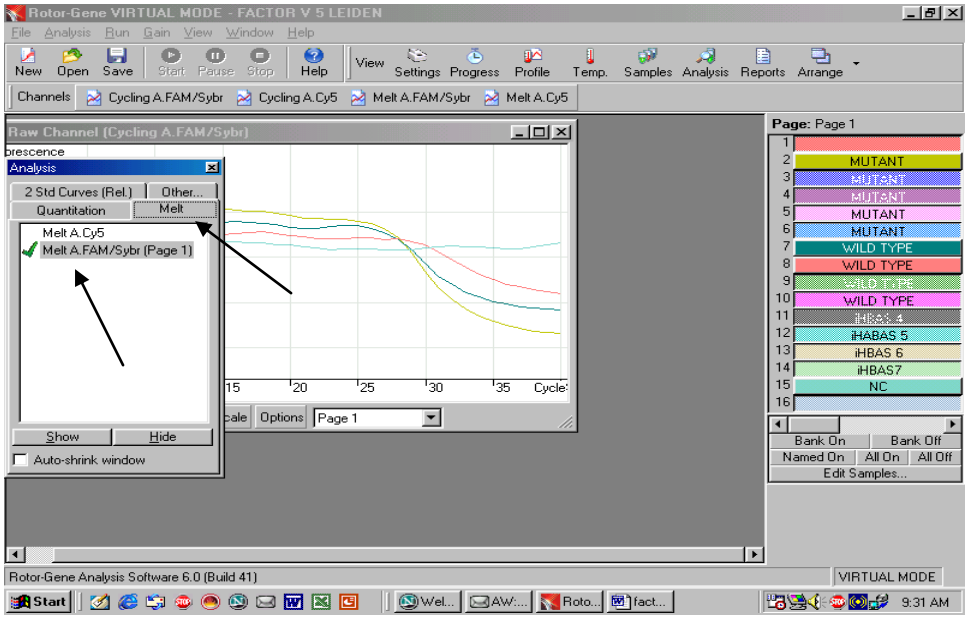


Fig. 47.

Once the right buttons have been pressed i.e. Melt and FAM as shown by arrows then press show. The Lower window will appear once show is pressed.

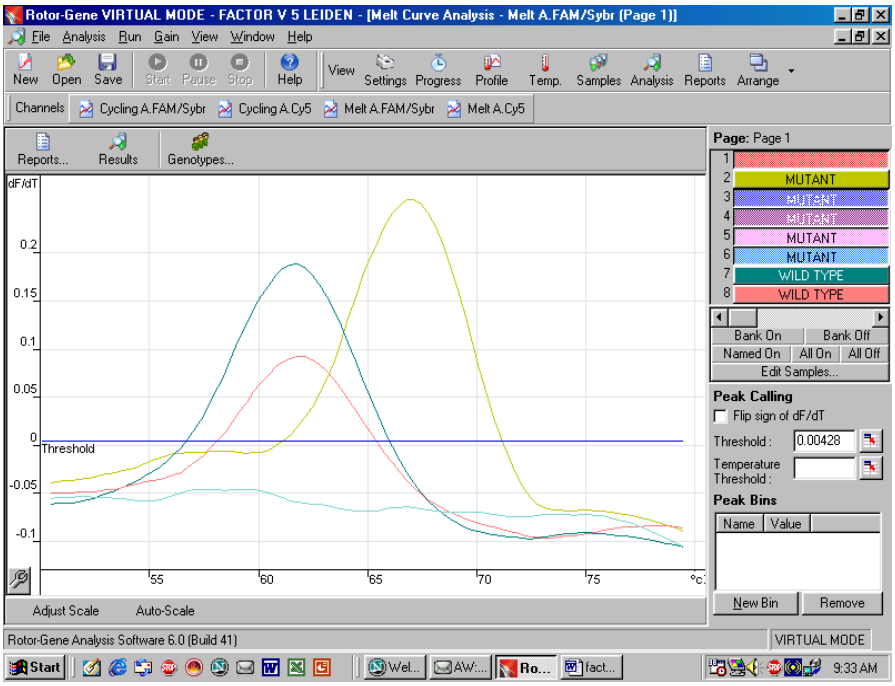


Fig. 48.

