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# Product Manual

Of

## Leukemia Q-Fusion Screening Kit

For simultaneous detection of 30 fusion genes in one real-time PCR reaction

*Note: Research Use Only. Not intended for use in diagnostic procedures.*

For the following products:

Cat. No. 1-100-10010 (10 Tests)

Cat. No. 1-100-10020 (20 Tests)

*Note: 'Q-Fusion' and 'Yin-Yang Probes' are trademarks belonging to QuanDx Inc. These products and the methods employed are covered by United States Patent Number 7,799,522; EU Patent Number 1339732; Japan Patent Number 3999653; Australia Patent Number 2001296647; China Patent Number 01817012.9; India Patent Application Number 1230/DELNP/2005; Brazil Patent Application Number PI0114575-4, and Canada Patent Application Number 2,424,856. QuanDx owns all of these patents.*

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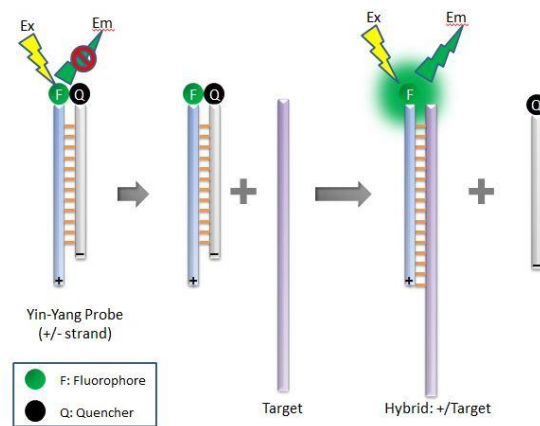
## PURPOSE OF THE TEST

The Q-Fusion Screening Kit is a novel, multiplex reverse transcription real-time PCR (RT-qPCR) system for the simultaneous detection of 30 fusion genes. The test detects more than 140 breakpoints for the 30 fusion genes. Sample fusion gene and control gene transcripts are co-amplified in each reaction, and identified by specific fluorescent probes. Rapid results can be obtained within 2-3 hours. The Q-Fusion system provides a sensitive, specific, reliable and cost-effective tool that makes it easy to perform routine screenings of leukemia fusion transcripts.

## PRINCIPLE OF THE TEST

The Q-Fusion Screening Kit is a RT-qPCR based assay for the detection of leukemia associated fusion gene transcripts found in total RNA from whole blood or bone marrow samples. Ready to use cDNA synthesis reagents and qPCR primer/probe mixes are included in the kit.

Q-Fusion Screening was developed based upon the platform of the 'Yin-Yang Probes', a novel technology for nucleic acid detection and related applications (Patent No. US7799522). The probe itself is non-fluorescent due to the close proximity of the fluorophore and quencher. When the Yin-Yang probe is in the presence of its target DNA, the target strand displaces the negative strand along with its quencher, and the fluorophore becomes fluorescent.



In the process of qPCR, the positive probes bind to the PCR products during the annealing step, and release fluorescence signals. Signals are collected in the annealing step.

cDNA is synthesized by adding purified RNA to the ready to use cDNA reaction mix. The resulting cDNA is divided into 8 tubes, and mixed with the appropriate specific PCR primers and probes mixes (as labeled A~H) for detection of fusion genes. qPCR is performed in a real-time qPCR instrument with optical filters for detection of FAM, HEX/JOE, ROX and Cy5 fluorescence. qPCR amplification plots and Ct (threshold cycle) values are used for identification of the translocation and fusion gene transcript using a simple interpretation table.

