Geno-Sen's

RARa/PML (APML) (Rotor Gene)

Real Time PCR Kit

Quantitative

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)

Genome Diagnostics Pvt. Ltd
Up Mohal Naryal, Khasra No. 427,
Old Timber Depot Road, Parwanoo,
Dist Solan Himachal Pradesh 173220
Ph: +91 9810037653 Fax: 01792-234286
Email: genome24@rediffmail.com

EC REP

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

Table of Contents

1.	Contents of Kit.	Page 3
2.	Storage of the kit.	Page 3
3.	RARa/PML information	Page 4
4.	Precautions for PCR	Page 5
5.	Additionally required Materials & Devices	Page 5
6.	Principle of Real Time PCR	Page 6
7.	Description of the Product.	Page 6
8.	Procedure	Page 6
	8.a RNA Extraction	Page 6
	8.b Reference Gene	Page 7
	8.c Quantitation	Page 7
	8.d Preparation for PCR	Page 8
	8.e Preparation for PCR amplification	Page 8
	8.f Programming of the Rotor Gene 2000/3000	Page 10
	8.g Programming of the Rotor Gene 6000	Page 18
9.	a) Generated Data Interpretation & Analysis 2000/3000	Page 28
	b) Generated Data Interpretation & Analysis 6000	Page 29
10.	a) Trouble shooting 2000/3000	Page 31
	b) Trouble shooting 6000	Page 32
11.	Specifications	
	11.a Sensitivity & Reproducibility	Page 33
	11.b Specificity	Page 33
12.	Warranty	Page 34
13.	Limitations of product use.	Page 34
14.	List of GENO-SEN'S range of Real Time PCR kits	Page 35

RARa/PML 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	Cat. No. 91116014 100 rxns	Cat. No. 91116015 50 rxns	Cat. No. 91116016 25 rxns
R1 Blue	RARa-Bcr-1/2 Super Mix RG.	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R2 Blue	RARa Bcr-3 Super Mix RG .	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R3 Blue	Abl Super Mix . RG	25 rxns x 4 Vials	25 rxns x 2 Vials	25 rxns x 1 Vials
R4 Yellow	RARa/abl Mg Sol RG	3 vials	2 vial	1 vial
RARa Bcr-1/2-S1 Red	*RARa* Bcr-1/2 *Standard 1* *1 X 10^6 copies/µl*	1 Vial of 200µl	1 Vial of 200µl	1 Vial of 200µl
RARa-Bcr-1/2-S2 Red	*RARa* Bcr-1/2 *Standard 2* *1 X 10^5 copies/µl*	1 Vial of 200µl	1 Vial of 200µl	1 Vial of 200µl
RARa-Bcr-1/2-S3 Red	*RARa* Bcr-1/2 *Standard 3* *1 X 10^4 copies/µl*	1 Vial of 200µl	1 Vial of 200µl	1 Vial of 200µl
RAR -Bcr-1/2-S4 Red	*RARa* Bcr-1/2 *Standard 4* *1 X 10^3 copies/µl*	1 Vial of 200µl	1 Vial of 200µl	1 Vial of 200µl
RAR-Bcr-1/2-S5 Red	*RAR* Bcr-1/2 *Standard 5* *1 X 10^2 copies/µl*	1 Vial of 200µl	1 Vial of 200µl	1 Vial of 200µl
Abl-S1 Red	*abl Standard 1* *1 X 10^6 copies/µl*	1 Vial of 200µl	1 Vial of 200µl	1 Vial of 200µl
Abl-S2 Red	*abl Standard 2* *1 X 10^5 copies/µl*	1 Vial of 200µl	1 Vial of 200µl	1 Vial of 200µl
Abl-S3 Red	*abl Standard 3* *1 X 10^4 copies/µl*	1 Vial of 200µl	1 Vial of 200µl	1 Vial of 200µl
Abl-S4 Red	*abl Standard 4* *1 X 10^3copies/µl*	1 Vial of 200µl	1 Vial of 200µl	1 Vial of 200µl
Abl-S5 Red	*abl Standard 5* *1 X 10^2 copies/µl*	1 Vial of 200µl	1 Vial of 200µl	1 Vial of 200µl
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.

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

3. RARa/PML Information

Application

Acute promyelocytic leukemia (APL) known as acute myeloid leukemia-3, AML3, or M3 according to the French-American-British (FAB) classification is characterized by a predominance of malignant promyelocytes that carry a reciprocal translocation between the long arms of chromosomes 15 and 17: t(15;17)(q22;q11.2-q12). This translocation is diagnostic for APL as it is present in almost 100% of cases. De The et al. showed that the (15;17) translocation fuses the retinoic acid receptor alpha (*RARA*) locus to a gene, initially called myl and then renamed *PML*. Retinoic acid, a metabolite of vitamin A, can overcome the block of maturation at the promyelocytic stage and induce the malignant cells to terminally mature into granulocytes resulting in complete albeit transient disease remission.Comparison of the cDNA structures obtained by multiple groups showed variation in the amount of PML sequences included in the fusion protein. The *RARA* portion was invariant, containing the DNA-binding and ligand-binding motifs. The *PML* sequence variation seen among patients was generated by heterogeneous breakpoint cluster regions as well as by alternative splicing.

Bcr1 is more 3' within the PML gene and includes sequences from PML exons 5 and 6. Breakage in bcr2 involves sites in and around exon 6 of PML and leads to an intermediate length of PML sequence. The most 5' breakpoint, bcr3, fuses PML exons 1 through 3 of PML to RARA exon 3. This breakpoint yields short PML-RARA fusion proteins. In general, about 50% of patients exhibit long PML-RARA fusion proteins associated with bcr1.

Many cases of leukemia are caused by translocations that result in the formation and expression of chimeric fusion genes. The majority of APL patients harbor a translocation between chromosomes 15 and 17, with resultant fusion of the PML gene, at 15q12, with the retinoic acid receptor α(RARα) gene, at 17q22. This gene fusion results in production of a leukemia-specific chimeric mRNA, PML-RARα. Acute promyelocytic leukemia is defined by a characteristic morphology (AML FAB M3/M3v), by the specific translocation t(15;17) and its molecular correlates (PML/RARa and RARa/PML). Thereby it can be separated from all other forms of acute leukemia. Now it is routinely possible to detect extremely small numbers of transcripts from these leukemia-associated fusion genes in the blood or bone marrow of affected patients with the help of Real Time PCR.

Acute promyelocytic leukaemia (APL; AML M3) is identified by a unique t(15;17) translocation which fuses the PML gene to the retinoic acid receptor alpha gene (RARA). It is known as PML-RARA when it is (15q+ derived) or RARA-PML when it is (17q- derived) chimaeric transcripts.

Geno Sen's RARa/PML Real Time PCR kit is a sensitive and quantitative assay that can expeditiously analyze large numbers of samples from the patients samples.

Fusion transcripts containing the PML/RARa can be measured with a distinct Geno Sen's Kit.

The kit is optimally suited for quantification of residual amounts of leukemia-specific Ph^+ cells and for the follow up of leukemia patients undergoing therapy. The increase or decrease of RARa/PML transcripts normalized to abl transcripts may be an additional tool for early treatment decisions.

The GenoSen RARa/PML cDNA Quantification assay is developed for laboratory scale or high-throughput quantitative transcript analysis by real time quantitative fluorescence PCR.

GenoSen standardized ready-to-use Control and Reaction mix allow fast processing of the samples.

Module specificity

In vitro quantification of RARa/PML fusion transcripts Bcr1/2 isoform & Bcr 3 isoform in whole RNA/mRNA samples which are reverse transcribed into cDNA using random hexanucleotides, optimized for transcripts prepared from crude or purified human white blood cells (WBC).

Samples which can be used for Extraction: Whole Blood or cultured cells.

4. Precautions for PCR

The following aspects should always be taken care of:

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

5. Additionally Required Materials and Devices

- RNA isolation kit (see **8.a. RNA extraction**)

- 0.2 ml PCR tubes for use with 36-well rotor (Corbett Research, Cat.-Nr.: SE-1003F) alternatively 0.1 ml PCR tubes for use with 72-well rotor (Corbett Research, Cat.-Nr.: ST-1001)
- Micro Pipettes Variable Volume 2-20µl, 10-100µl, 100-1000µl,
- Sterile pipette tips with aerosol barrier 2-20µl, 10-100µl, 100-1000µl,
- Disposable powder-free gloves
- Vortex mixer
- Centrifuge Desktop with rotor for 1.7 ml reaction tubes
- *Rotor Gene™ 2000/3000* or *Rotor Gene™ 6000*, Corbett Research (The Real time PCR Instrument)

6. Principle of Real-Time PCR

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

7. Description Of the Product.

The ***Geno-Sen's RARa/PML*** *PCR* Reagents constitute a ready to use system for detection and quantification of ***RARa/PML*** 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 either the Bcr 1/2 isoform or Bcr 3 isoform of RARa/PML and for the direct detection of the specific amplicon in fluorescence channel Cycling A.FAM of the *Rotor Gene 2000/3000/6000 & the Reference gene on* Cycling A.FAM. in a seperate reaction to determine ratio of RARa/PML Vs abl gene. External positive Standards (RAR Bcr-1 S 1-5) , & (abl S 1-5) are supplied which allow the determination of the gene load. For further information, please refer to section 8.c Quantitation.

8. Procedure

8.a RNA Extraction

RNA Extraction kits are available from various manufacturers. Sample volumes for the RNA Extraction procedure depend on the protocol used. Please carry out the

RNA Extraction according to the manufacturer's instructions. The recommended Extraction kits are the following:

Sample Material	**Nucleic Acid Isolation Kit**	**Cat. Number REF**	
Whole Blood or cultured cells.	QIAamp RNA Blood Mini extraction Kit (50) Geno Sen's® Whole Blood RNA Extraction Mini Kit	52304 98007, 98008	QIAGEN Genome Diagnostics.

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

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

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

The *RARa/PML Rotor Gene PCR Reagents* should not be used with phenol based isolation methods.

8.b *Reference gene:*

Reference Gene i.e. **abl** allows the user to determine the RARa/PML Vs abl ratio & simultaneously control possible PCR inhibition. The Reference gene control reagents are provided separately and has to be run separately on FAM channel itself. There is no need to add separate internal control in the reaction mix.

8.c Quantitation

The quantitation standards provided in the kit *(RARa Bcr-1/2- S 1-5), & (abl S 1-5)* are treated in the same way as extracted samples and the same volume is used i.e. (2µl) instead of the sample. To generate a standard curve in the *Rotor Gene™ 2000/3000/6000*, all 5 Standards should be used as defined in the menu window *Edit Samples* of the *Rotor Gene™* software. The same should also be defined as standards with the specified concentrations (see *Rotor Gene™* Manual).

The standard curve generated as above can also be used for quantitation in subsequent runs, provided that at least one standard is used in the current run. For this purpose, the previously generated standard curve needs to be imported (see *Rotor Gene™ 2000/3000/6000 Manual*). However, this quantitation method may lead to deviations in the results due to variability between different PCR runs & due to varying Reaction efficiencies.

Attention: The standards are defined as copies/µl. The following formula has to be applied to convert the values determined using the standard curve into copies/ml of sample material:

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

Or else direct conversion can be done keeping in mind the starting volume of the sample & the final eluted Volume e.g.

If the starting volume of the sample while using the Qiagen QIAamp RNA Blood Mini extraction kit is 1.5 ml & the final Eluted Volume is 40µl then to obtain the direct values i.e. copies/ml for the patient samples the following values for the standards can be fed directly into the operating software which is again based on the above formula and will yield direct conversion of per ml of patient sample data.