Do the following:

1. First with the help of help of right button of your mouse if required change the digital filter to Medium or heavy if required.
2. Now set the threshold manually.
3. Now define the bins by clicking on the New bin and placing it at the right place. As shown in the figure below.

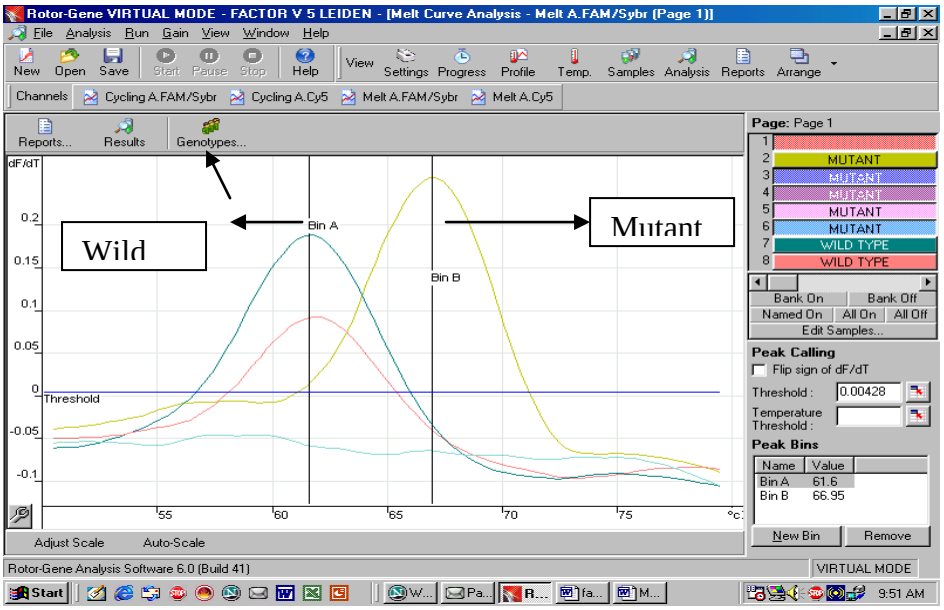


Fig. 49.

Now Click on the Genotyes ICON to define the respective Genotype. The following window will appear.

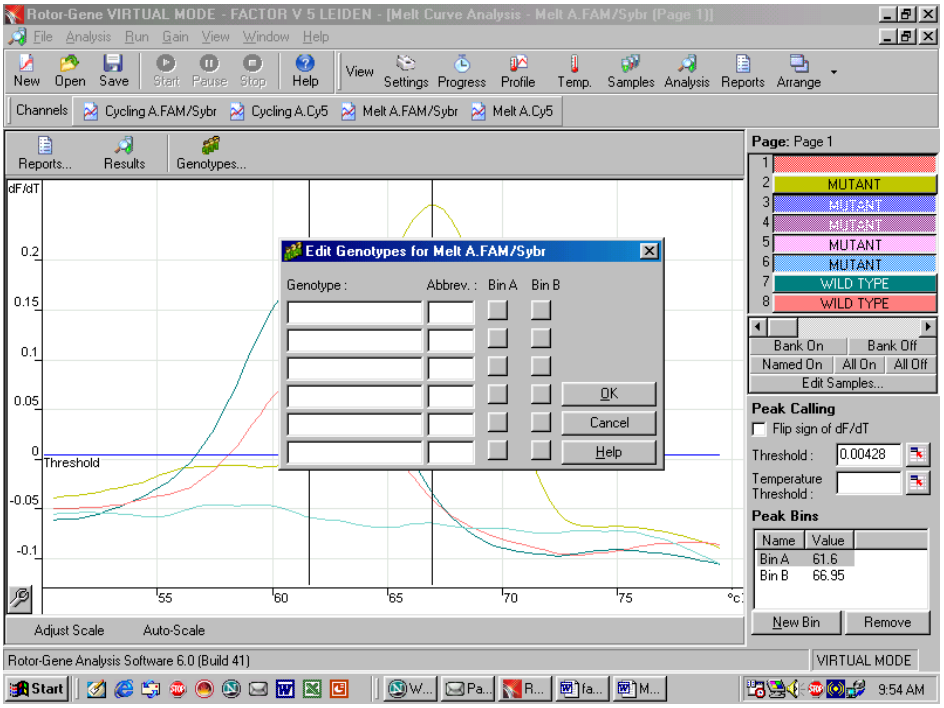


Fig. 50.