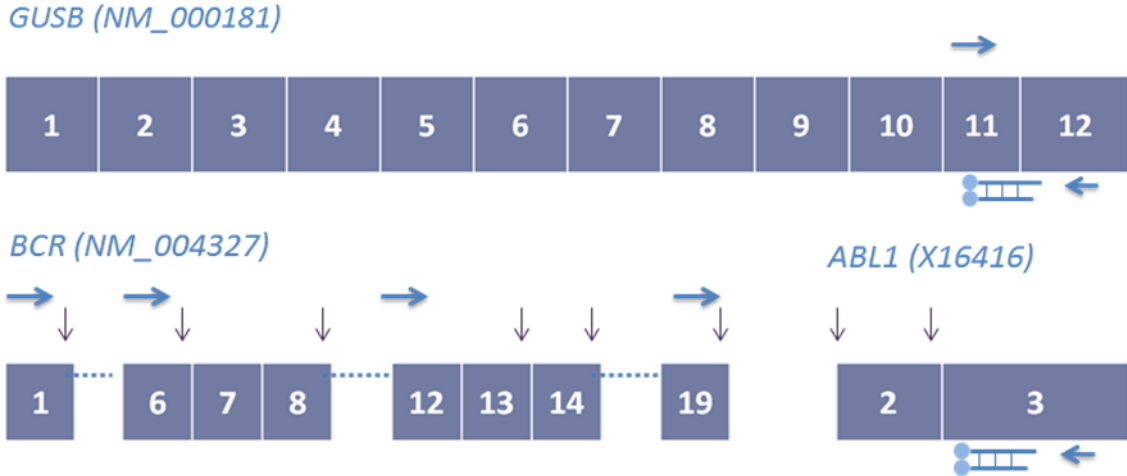


Figure 1: Schematic of the primers and probes designed in the Q-Fusion Screening Kit. The schematic on the top shows an example of the GUSB (internal control). The schematic on the bottom shows the detection of BCR-ABL1. Primers are depicted by horizontal blue arrows; breakpoints are shown by vertical arrows.

### KIT COMPONENTS AND STORAGE

The kit must be stored at -20°C away from light. Avoid frequent/unnecessary freeze-thaw cycles of the kit.

		10-test kits	20-test kits
REVERSE TRANSCRIPTION REAGENTS	RT buffer*	1 x 20 µL	1 x 40 µL
	RT Enzyme	1 x 5 µL	1 x 10 µL
	RT primer	1 x 20 µL	1 x 40 µL
	DEPC H <sub>2</sub> O	1 x 20 µL	1 x 20 µL
PCR REAGENTS	LF PCR Mix A~H	8 x 198 µL	8 x 396 µL
	LF Polymerase	1 x 16 µL	1 x 32 µL
CONTROLS	LF Positive Control	1 x 40 µL	1 x 40 µL
	LF Negative Control	1 x 100 µL	1 x 100 µL

\*RT buffer contains dNTP mix and Mg<sup>2+</sup>

## REQUIRED EQUIPMENT

- Table centrifuge
- Thermal heating block
- qPCR instrument with filters for FAM (Abs 495 nm, Em 520 nm), HEX (Abs 535nm, Em 556 nm) , ROX (Abs 585 nm, Em 605 nm) and Cy5 (Abs 650 nm, Em 670 nm).
- Pipettes and sterile RNase free filter tips.

## PRECAUTIONS

Due to the inherent instability of RNA, use fresh patient samples or otherwise samples that have been stored at 2-8°C within 24 hours after extraction. When working with RNA, always wear gloves to avoid RNase from the hands. Extracted RNA should be treated for cDNA synthesis, or stored at -70 °C for no more than 3 months.

## Test PROCEDURE

### *RNA preparation*

Total RNA is prepared from whole blood, cell lines, or bone marrow cells. Heparin must **NOT** be used for stabilization of blood samples as it inhibits the PCR reaction.

Extracted RNA should be analyzed for concentration and quality control.

$A_{260}/A_{280}=1.9\sim 2.1$ .  $A_{260}/A_{230} \geq 2.0$ .

A minimum concentration of 20ng/ $\mu$ L RNA is needed.

Total RNA can be stored at -80°C before use in cDNA synthesis.