S1: 10^6 Copies/µl = 26660000 Copies/ml
S2: 10^5 Copies/µl = 2666000 Copies/ml
S3: 10^4 Copies/µl = 266600 Copies/ml
S4: 10^3 Copies/µl = 26660 Copies/ml
S5: 10^2 Copies/µl = 2666 Copies/ml

8.d Preparation for PCR

8. e. 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 X 3 (in triplicate). Make sure that the tubes for standards & at least one negative control (*Water, PCR grade*) are included per PCR run. To generate a standard curve, use all supplied Standards *(PML Bcr-1*

S 1-5) & (abl S 1-5) for each PCR run. Before each use, all reagents need to be thawed completely and mixed (by pipeting 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

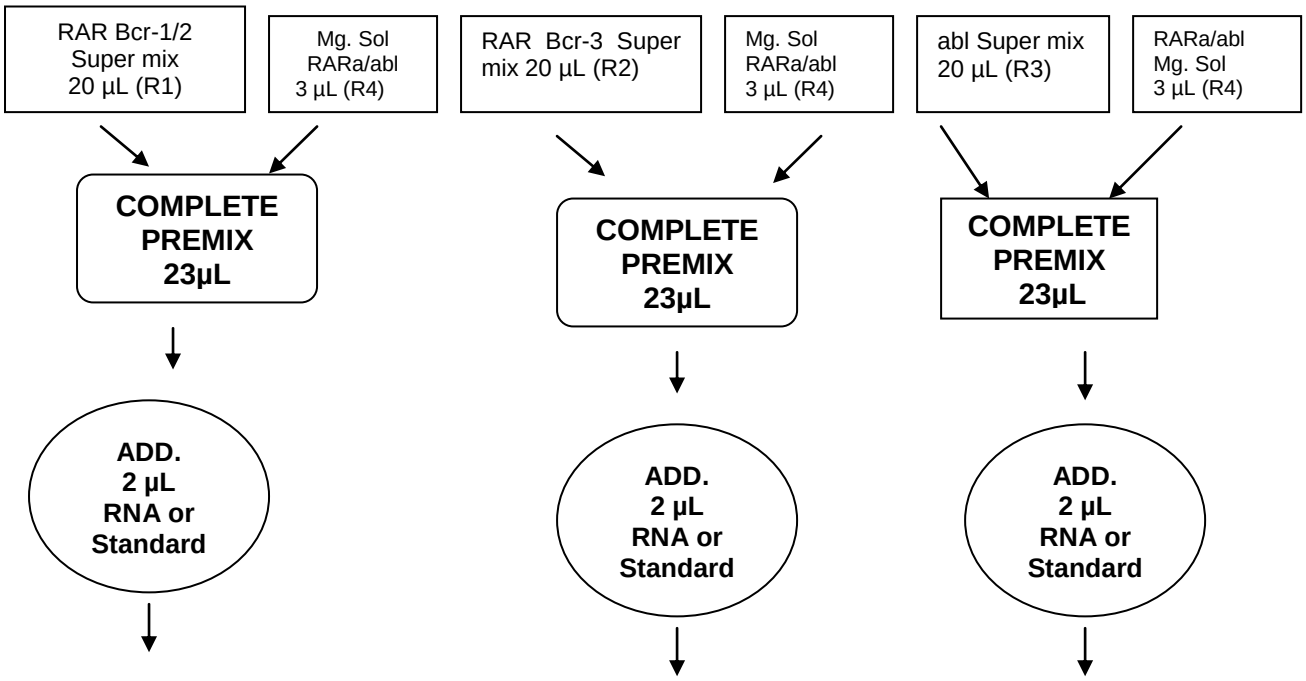

Fig. 4.

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

For Bcr 1/2 isoform

Bcr	1 rxns.	10 rxns.
Bcr Super Mix (R1)	20 µL	200 µL
Bcr Mg Sol. (R4)	3 µL	30 µL
Total	23µL	230µL

For Bcr 3 isoform

Bcr	1 rxns.	10 rxns.
Bcr Super Mix (R2)	20 µL	200 µL
Bcr Mg Sol. (R4)	3 µL	30 µL
Total	23µL	230µL

For abl

abl	1 rxns.	10 rxns.
abl Super Mix (R3)	20 µL	200 µL
RARa/PML Mg Sol. (R4)	3 µL	30 µL
Total	23µL	230µL

Fig. 5.

Pipette 23 µl of the Master Mix into each PCR tube labelled separately for Bcr 1 isoform, Bcr 3 isoform & ***abl*** respectively. Then add 2 µl of extracted RNA to each sample tube in both sets and mix well by pipeting up and down. Correspondingly, 2 µl of the Standards (RARA Bcr-1/2 *S1-5*), & (abl S 1-5) must be used as a positive control and 2 µl of water (*Water, PCR grade*) as a negative control. Close the PCR tubes and transfer the RARA tubes into the rotor of the *RotorGene*™ instrument whereas that of abl into the deep freezer at –20°C so that the same can be run later if required or else if the sample batch is small then the same can be run simultaneously in the same batch. 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.f. Programming the *RotorGene*™ 2000/3000

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

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

Program the *RotorGene*™ *2000/3000* for these 5 steps according to the parameters shown in Fig. 6-20. All specifications refer to the *RotorGene*™ software version 6.0.33. Please find further information on programming the *RotorGene*™ in the *RotorGene*™ *2000/3000 Operator's Manual*,. In the illustrations these settings are shown by arrows

Setting of general assay parameters & Reaction volume.

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

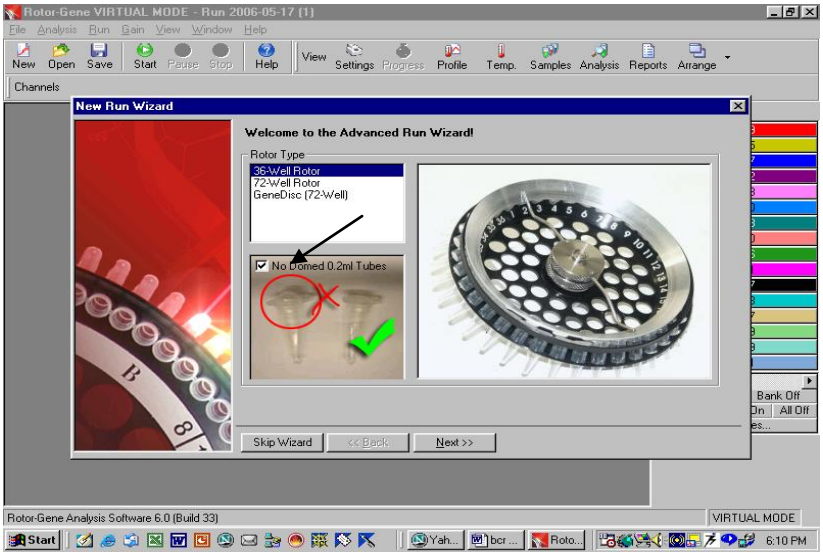

Fig. 6.

Confirmation of Reaction Volume as follows.

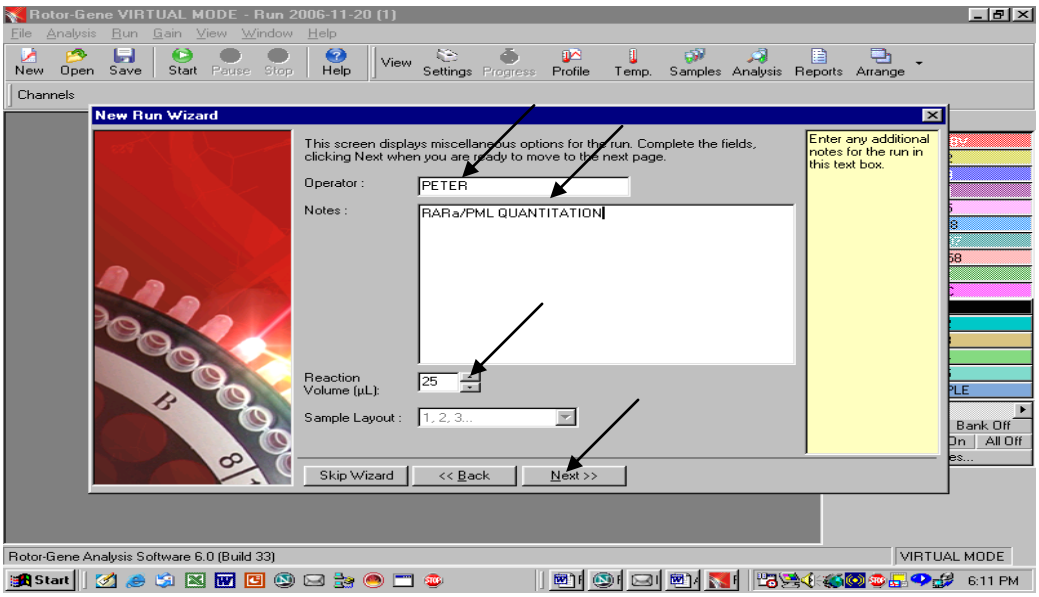

Fig. 7. Setting of general assay parameters.

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

THERMAL PROFILE & CALIBRATION:

Here the thermal profile for the assay will be defined.

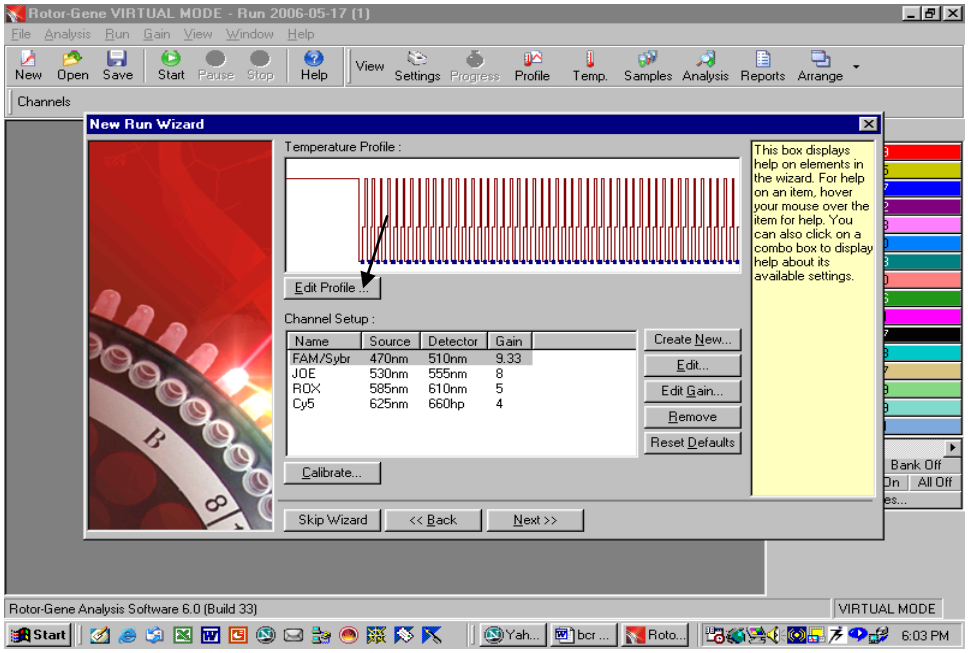

Fig. 8.

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

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

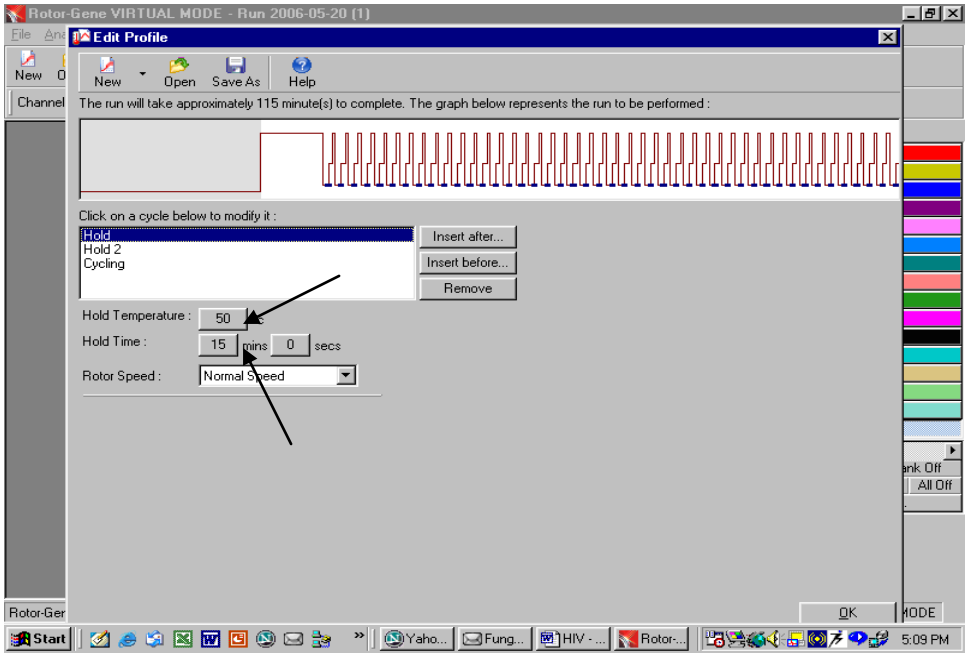

Fig. 9.

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

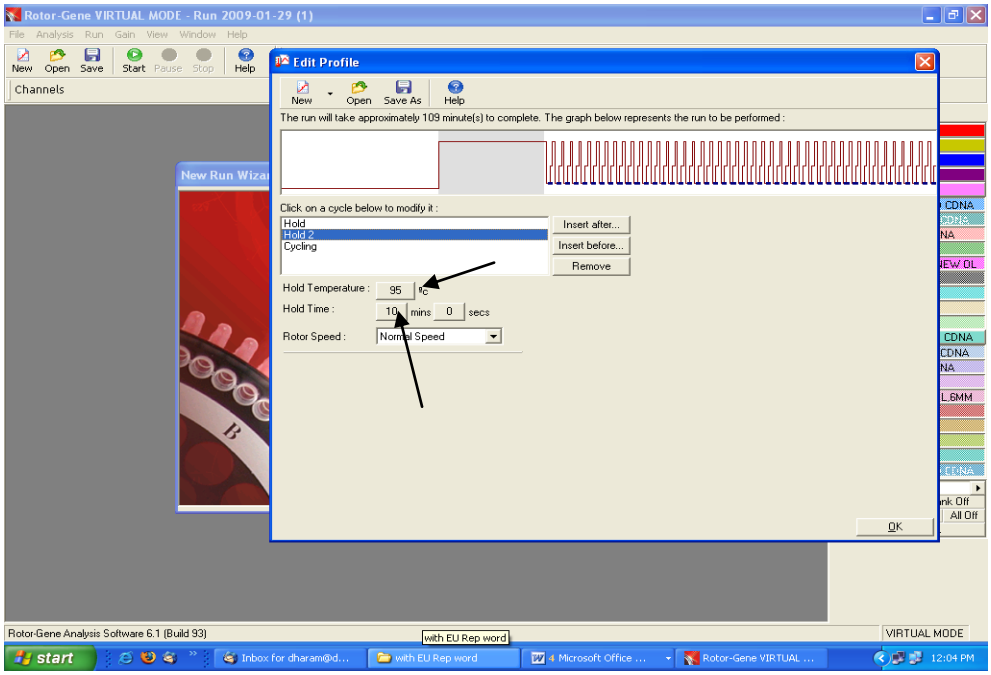

Fig. 10. Initial activation of the Hot Start enzyme.