Now define the Genotypes as shown in the window below.

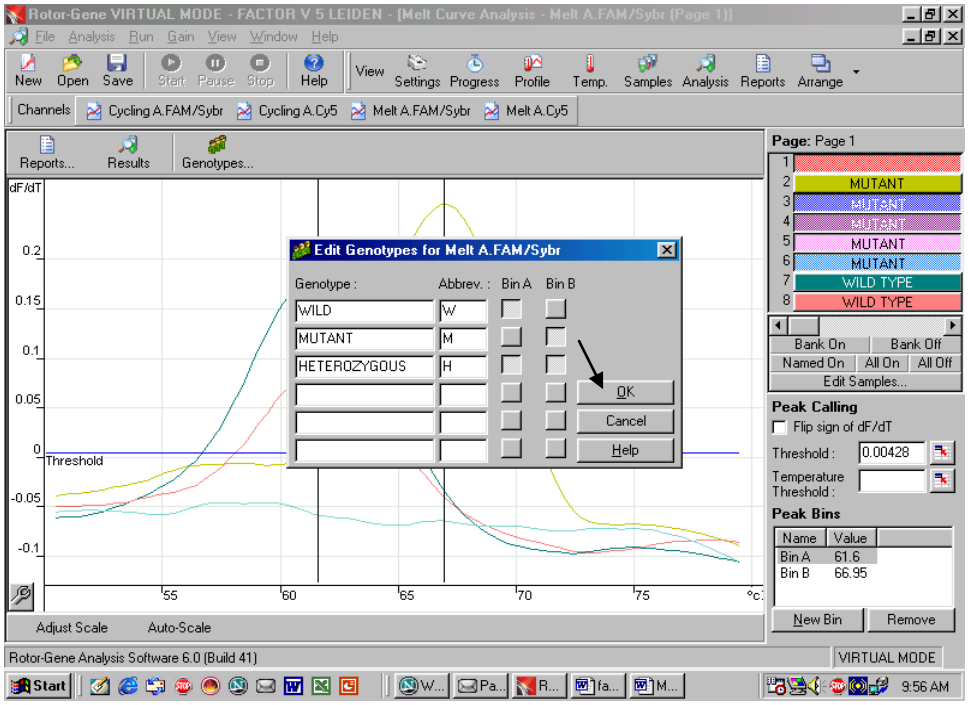


Fig. 51.

then Press O.K. as shown by arrow.

Now the software will automatically define the samples and will provide the results.

The following results are possible: The results mentioned below are with the heavy Filter.

**1. Only one Peak is detected in Melt channel FAM at 61.6 ± 0.5**

The sample is of wild type.

**2. Only one Peak is detected in Melt channel FAM at 66.95± 0.5**

The sample is of Mutant type.

**3. Two Peak's are detected in Melt channel FAM at both 61.6± 0.5**

**& 66.95 ± 0.5**

The sample is Heterozugous.

**4. . No Peak's are detected in Melt channel FAM at both 61.6 ± 0.5**

**& 66.95 ± 0.5**

The sample does not contain any Factor V DNA hence should be checked for PCR inhibition or else repeat extraction.

The software will automatically define the samples based on the above classification if the genotypes are Fed properly as shown earlier.