### *cDNA synthesis*

- Thaw reverse transcription reagent, the LF Positive Control and LF Negative Control to room temperature, vortex for 20 seconds and spin down at 3000 rpm for 10s.
- Take out 5  $\mu$ L LF Positive Control and 5  $\mu$ L LF Negative Control, incubate at 95°C for 5 min and spin them down (for reverse transcription with the sample RNA).
- Make the 10  $\mu$ L RT reaction mix on ice for each of the controls and RNA samples as the table below:

Reagents	Volume ( $\mu\text{L}$ ) per reaction	Final concentration
RT buffer	2	1X
RT primer	2	200 $\mu\text{mol}$
RT Enzyme	0.5	
Total RNA *	5	
DEPC H <sub>2</sub> O	0.5	

\* Total RNA amount should be 0.1  $\mu\text{g}$  to 1  $\mu\text{g}$  in the 10 $\mu\text{L}$  reverse transcription reaction.

- Incubate the cDNA reactions at 37 °C for 15 minutes.
- Inactivate reverse transcriptase enzyme by heating at 85 °C for 5 seconds.
- Dilute with 40  $\mu\text{L}$  ddH<sub>2</sub>O for each of the controls and sample cDNA tubes. Each sample will be divided into 8 reaction tubes for the simultaneous fusion gene screening test.

#### Real-time qPCR

- Make the 8-PCR master mixes A~H: If the number of sample RNA that need to be detected is number n, the volume of each of the 8-PCR master mixes needs to be at a minimum  $(n+3) * 19.8 \mu\text{L}$  LF PCR mix +  $(n+3) * 0.2 \mu\text{L}$  LF polymerase. (The extra 3 are for one LF positive control, one LF negative control, and one spare tube in case of pipette error.)
- For every sample, pipette 20  $\mu\text{L}$  of each of the 8-PCR master mixes A~H to each reaction.
- Add 5  $\mu\text{L}$  cDNA sample to each of the 20  $\mu\text{L}$  8-PCR master mix A~H.
- Run the qPCR reaction with the following program:

	Thermal cycle	Cycle #
Preheating	95°C 3 min	1
Touchdown	95°C 20 sec	10
	65°C 1min (decrease 1°C/step)	
	72°C 1min	
PCR Cycle	95°C 20 sec	40
	56°C 32 sec (4 channel detection: FAM,HEX,ROX,Cy5)	
	72°C 1min	

### Analysis and interpretation

After the run of qPCR, a threshold line should be carefully set up to allow accurate Ct determination.

No signal should be detected for the LF Negative Control in any channels.

All reactions should give Cy5 signals for the LF Positive Control, and the Ct values should be below 25. In addition, reactions with *PCR Mix C* should also give FAM signal for the LF Positive Control, and the Ct values should be below 25.

The Ct values above 25 for Cy5 indicate that the quality of the patient RNA sample is too low to generate a valid test results; therefore the test should be repeated with fresh RNA.

Ct values for positive sample signals other than Cy5 should be below 30. Ct values above 30 might be non-specific amplification. Repeat the test with fresh RNA, if the second test is positive, the sample is positive for the corresponding fusion gene. Other diagnostic techniques are highly recommended to confirm the results from positive tests with Ct values above 30.