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

95°C for 15 seconds.

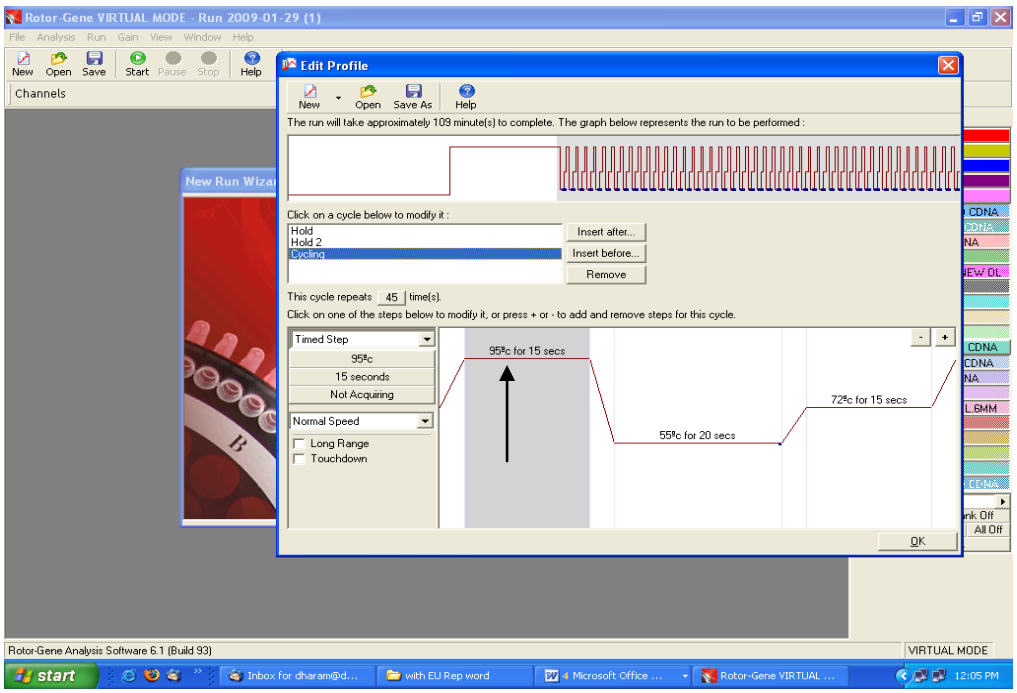

Fig. 11.

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

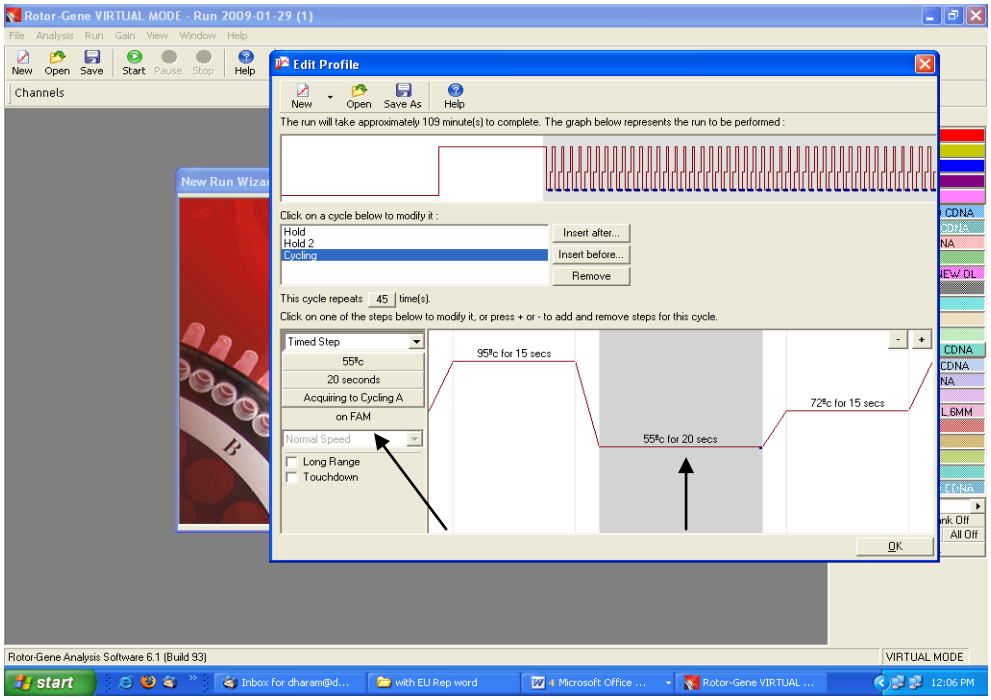

Fig. 12.

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

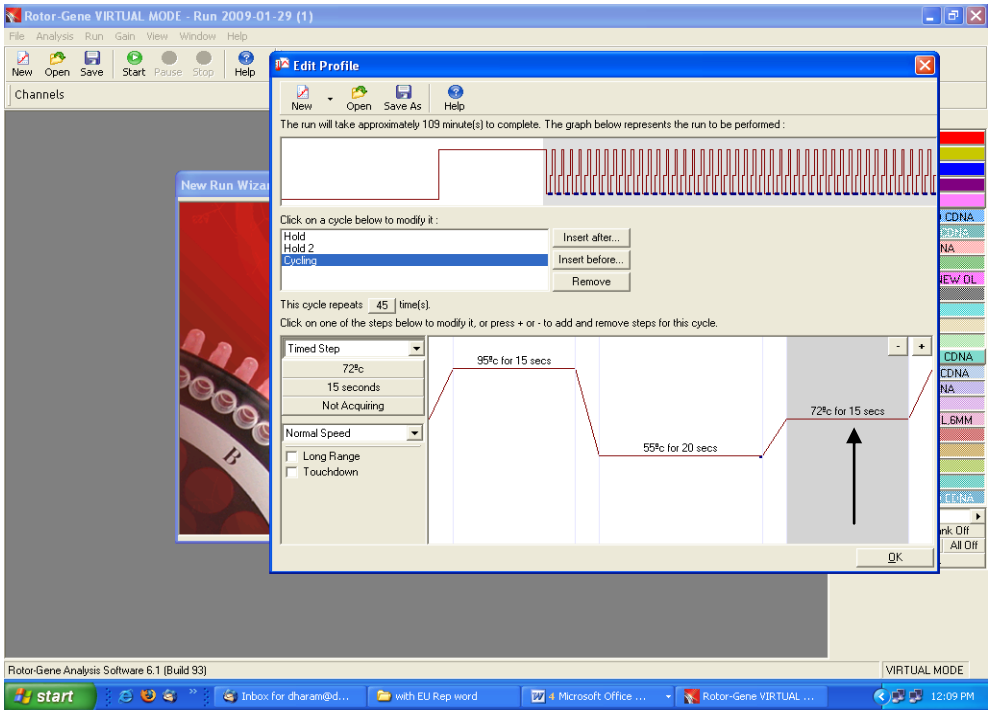

Fig. 13.

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

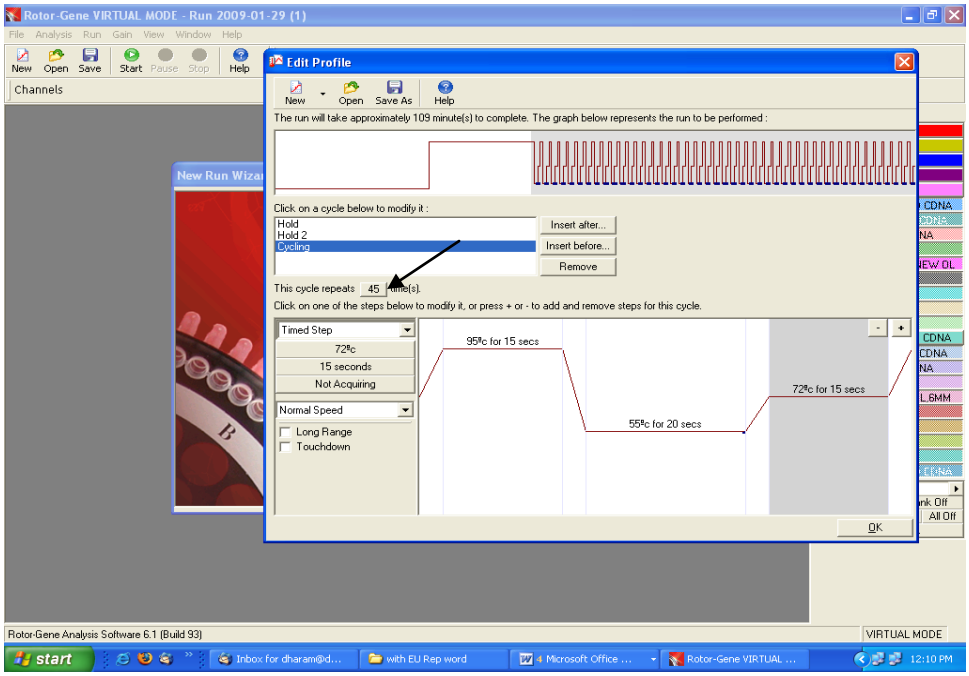

Fig. 14.

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

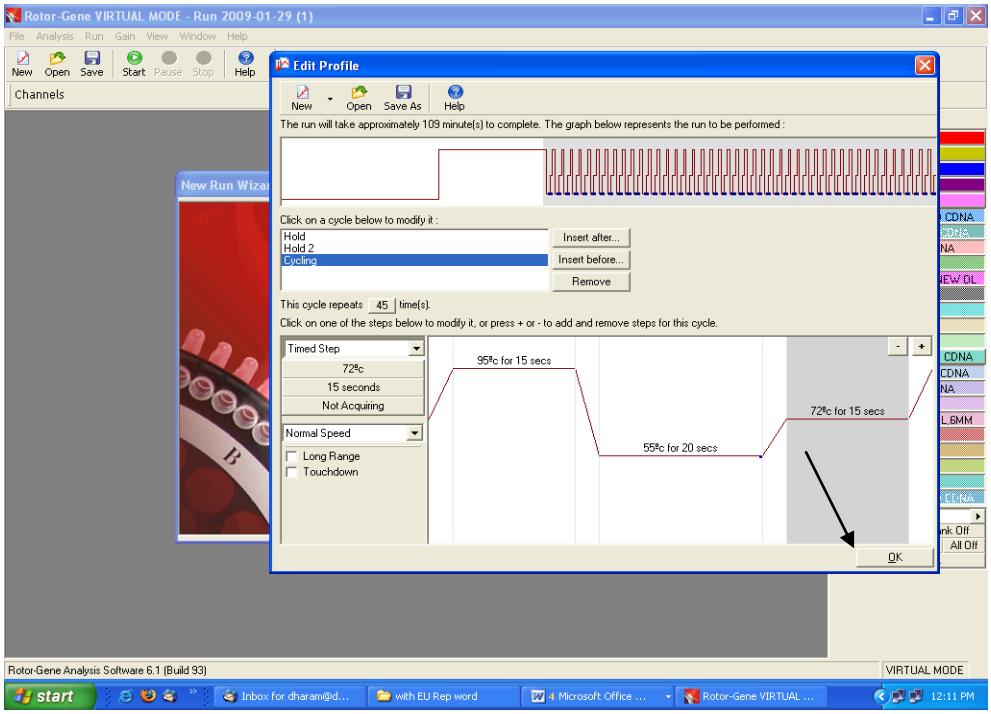

Fig. 15.

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

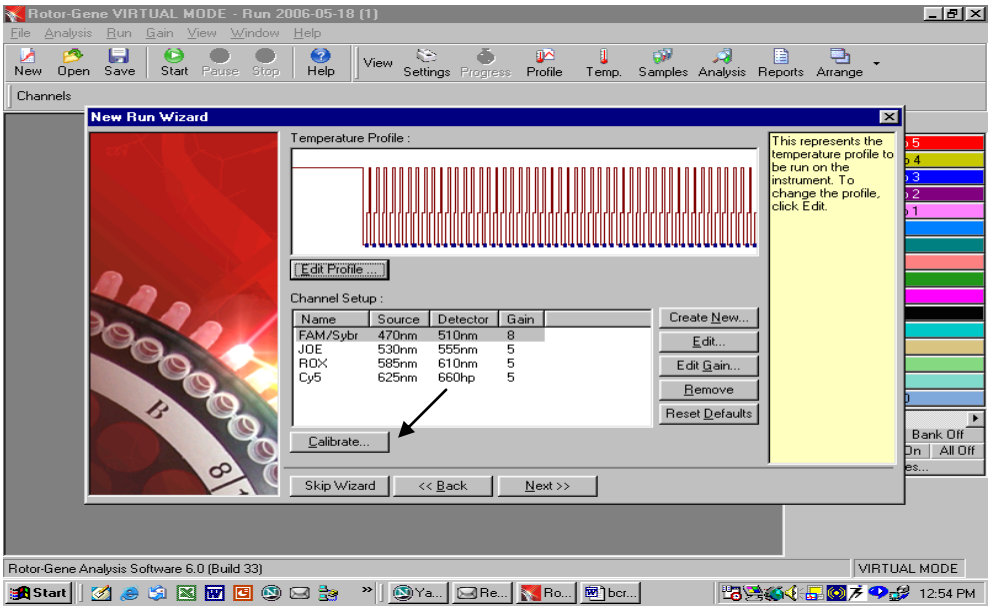

Fig. 16.