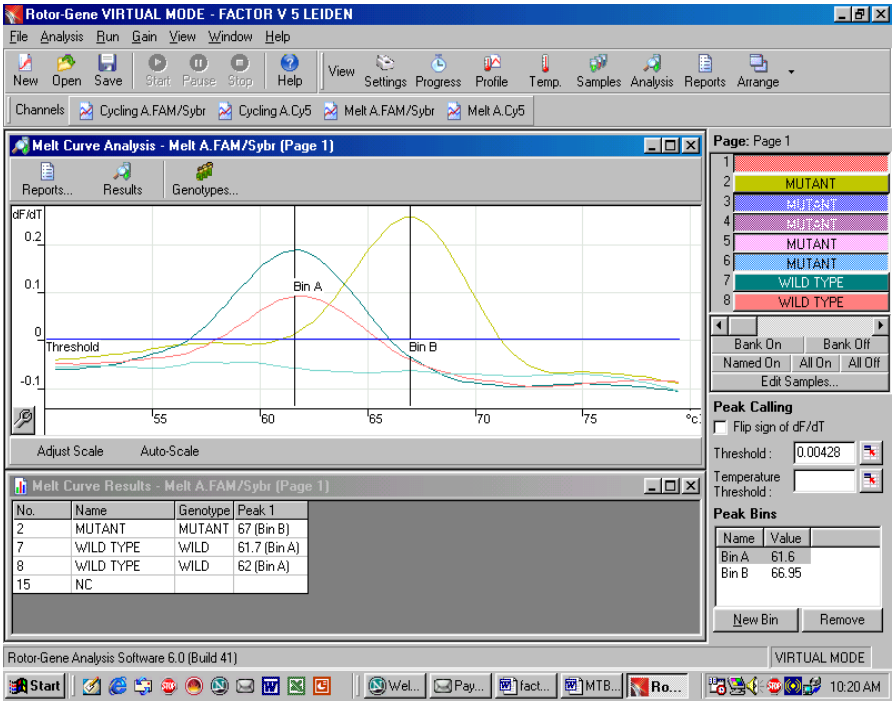


Fig. 52.

For your conveniance the above figure depicts two wild type, one mutant and one Negative sample for Factor V which is duly defined by the software as well in the window below the curves.

Important Note::: The above results and peaks are interpreted with the Heavy Filter. In case Light filter or Medium Filter is used, then the Melt peaks can have a shift towards a little higher side. In such a case the peaks have to be defined based on the Bin.

# 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).

However for convenience the procedure is detailed below.

2. Go to the Analysis part.

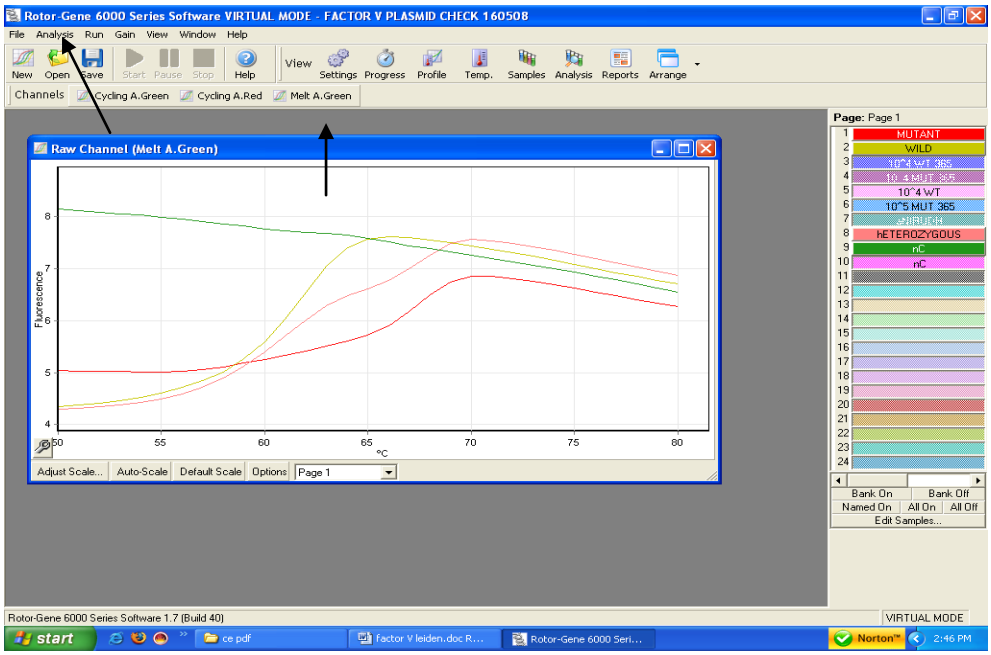


Fig. 53.

After the window is opened click on the following as shown in figure below.