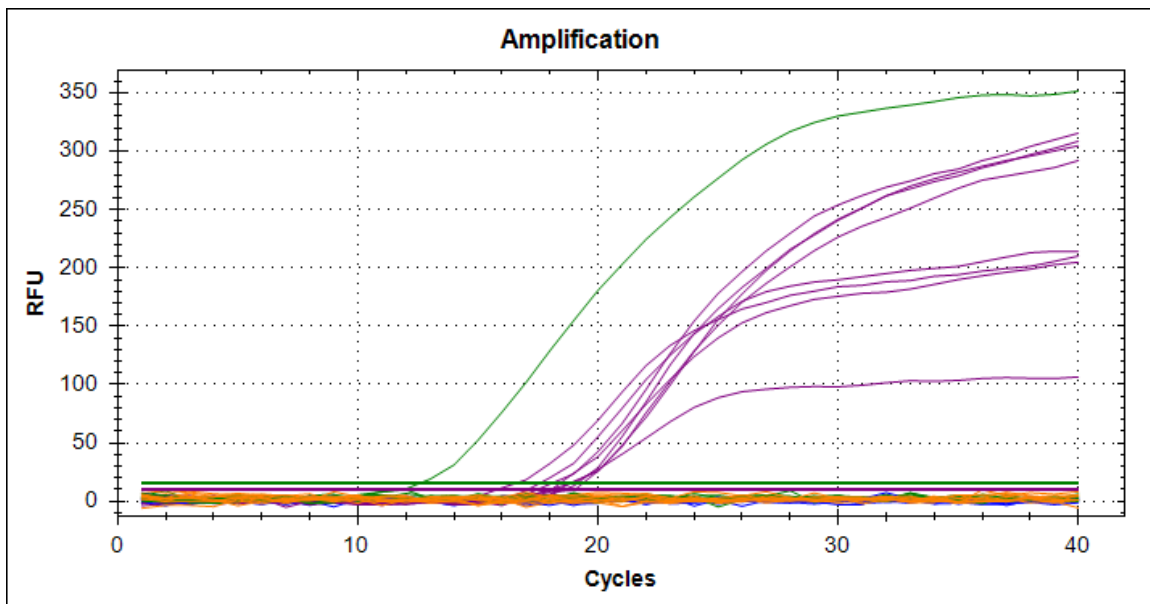


Figure 2: Example of the amplification curve for the LF Positive Control.  
Green: FAM, Purple: Cy5.

## INTERPRETATION TABLE

TUBE	FLUORESCENCE SIGNAL		FUSION GENE
A	HEX	CY5	MLL-AF9
	FAM	CY5	PML-RAR $\alpha$
	ROX	CY5	AML1-ETO
B	HEX	CY5	MLL-AF4
	FAM	CY5	TEL-AML1
	ROX	CY5	E2A-PBX1
C	HEX	CY5	MLL-ENL
	FAM	CY5	BCR-ABL1
	ROX	CY5	SIL-TAL1
D	HEX	CY5	MLL-AF10
	FAM	CY5	CBF $\beta$ -MYH11
	ROX	CY5	AML1-MDS1/EVI1
E	HEX	CY5	FIP1L1-PDGFR $\alpha$
	ROX	CY5	E2A-HLF
	FAM	CY5	SET-CAN
	ROX+FAM	CY5	DEK-CAN
F	HEX	CY5	MLL-SEPT6
	ROX	CY5	TEL-PDGFR $\beta$
	FAM	CY5	TLS-ERG
	HEX+ROX	CY5	MLL-ELL
G	HEX	CY5	MLL-AF17
	ROX	CY5	NPM-MLF
	FAM	CY5	NPM-RAR $\alpha$
	HEX+ROX	CY5	MLL-AF1q
	HEX+FAM	CY5	PLZF-RAR $\alpha$
H	HEX	CY5	MLL-AF1p
	ROX	CY5	AML1-MTG16
	FAM	CY5	TEL-ABL
	HEX+ROX	CY5	MLL-AF6
	ROX+FAM	CY5	AML1-EAP



## APPENDIX

### ***Program set-up for commonly used real-time qPCR instruments:***

ABI 7500, Agilent Mxpro 3005, and Bio-Rad CFX96.

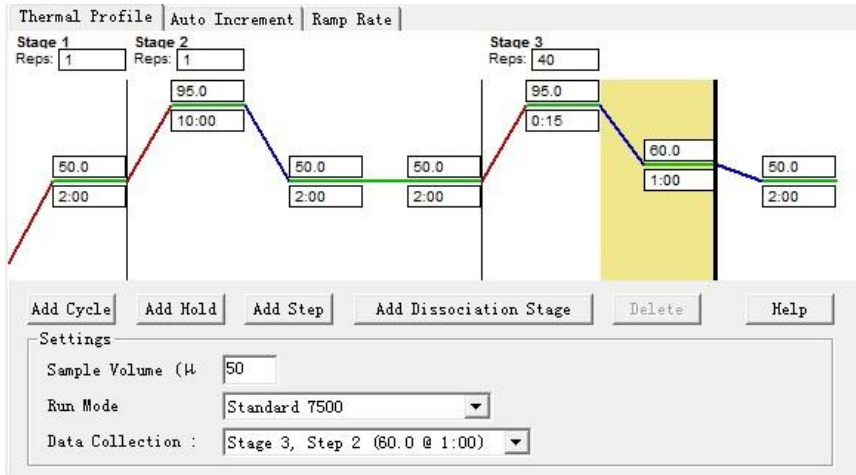
#### *qPCR program set-up for ABI 7500*

1. Log in to 7500 System SDS Soft , choose the right channel, click on INSTRUMENT, set up the program in the blank area as shown below:

The screenshot displays the 7500 System SDS Software interface. The 'Instrument' tab is selected and circled in red. The 'Instrument Control' section includes buttons for Start, Stop, Disconnect, and Extend, along with fields for Estimated Time Remaining and Status. The 'Thermal Cycler Protocol' section shows a thermal profile graph with three stages: Stage 1 (50.0, 2:00), Stage 2 (95.0, 10:00), and Stage 3 (95.0, 0:15). A yellow shaded area highlights a step at 60.0 for 1:00. The 'Settings' section includes fields for Sample Volume (50), Run Mode (Standard 7500), and Data Collection (Stage 3, Step 2 (60.0 @ 1:00)).

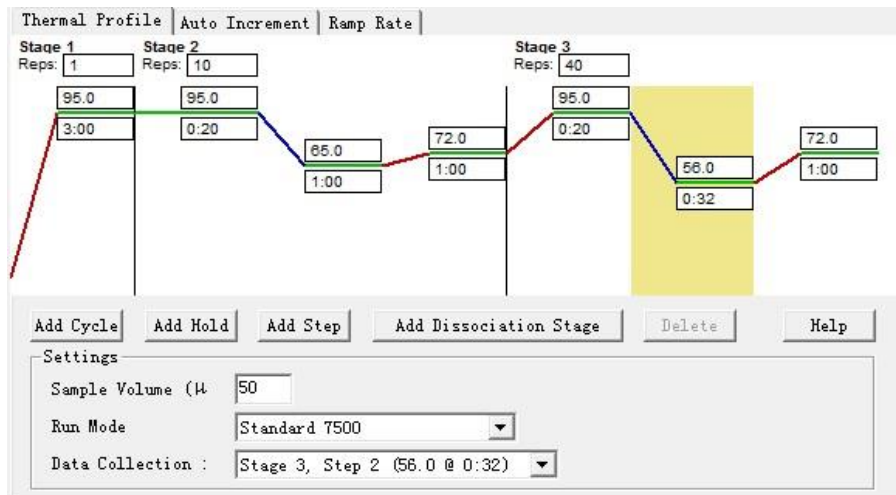
Stage	Temp (°C)	Time	Reps
Stage 1	50.0	2:00	1
Stage 2	95.0	10:00	1
Stage 3	95.0	0:15	40
Stage 3, Step 2	60.0	1:00	-

- Click on Stage 2, a black line will show up on the right side, then double click on Add Step for additional two steps. Also add one more step for Step 3.