The detection range of the fluorescence channels has to be determined according to the fluorescence intensities in the PCR tubes. This adjustment is done in the menu window *Auto Gain Calibration Setup* (activation in menu window *New Experiment Wizard* under *Calibrate*). Please set the calibration temperature to the annealing temperature of the amplification program (compare Fig. 17).

Adjustment of the fluorescence channel sensitivity as shown below.

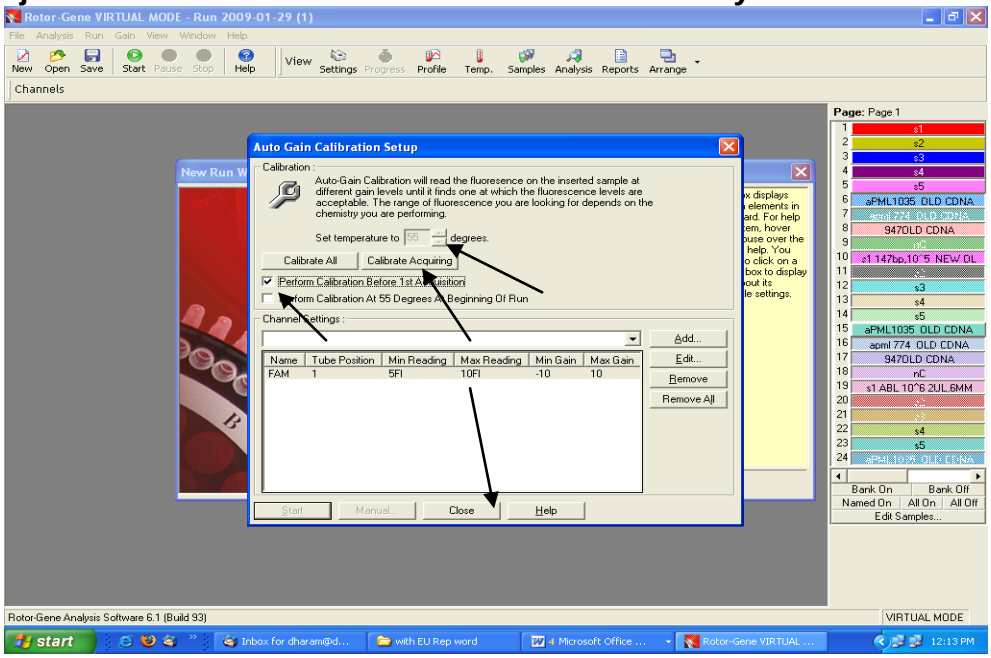

Fig. 17.

Please do not forget to click on the box against " Perform calibration before 1st, acquisition." After that press Close and a new window will open as shown below.

The gain values determined by the channel calibration are saved automatically and are listed in the last menu window of the programming procedure (Fig. 8).

PRESS NEXT

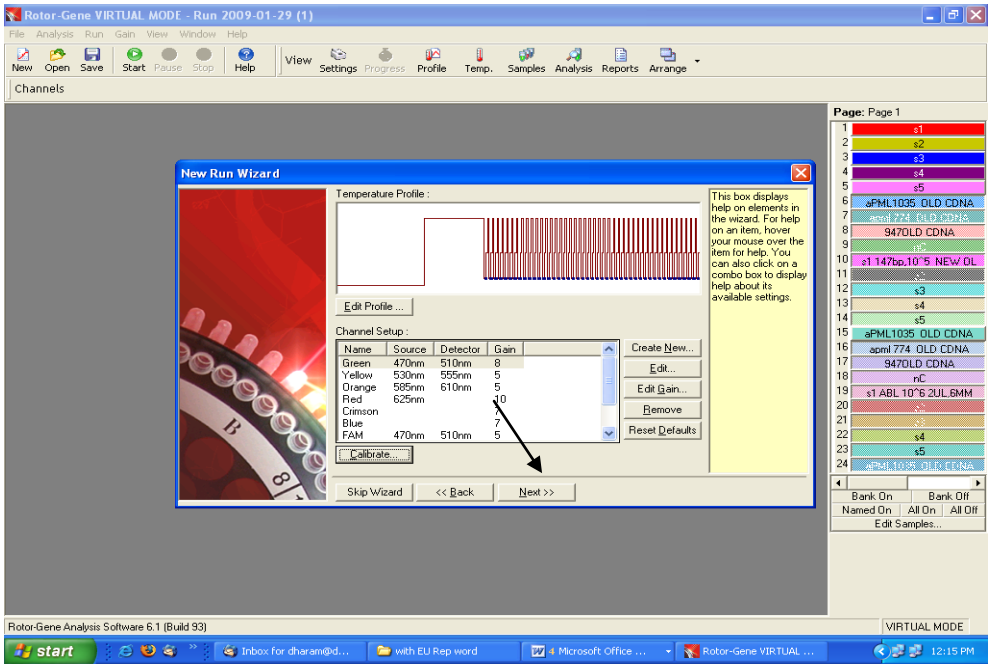

Fig. 18.

Starting of the *Rotor Gene*™ run. **FIG: AMPLIFICATION OF THE cDNA**

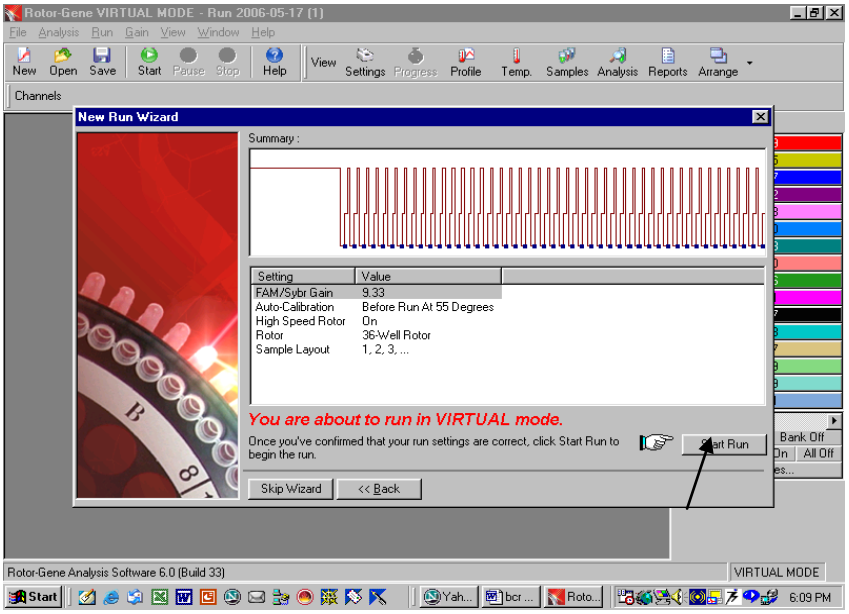

Fig. 19: Press Start Run Button.

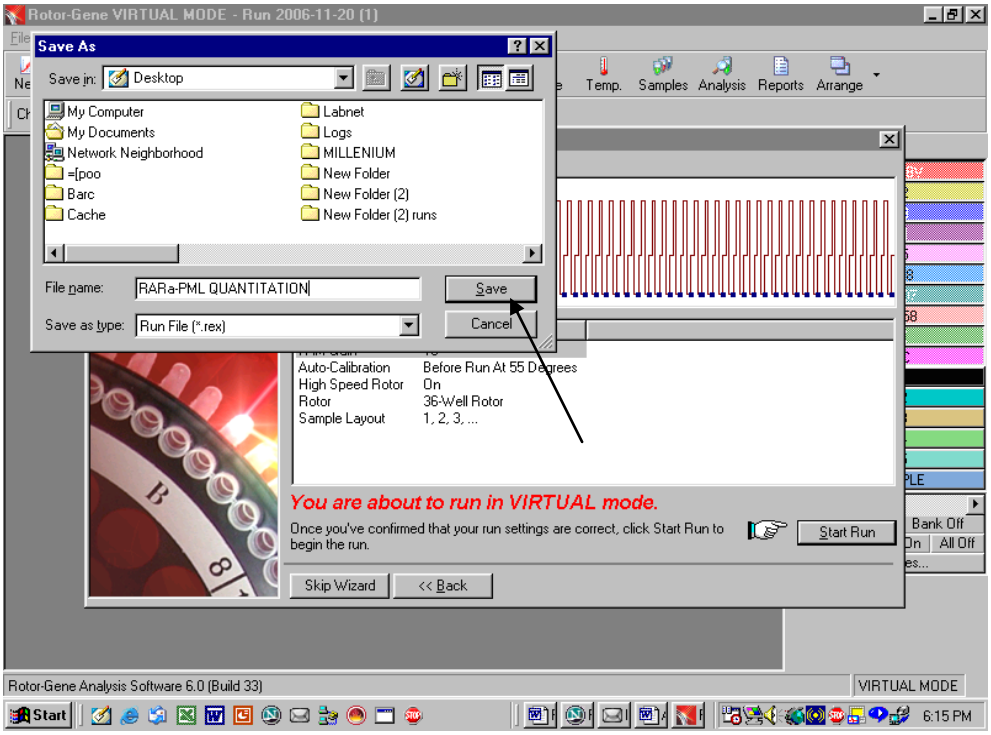

Fig. 20.

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.g. Programming the *Rotor Gene*™ 6000

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

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

Program the *RotorGene*™ 6000 for these 5 steps according to the parameters shown in Figures 21-38 below All specifications refer to the *RotorGene*™ 6000 software version 1.7 Please find further information on programming the *RotorGene*™ in the *RotorGene*™ 6000 *Operator's Manual*,. In the illustrations these settings are shown by arrows

g) Setting of general assay parameters & Reaction volume.

Please see to it that you 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.

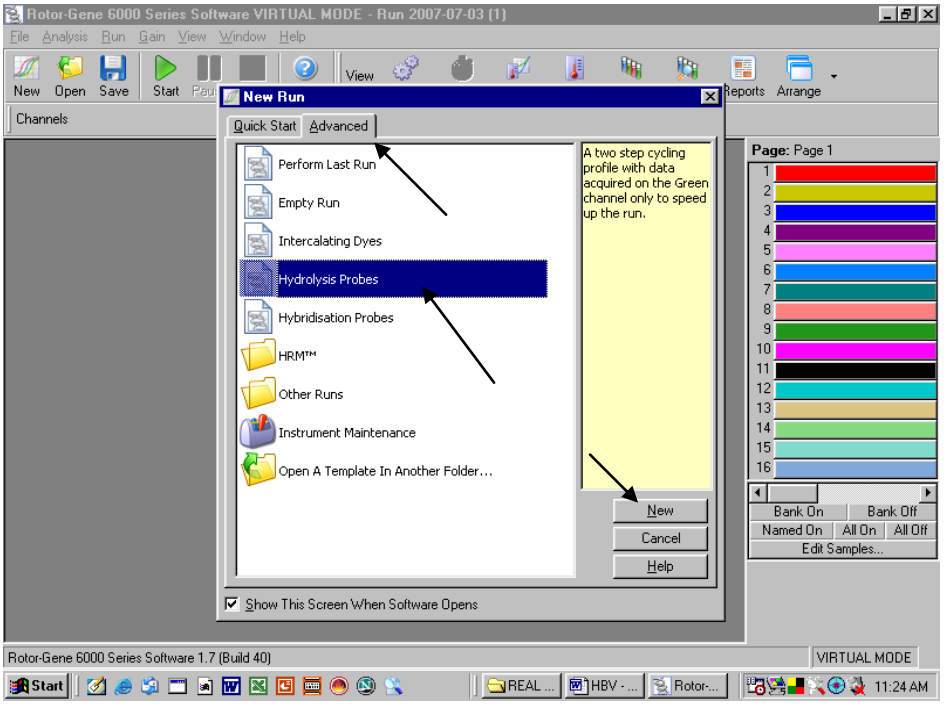

Fig. 21.