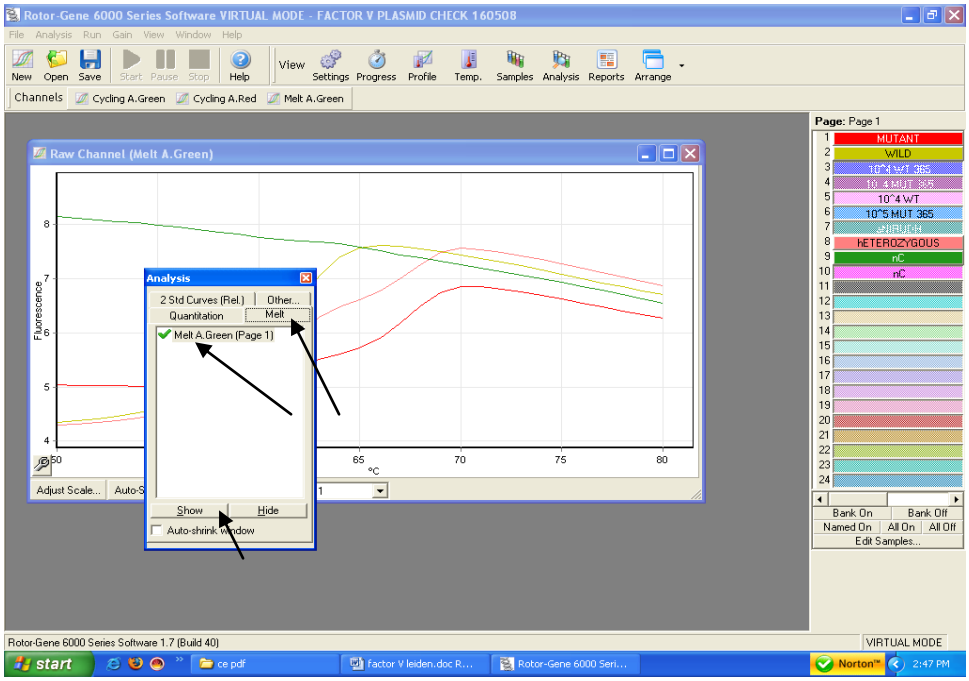


Fig. 54.

Once the right buttons have been pressed i.e. Melt and Green as shown by arrows then press show.

The Lower window will appear once show is pressed.

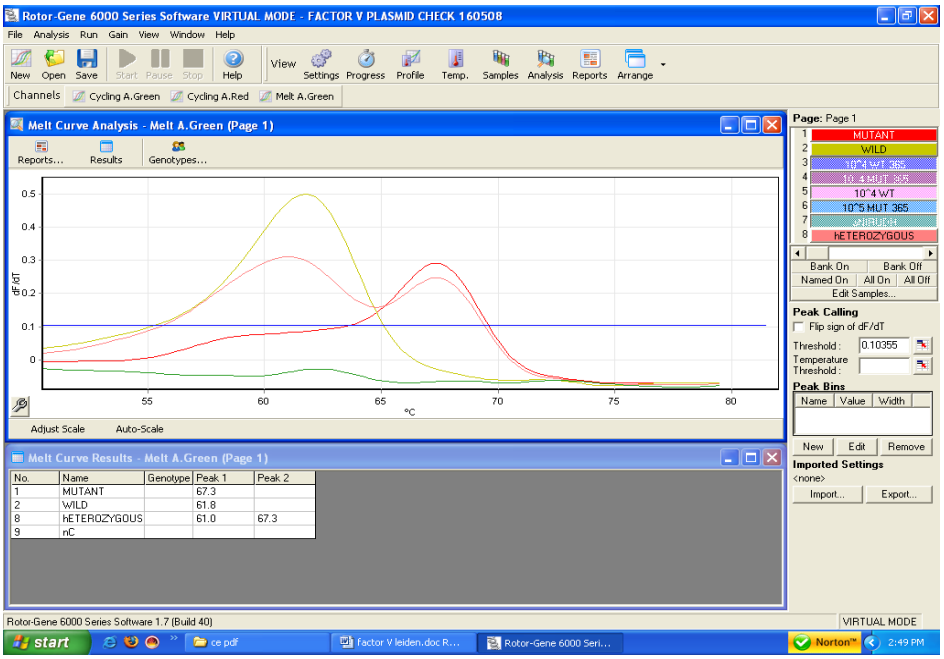


Fig. 55.

Do the following:

4. First with the help of help of right button of your mouse if required change the digital filter to Medium or heavy if required.
5. Now set the threshold manually.
6. Now define the bins by clicking on the New bin and placing it at the right place. As shown in the figure below.