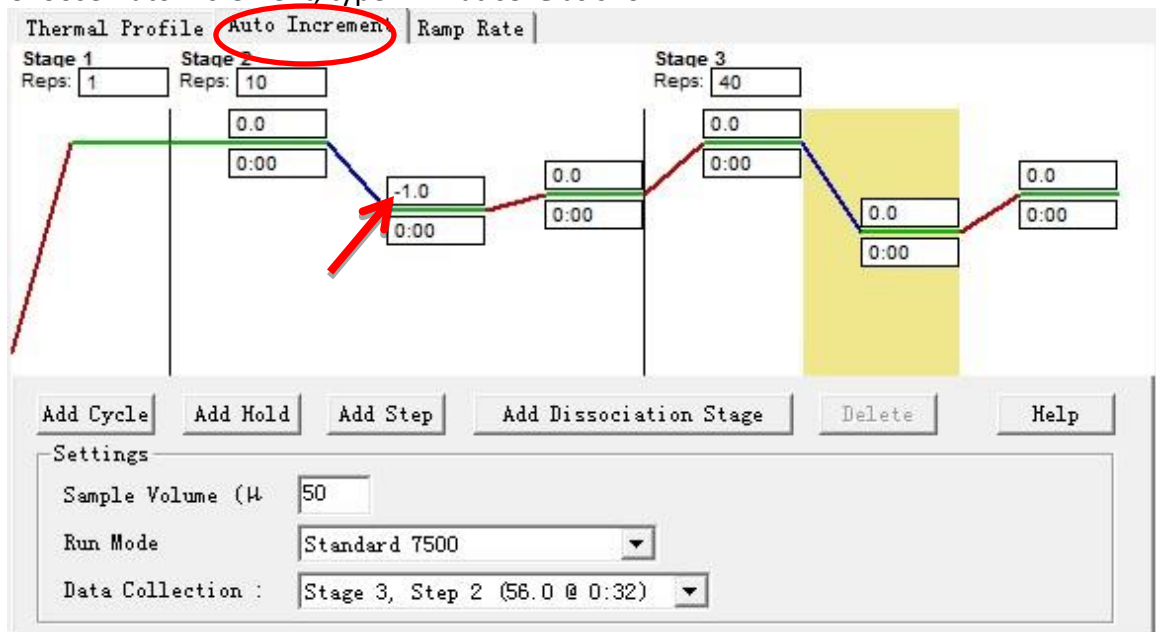
- Program modification

	Thermal cycle	Reps
Stage 1	95°C 3 min	1
Stage 2	95°C 20 sec	10
	65°C 1min	
	72°C 1min	
Stage 3	95°C 20 sec	40
	56°C 32 sec	
	72°C 1min	