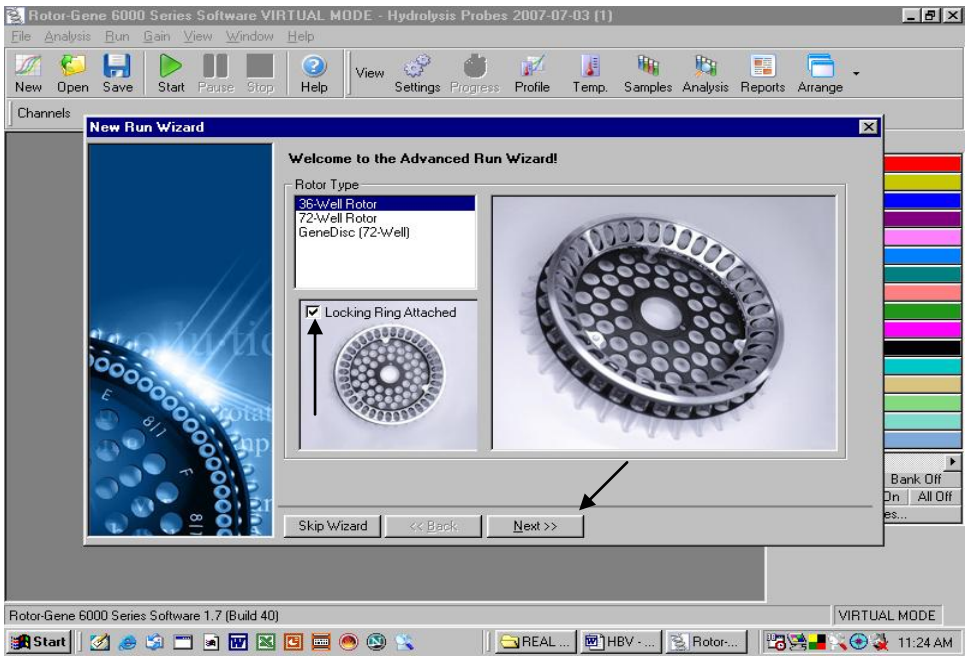

Fig. 22.

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.

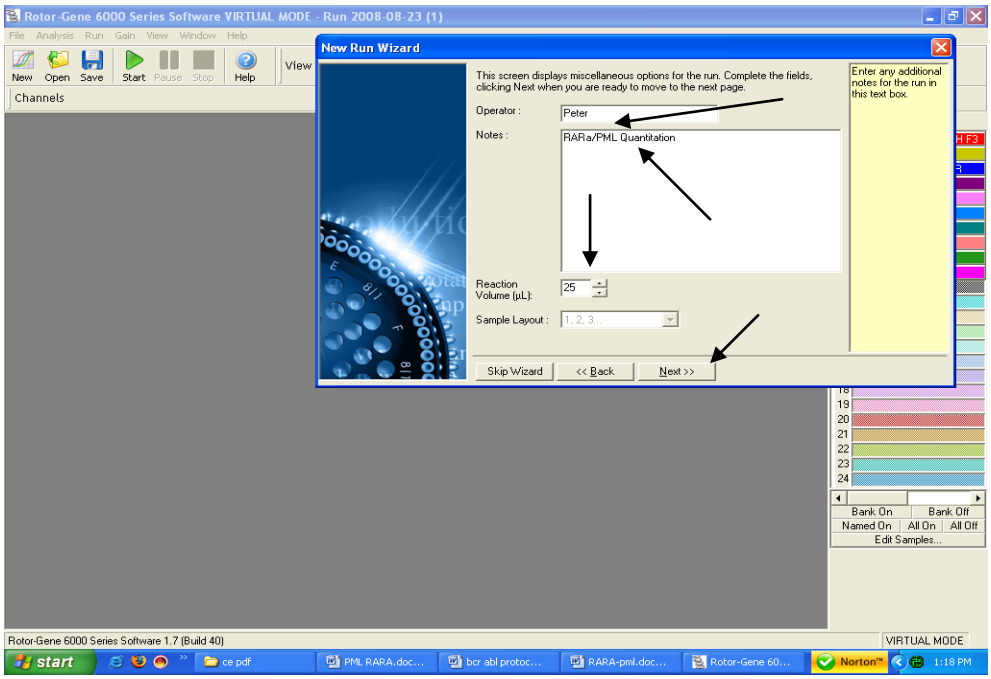

Fig. 23.

- 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.

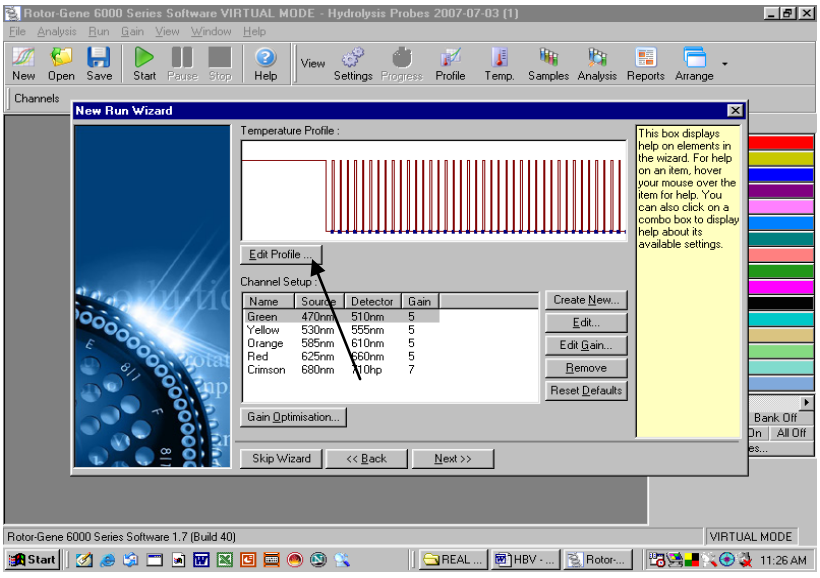

Fig. 24.

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

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

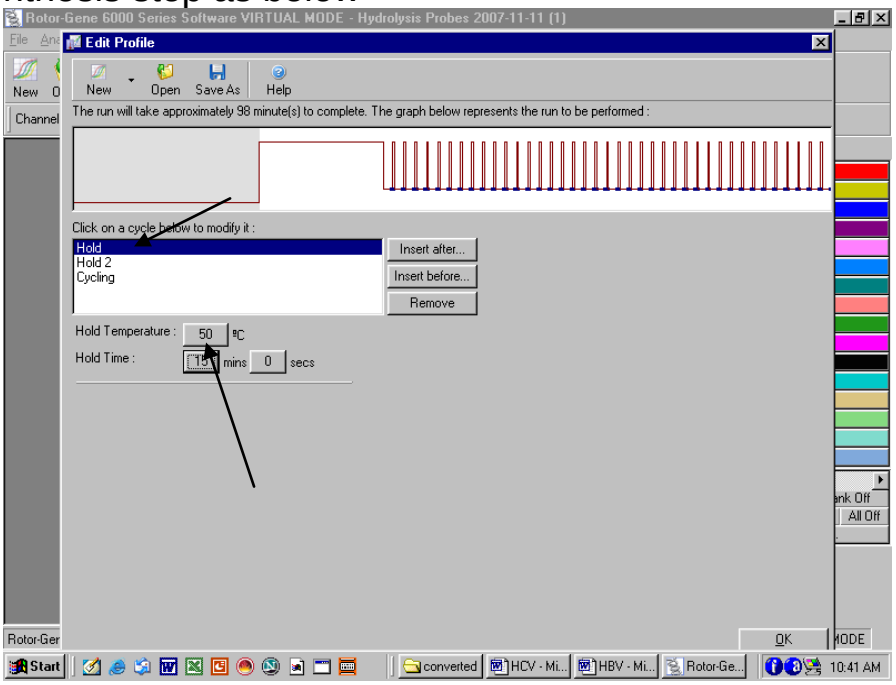

Fig. 25.

Second hold 95°C for 10 minutes as below

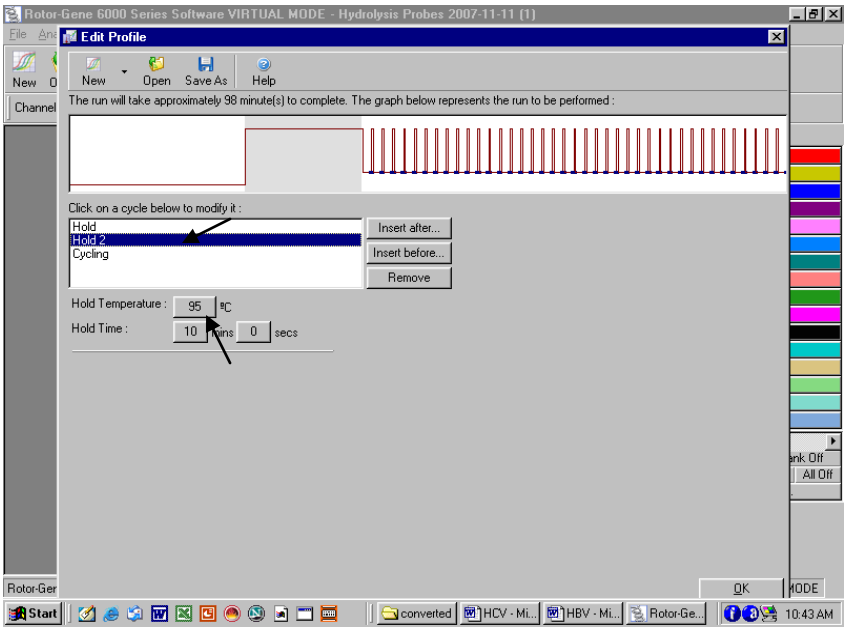

Fig. 26. Initial activation of the Hot Start enzyme. Generally the window will open with the Hold Temp as 95°C and the Hold Time as 10 minutes. In case it is different then set the same to as shown above.

j) Setting the Cycling and acquisition.

When clicked on Cycling the window will open as below.

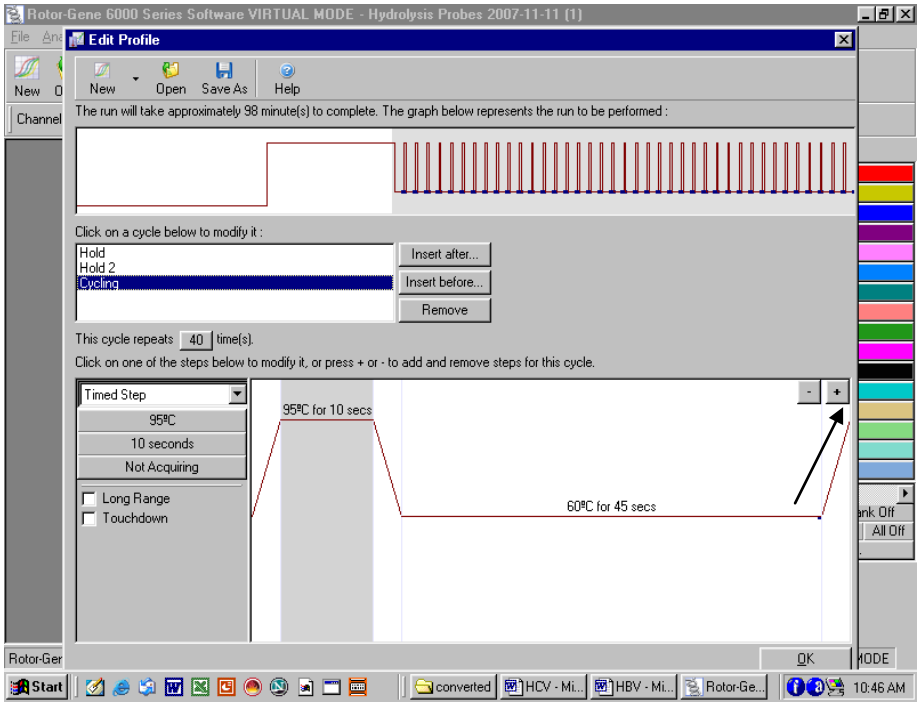

Fig. 27.

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.

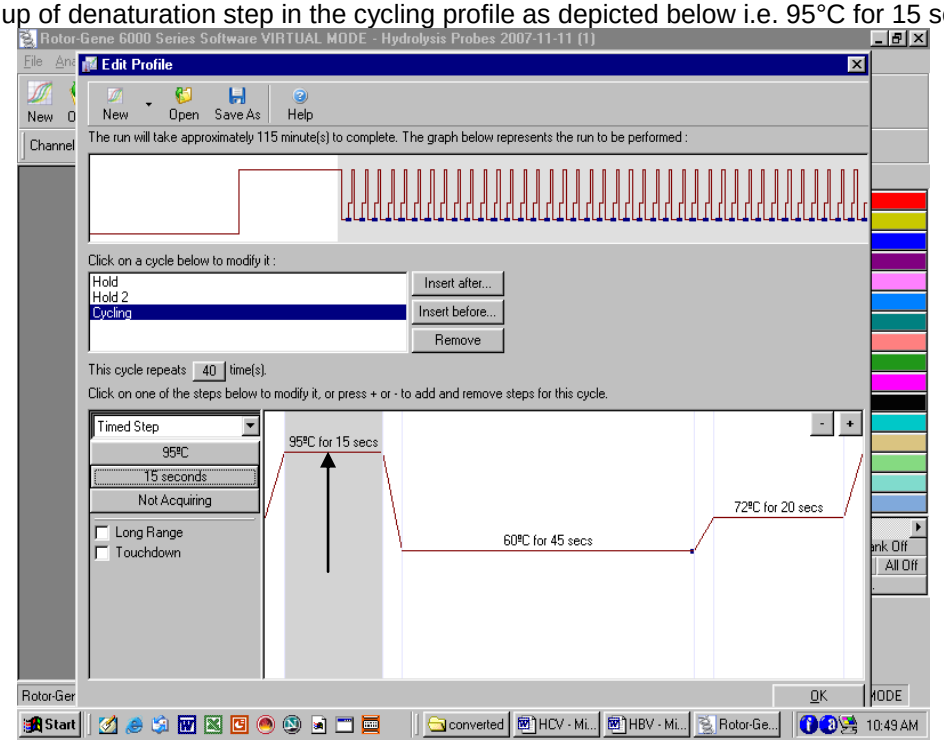

Fig. 28.