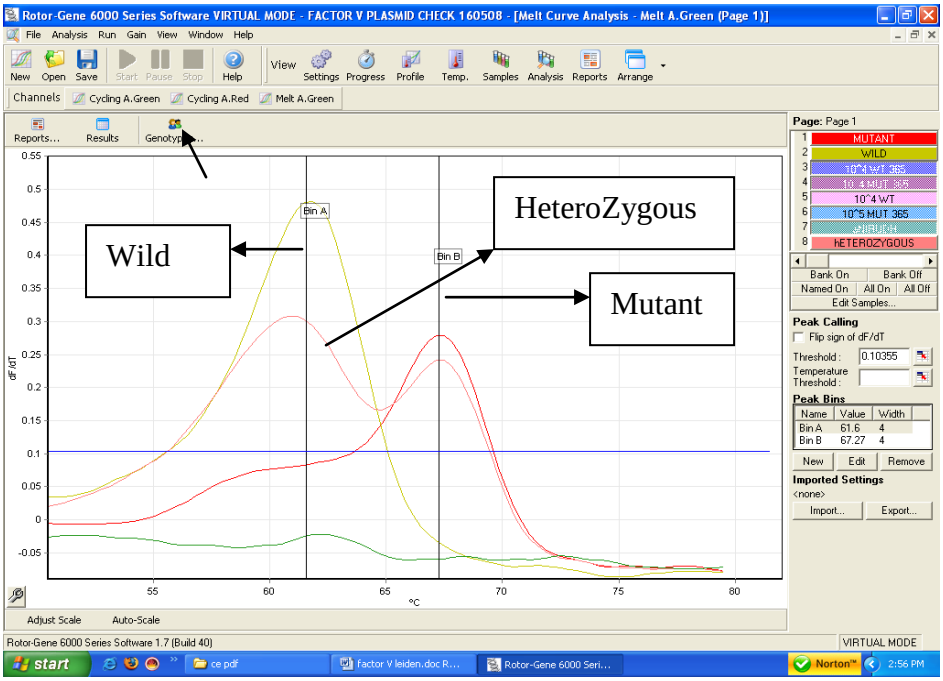


Fig. 56.

Now Click on the Genotypes ICON to define the respective Genotype. The following window will appear.

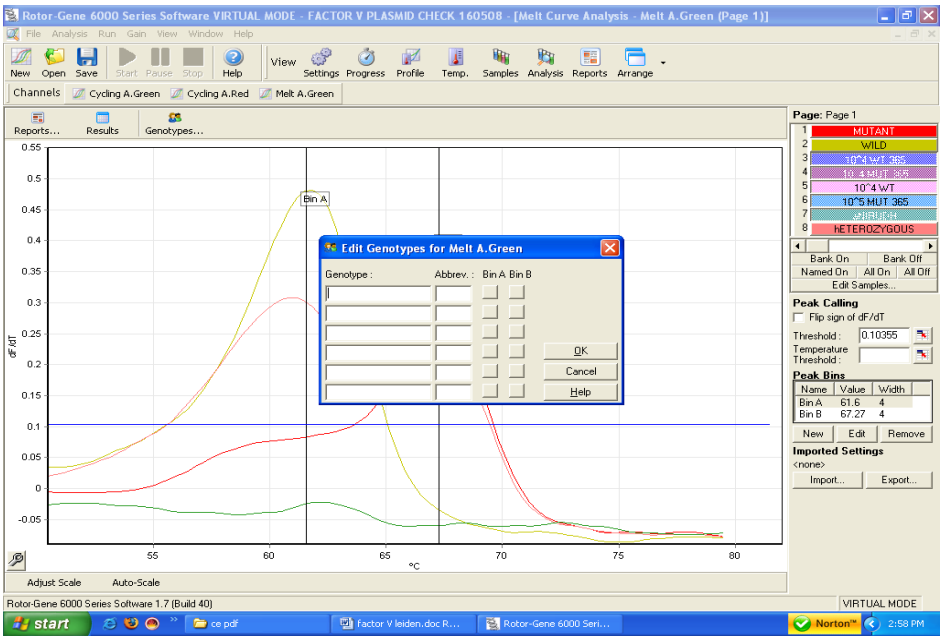


Fig. 57.

Now define the Genotypes as shown in the window below.

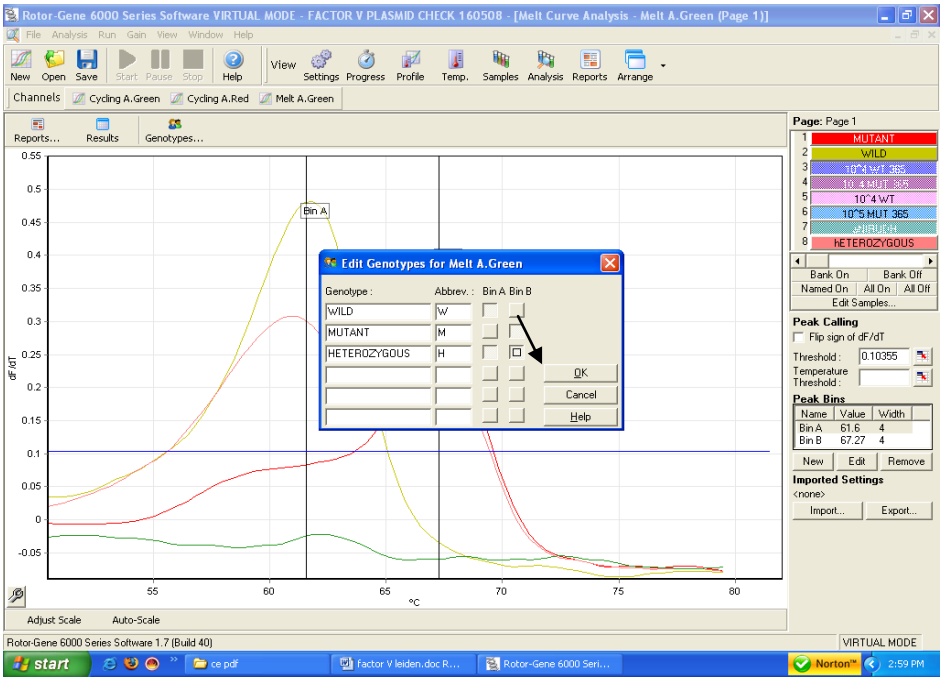


Fig. 58.

then Press O.K. as shown by arrow.

Now the software will automatically define the samples and will provide the results.

The following results are possible: The results mentioned below are with the Medium Filter.

**5. Only one Peak is detected in Melt channel Green at 61.6 ± 0.5**

The sample is of wild type.

**6. Only one Peak is detected in Melt channel Green at 66.95± 0.5**

The sample is of Mutant type.

**7. Two Peak's are detected in Melt channel Green at both 61.6± 0.5**

**& 66.95 ± 0.5**

The sample is Heterozygous.

**8. . No Peak's are detected in Melt channel Green at both 61.6 ± 0.5**

**& 66.95 ± 0.5**

The sample does not contain any Factor V DNA hence should be checked for PCR inhibition or else repeat extraction.

The software will automatically define the samples based on the above classification if the genotypes are Fed properly as shown earlier.