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

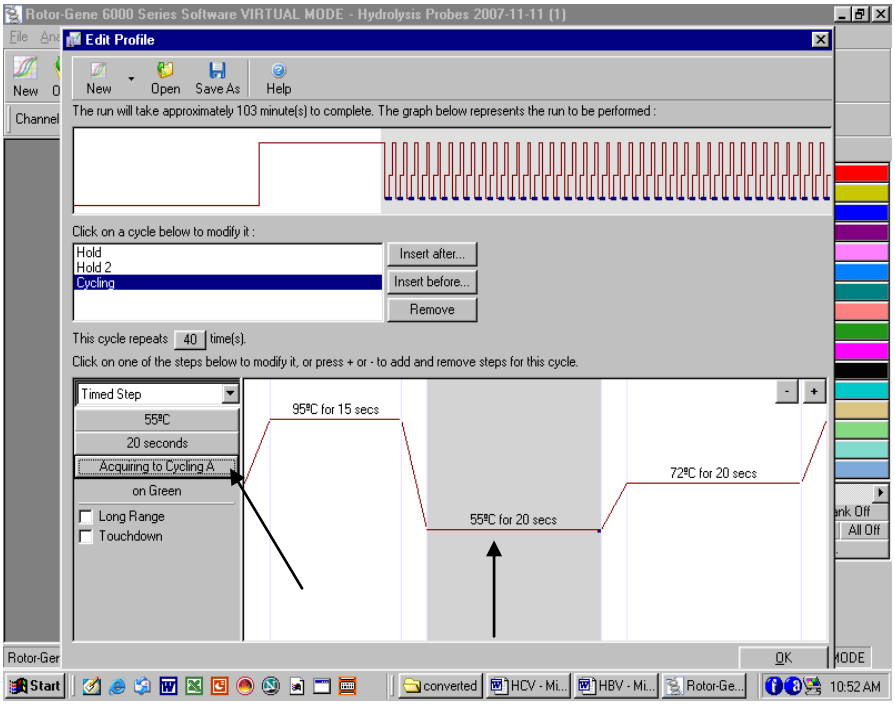

Fig. 29.

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

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

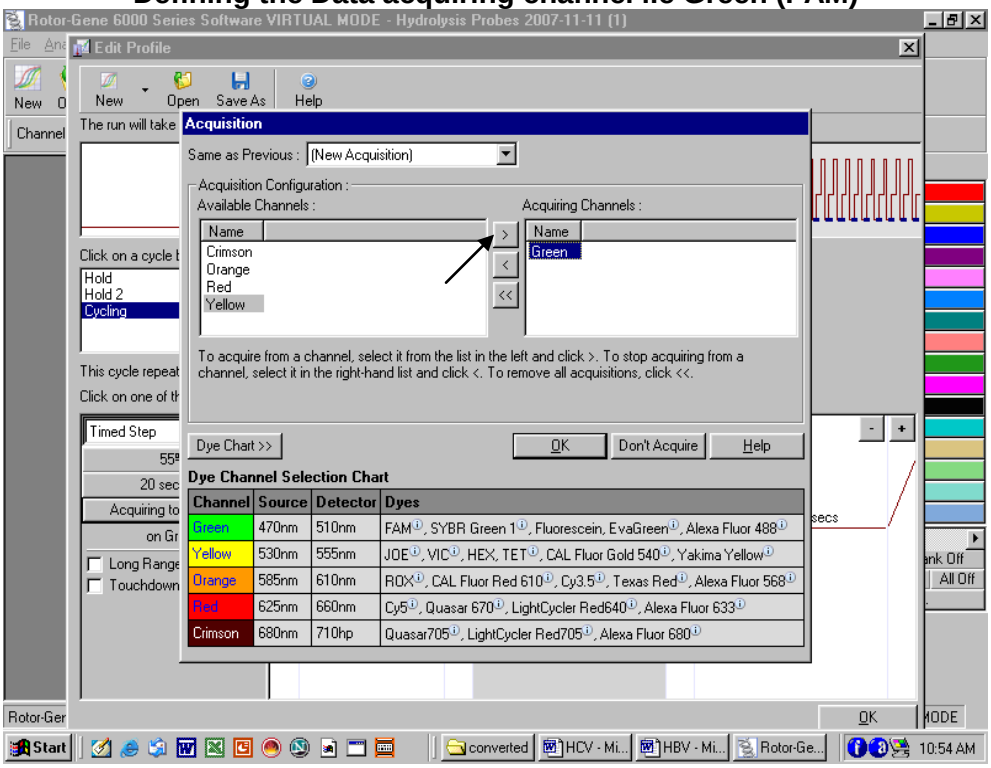

Fig. 30.

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.

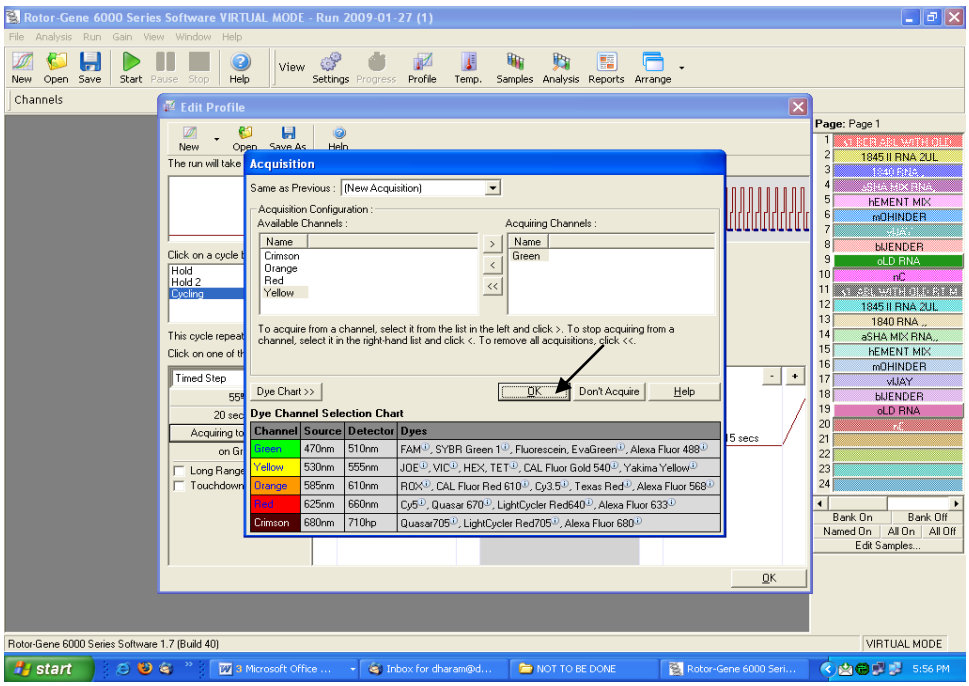

Fig. 31.

Once the 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

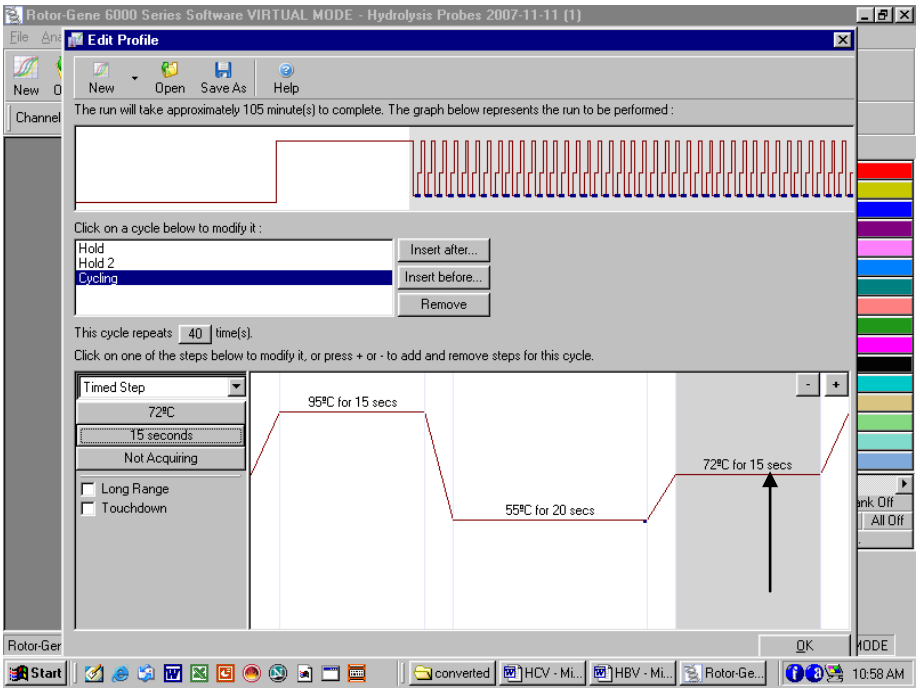

Fig. 32.

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

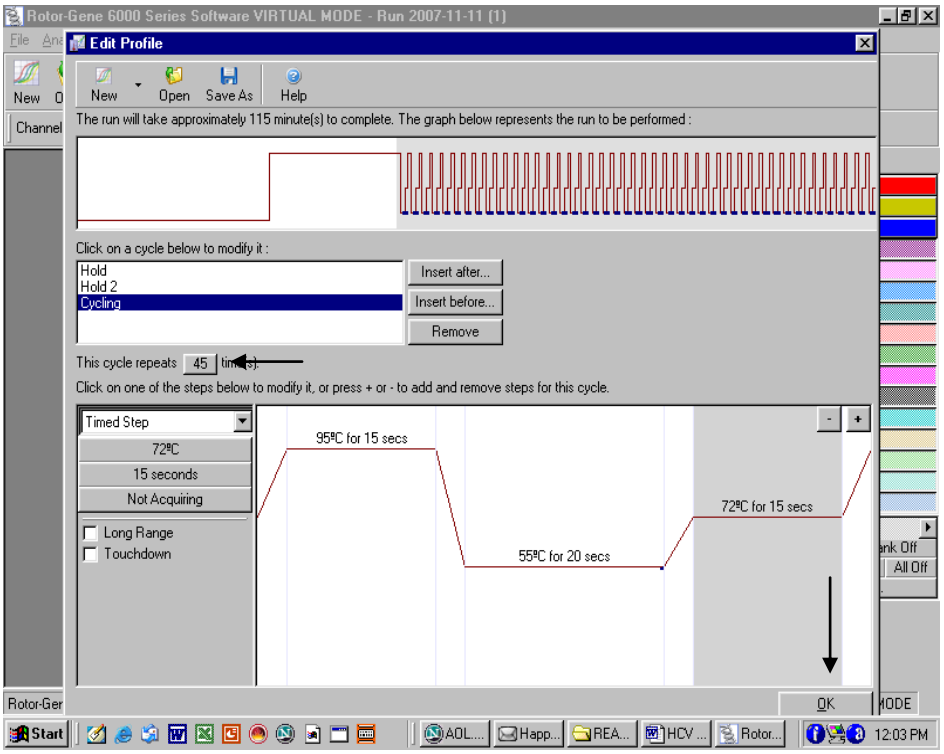

Fig. 33.

After setting the number of Cycles Press OK.

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

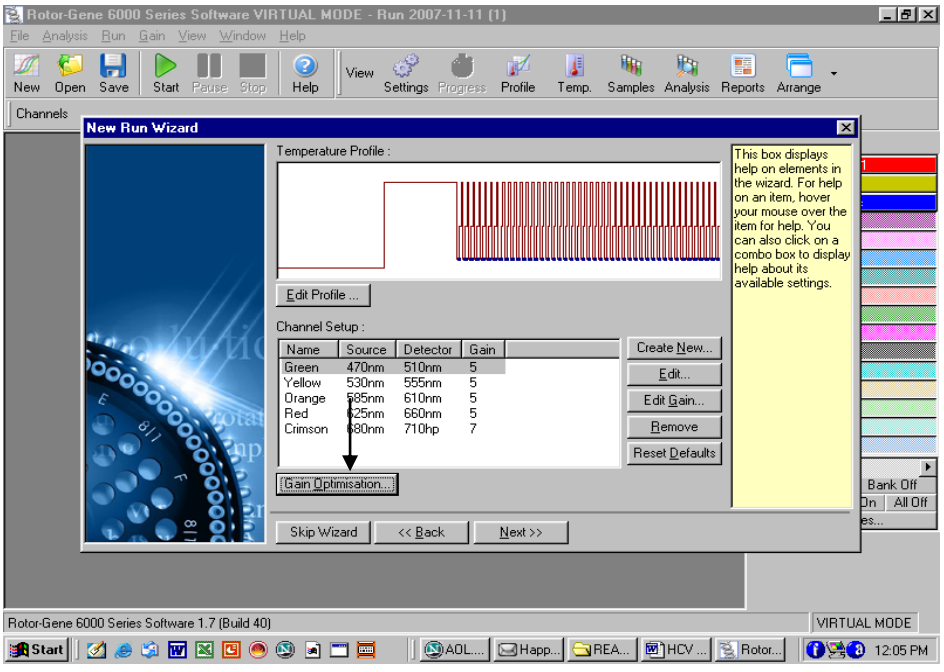

Fig. 34

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.35 .