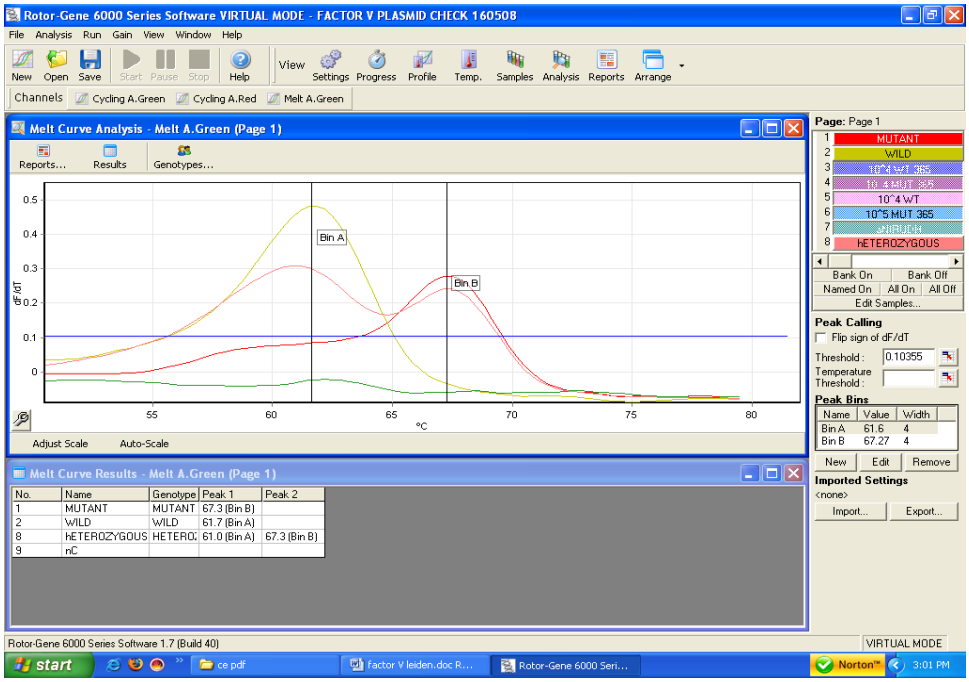


Fig. 59.

For your conveniance the above figure depicts One wild type, one mutant, One heterozygous and one No template control sample for Factor V which is duly defined by the software as well in the window below the curves.

Important Note:: The above results and peaks are interpreted with the Medium Filter. In case Light filter or Heavy Filter is used, then the Melt peaks can have a shift towards a little higher side or lower side. In such a case the peaks have to be defined based on the Bin.

## 10. a) Troubleshooting For Rotor Gene 2000/3000

### 1. No Peak's with Positive Controls (Factor V leiden) in Melt channel FAM.

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

➔ Repeat the PCR with corrected settings.

**2. Weak or no signal in Melt Channel FAM:**

- The PCR conditions do not comply with the protocol.

  ➔ Repeat the PCR with corrected settings.

- The FACTOR V LEIDEN Super Mix *R1* has been thawed and frozen too often.
- The FACTOR V LEIDEN 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 Factor V leiden 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 Rotor Gene 6000

**1. No Peak's with Positive Controls (Factor V leiden) in Melt channel Green.**

- Incorrect programming of the *Rotor-Gene*™ 6*000.*

  ➔ Repeat the PCR with corrected settings.

**2. Weak or no signal in Melt Channel Green:**

- The PCR conditions do not comply with the protocol.

  ➔ Repeat the PCR with corrected settings.

- The FACTOR V LEIDEN Super Mix *R1* has been thawed and frozen too often.
- The FACTOR V LEIDEN 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 Factor V leiden 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*** *Factor V leiden Real Time PCR Kit*, a dilution series has been set up from $10^6$ down to $10^0$ Copies/μl of Factor V leiden DNA and analyzed with the ***Geno-Sen's*** *Factor V leiden 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*** *Factor V leiden Real Time PCR Kit* is consistently 220 copies/ml. This means that there is 95% probability that 220 copies/ml will be detected.

### 11.b Specificity

In order to check the specificity of the ***Geno-Sen's*** Factor V leiden Real Time PCR kit, different DNA & DNA listed below were analyzed with ***Geno-Sen's*** FACTOR V LEIDEN Real Time PCR Kit. None of these led to a positive signal with the ***Geno-Sen's*** Factor V leiden Real Time PCR kit. Gene sequence analysis of the amplified region of Factor V leiden shows a pronounced homology among the various Factor V leiden 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 | Hepatitis B Virus | N. Meningitis |
| Human Herpes Virus 1 & 2 | Hepatitis C Virus | S. Pneumonia |
| Epstein barr Virus | Hepatitis E Virus | JEV |
| 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 | Malaria | Scrub typhus |
| B.pseudomallie | EnteroVirus | 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 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. 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. |
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| 12 | Hanta Virus RG quantitative Real time PCR kit. |
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| 13 | Measles Virus RG quantitative Real time PCR kit. |
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| 14 | West Nile Virus RG quantitative Real time PCR kit. |
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| 15 | H5 N1 (Bird Flu) RG quantitative Real time PCR kit. |
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| 16 | Chikungunya RG quantitative Real time PCR kit. |
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| 17 | TTV RG quantitative Real time PCR kit. |

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| 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. |

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| **42** | **Rabies RG quantitative Real time PCR kit.** |
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| **43** | **Factor V Leiden detection RG Real time PCR kit.** |

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