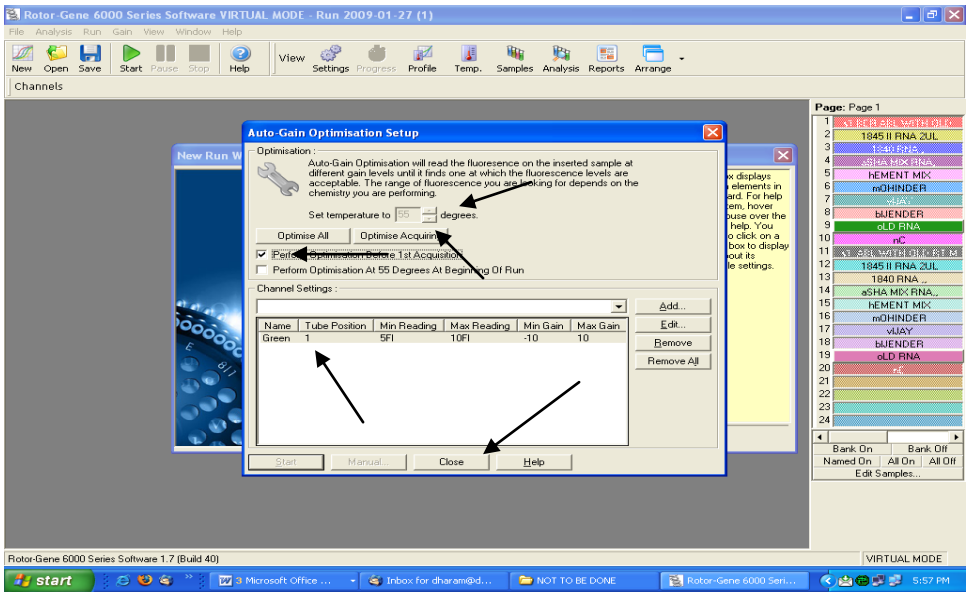

Fig. 35.

The following needs to be done.

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

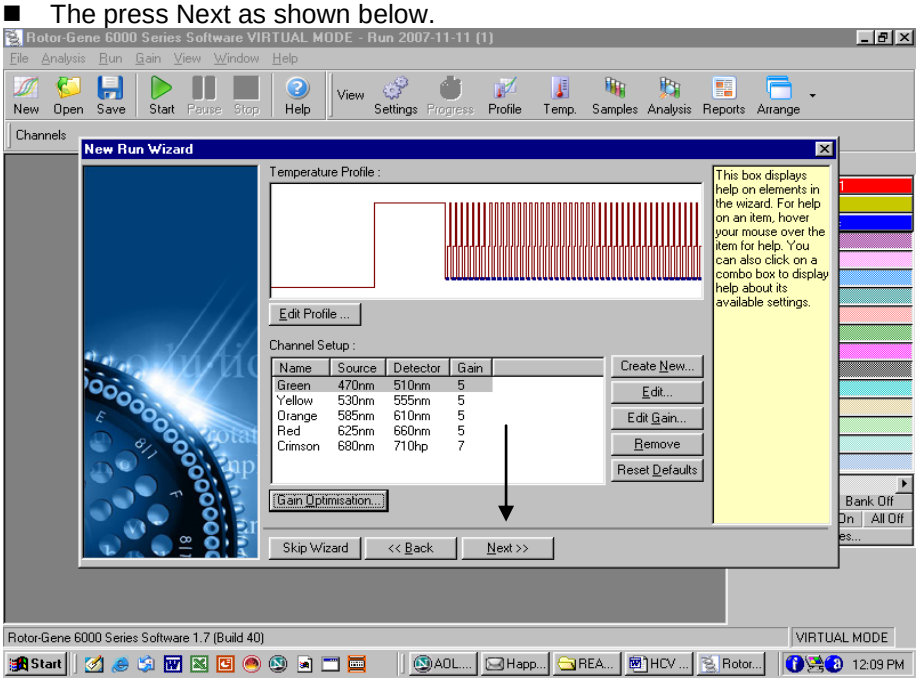

Fig. 36.

N) PRESS Start RUN

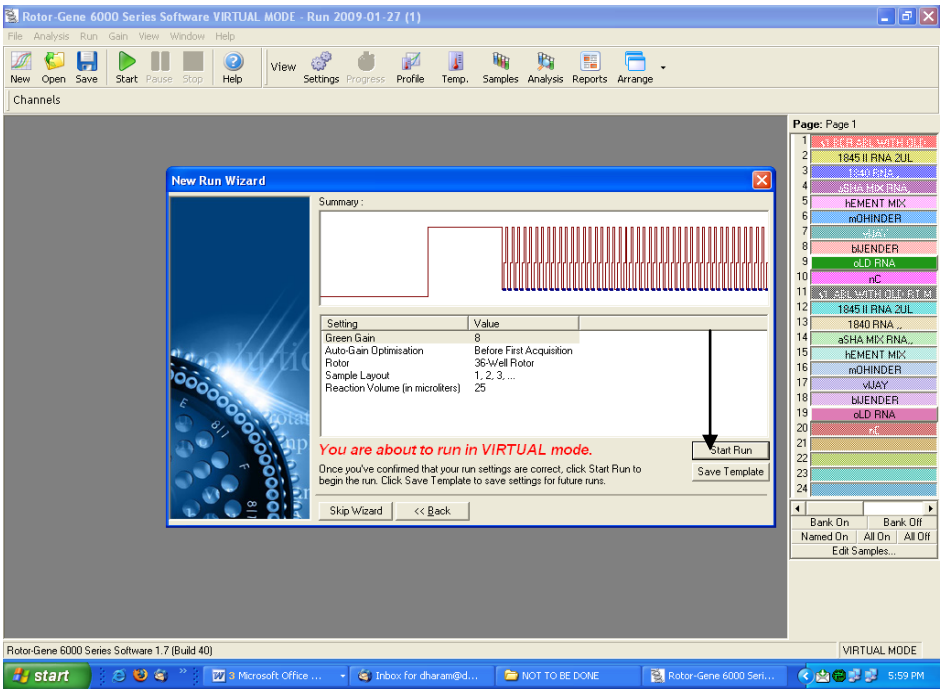

Fig. 37.

Saving the RUN File.

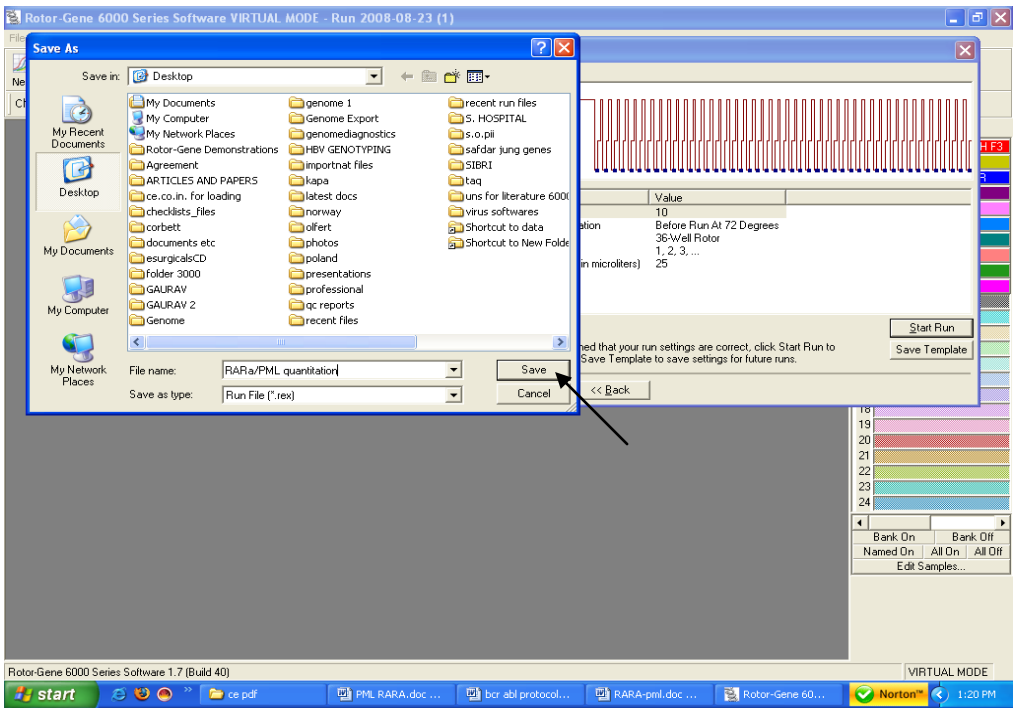

Fig. 38.

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 RotorGene 2000/3000

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

The following results are possible:

Important Note:

Please turn off rest of the tubes while reading the results of Bcr-1 including other standards as well. Only the Standards & the samples run in the Bcr-1 tubes & Bcr-3 should be read. Similar procedure should be followed for Abl while reading the results.

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

The result of the analysis is positive: The sample contains RARa/PML Bcr-1/2 cDNA or RARa/PML Bcr-3 cDNA depending upon the tubes it has amplified. Please look for the results in respective Bcr-1/2 or Bcr-3 tubes.

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

No RARa/PML cDNA detectable. It can be considered negative if there is amplification in the abl run. Please refer for details on PCR inhibition in the section PCR inhibition.

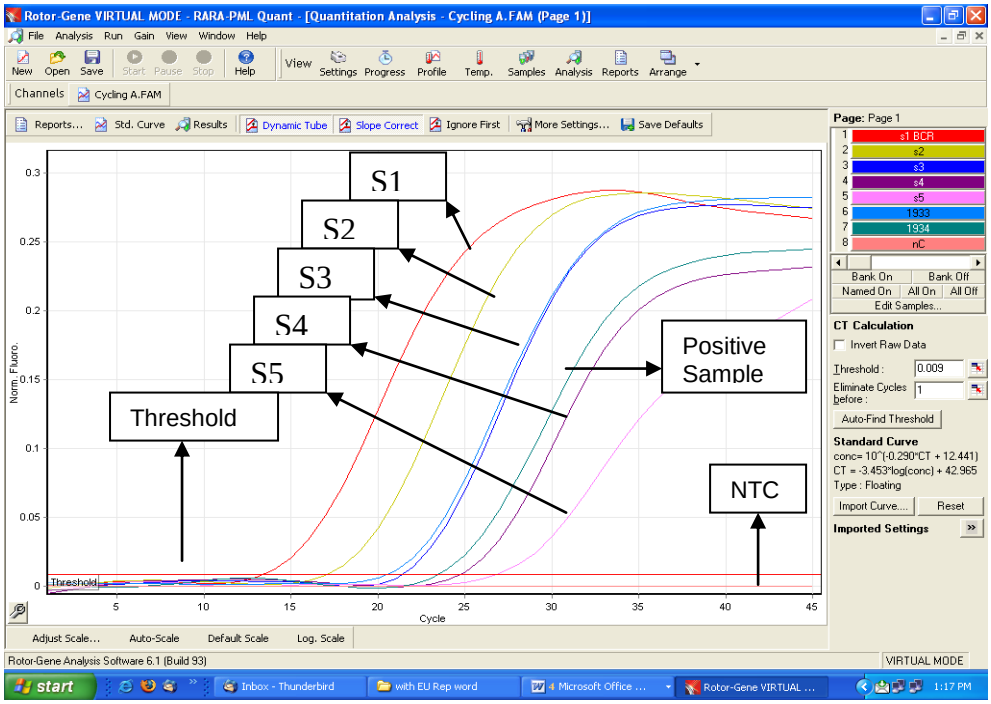

Fig. 39: Detection of the quantitation standards in fluorescence channel Cycling A.FAM. NTC: non-template control.

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

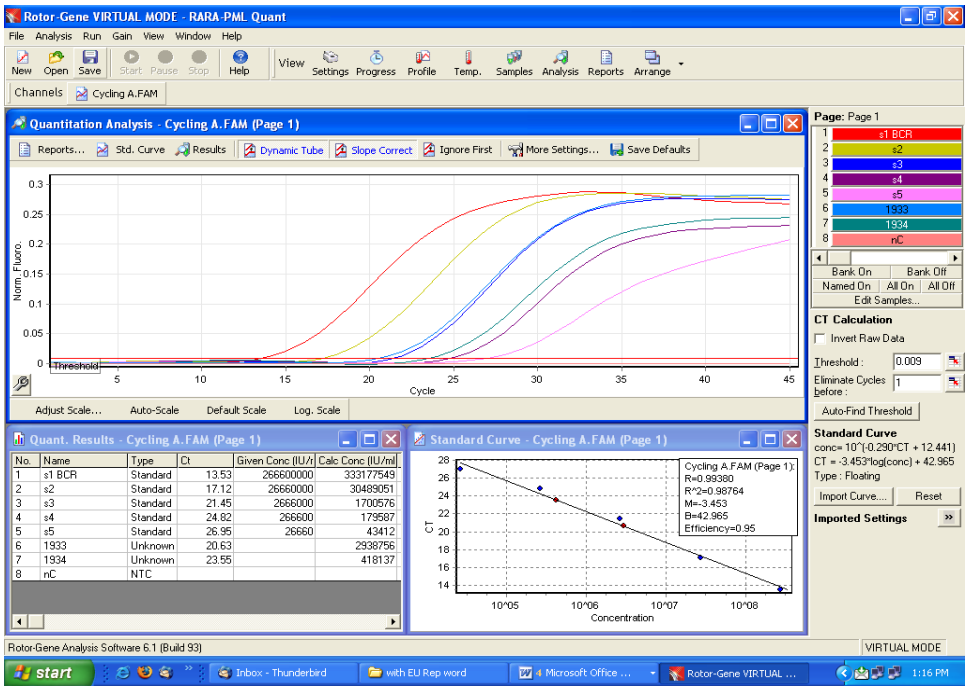

Fig. 40.

Example of analysed data for RARa/PML where all the amplification curves can be seen as well as the standard curve, reaction efficiency, M value etc. can be seen and the quantitative data for the samples can be seen.

Abl reference gene amplification.

Run the diluted samples for abl stored at –20 **°C in exactly the similar fashion as described for RARa/PML so far. After the run the quantitative results for the abl gene can be obtained & then the ratio of RARa-PML/ABL can be determined from the values obtained. These can also be run simultaneously in the same run as Bcr in case there is additional space in the Rotor to do so.**

PCR Inhibition

A signal is detected in fluorescence channel Cycling A. FAM in **abl**: **No PCR inhibition**

In fluorescence channel Cycling A. FAM in **abl** no signal is detected.

A possible PCR inhibition has occurred.

9. b) Generated Data Interpretation & Analysis for RotorGene 6000

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

The following results are possible:

Important Note:

Please turn off rest of the tubes while reading the results of Bcr-1 & Bcr-3 including other standards as well. Only the Standards & the samples run in the Bcr-1 tubes should be read. Similar procedure should be followed for Abl while reading the results.

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

The result of the analysis is positive: The sample contains RARa/PML Bcr-1/2 cDNA or RARa/PML Bcr-3 cDNA depending upon the tubes it has amplified. Please look for the results in respective Bcr-1/2 or Bcr-3 tubes.

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

No RARa/PML cDNA detectable. It can be considered negative if there is amplification in the abl run. Please refer for details on PCR inhibition in the section PCR inhibition.

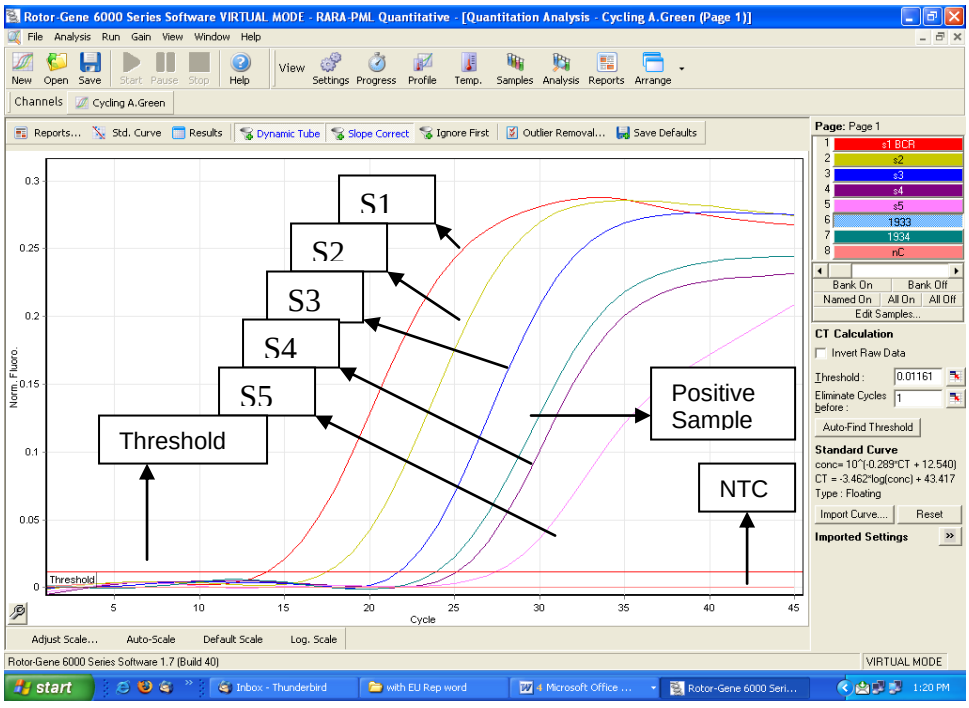

Fig. 41: Detection of the quantitation standards in fluorescence channel Cycling A.Green. NTC: non-template control.

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

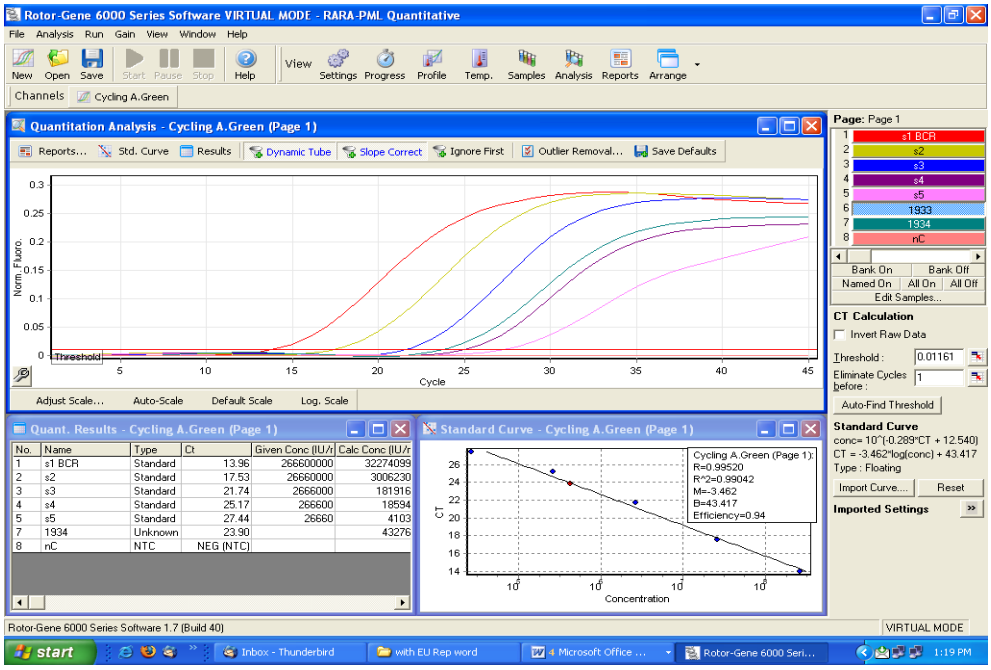

Fig. 42.

Example of analysed data for RARa/PML where all the amplification curves can be seen as well as the standard curve, reaction efficiency, M value etc. can be seen and the quantitative data for the samples can be seen.

Abl reference gene amplification.

Run the diluted samples for abl stored at –20 **°C in exactly the similar fashion as described for RARa/PML so far. After the run the quantitative results for the abl gene can be obtained & then the ratio of RARa-PML/ABL can be determined from the values obtained. These can also be run simultaneously in the same run as Bcr in case there is additional space in the Rotor to do so.**

PCR Inhibition

A signal is detected in fluorescence channel Cycling A. Green in **abl**: **No PCR inhibition**

In fluorescence channel Cycling A. Green in **abl** no signal is detected.

A possible PCR inhibition has occurred.

10. a) Troubleshooting for RotorGene 2000/3000

1. No signal with positive Standards (RAR Bcr-1/2, RAR Bcr-3/, abl *S 1-5*) in fluorescence channel Cycling A.FAM.

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

  ➔ Repeat the PCR with corrected settings.

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

- The PCR conditions do not comply with the protocol.

  ➔ Repeat the PCR with corrected settings.

- The Super Mix *R1, R2, R3* has been thawed and frozen too often.
- The Super Mix R1, R2, R3 has been kept at +4°C for longer than 5 hours.

  ➔ Please mind the storage conditions given in the **Storage.**

  ➔ Repeat The steps with using a new RARa/PML Super mix R1, *R2 & abl R3 super Master Mix*.

- **The PCR was inhibited.**

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

10. b) Troubleshooting for RotorGene 6000

1. No signal with positive Standards (RAR Bcr-1/2, RAR Bcr-3/, abl *S 1-5*) in fluorescence channel Cycling A.Green.

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

  ➔ Repeat the PCR with corrected settings.

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

- The PCR conditions do not comply with the protocol.

  ➔ Repeat the PCR with corrected settings.

- The Super Mix *R1, R2, R3* has been thawed and frozen too often.

- The Super Mix R1, R2, R3 has been kept at +4°C for longer than 5 hours.

  ➔ Please mind the storage conditions given in the **Storage.**

  ➔ Repeat The steps with using a new RARa/PML Super mix R1, *R2 & abl R3 super Master Mix*.

- **The PCR was inhibited.**

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

11. Specifications

11.a Sensitivity and Reproducibility

In order to determine the sensitivity of the ***Geno-Sen's RARa/PML Real Time PCR Kit***, a dilution series has been set up from 10^7 down to 10^0 Copies/µl of RARa/PML cDNA and analyzed with the ***Geno-Sen's RARa/PML 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 RARa/PML Real Time PCR Kit* is consistently 110 copies/ml. This means that there is 95% probability that 110 copies/ml will be detected.

Analytical Sensitivity

Analytical Sensitivity in Conjunction with the Geno Sen's® Whole Blood RNA Extraction Mini Kit — for RNA purification (Cat. No. 98007, 98008) of the Geno Sen's® Geno-Sen's RARA PML Real Time PCR RG Kit on ROTOR GENE 3000/6000 was determined by Spiking a known negative blood to a nominal 95 copies/ml. This was subjected to extraction using the Geno-Sen's® Whole Blood RNA Extraction Mini Kit — for RNA purification (Cat. No. 98007, 98008) eight times with starting volume of 1.5 ml & elution volume of 40µl.

All the Eight extractions were then analyzed with the Geno-Sen's® RARA PML Real Time PCR RG Kit along with the standards & the NTC.

All the eight extractions were amplified correctly & the mean value obtained for the eight replicates was 92 copies/ml.

Hence Analytical Sensitivity in Conjunction with the Geno Sen's® Whole Blood RNA Extraction Mini Kit — for RNA purification (Cat. No. 98007, 98008) of the Geno Sen's® RARA PML Real Time PCR RG was determined to be 95 copies/ml.

11.b Specificity

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

TNF α
IFN γ
Bcr Major
Bcr Minor
h-tert

In order to validate the Specificity further with wet lab testing the following Known clinical samples were analyzed using the ***Geno Sen's***® RARa/PML Real Time PCR RG kit on ROTOR GENE 3000/6000 machine. The extraction was carried out with the QIAamp RNA Blood Mini extraction Kit **(Cat. No. 52304)**

Sample Type	WHOLE BLOOD	CELL CULTURE
High +ve's	5	5
Medium +ve's	5	5
Low +ve's	8	3
Extremely low +ve's	4	3
Negative samples.	4	1
	26	17

All the above samples were correctly identified by the ***Geno Sen's***® RARa/PML Real Time PCR RG kit & all the 7 extremely low samples were accurately detected by the ***Geno Sen's***® RARa/PML Real Time PCR RG kit & exhibited copies around 110 copies /ml or less than 110 copies/ml.

Further studies are underway on this aspect.

12. Warranty:

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

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

13. Limitations of product use:

a.) All reagents may exclusively be used for *in vitro* diagnostics.
b.) The product is to be used by personnel specially instructed and trained in for the *in-vitro* diagnostics procedures only.
c.) It is important to pipet the indicated quantities, and mix well after each reagent addition. Check pipettes regularly.
d.) Instructions must be followed Correctly in order to obtain correct results. If the user has any questions, please contact our Technical Dept. (dharam@vsnl.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 RNA depends on the number of RNA 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.
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.

GENOME DIAGNOSTICS PVT. LTD.
(AN ISO 13485 :2012,9001 : 2008 CERTIFIED COMPANY.)
Up Mohal Naryal, KHASRA NO : 427,Opp. Divya Packers,
Old Timber Depot Road, Near Sector 4,Parwanoo
Distt. Solan, Himachal Pradesh-173220 ,INDIA.
Tel No : 00-91-1792-234285, +91 9810037653 Fax : 00-91-1792-234286
E-mail: genome24@rediffmail.com
dharam@vsnl.com
pbpl@vsnl.net
Version : 005
Websites :
www.genomediagnostics.in
www.genome-diagnostics.com
www.genomediagnostics.co.in
www.diagnosticsgenome.com

EC REP

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