4. Set up Touchdown

Choose Auto Increment, type in -1 at 65°C as shown

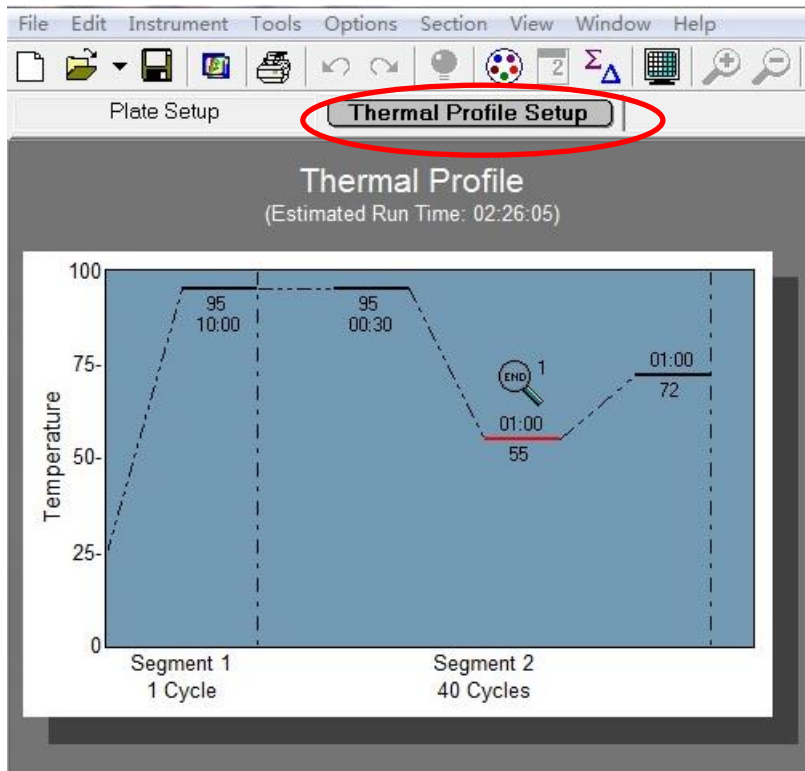


5. Set Sample Volume as 25, make sure the Data Collection is at Stage 3, Step 2.

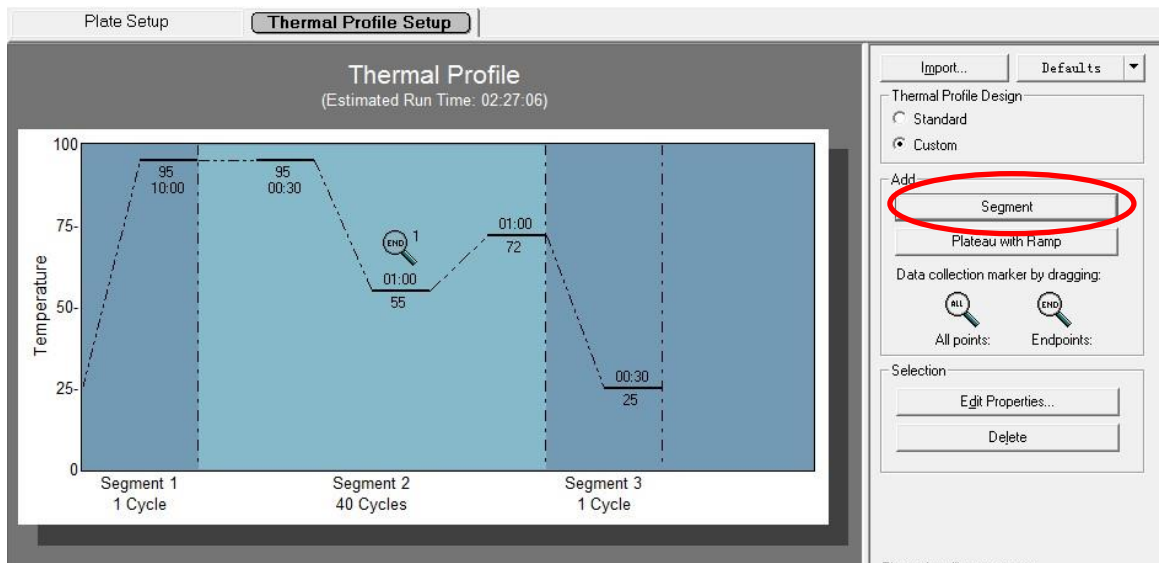
6. Save and Run

## qPCR program set-up for Mxpro 3005

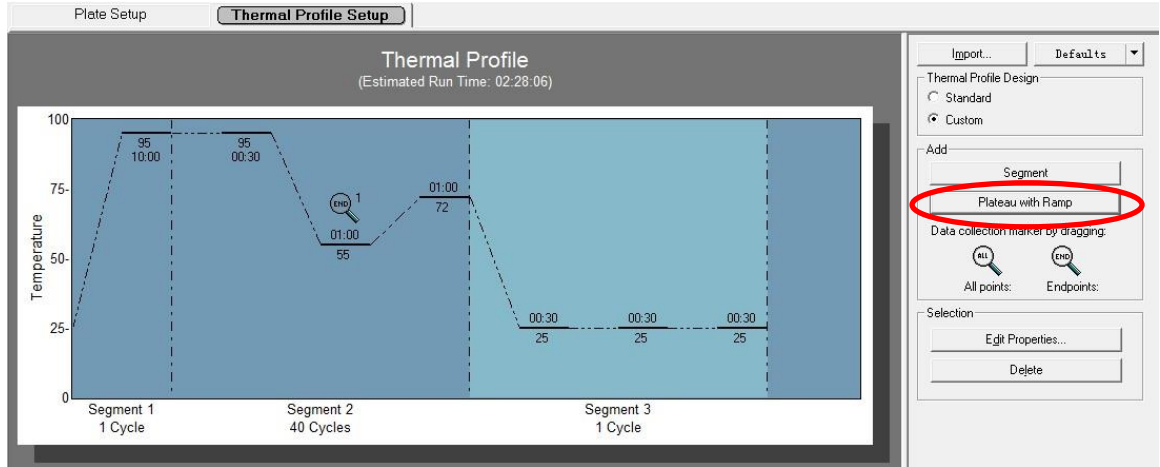
### 1. Choose Thermal Profile Setup



### 2. Choose Segment 2, click on Add Segment.



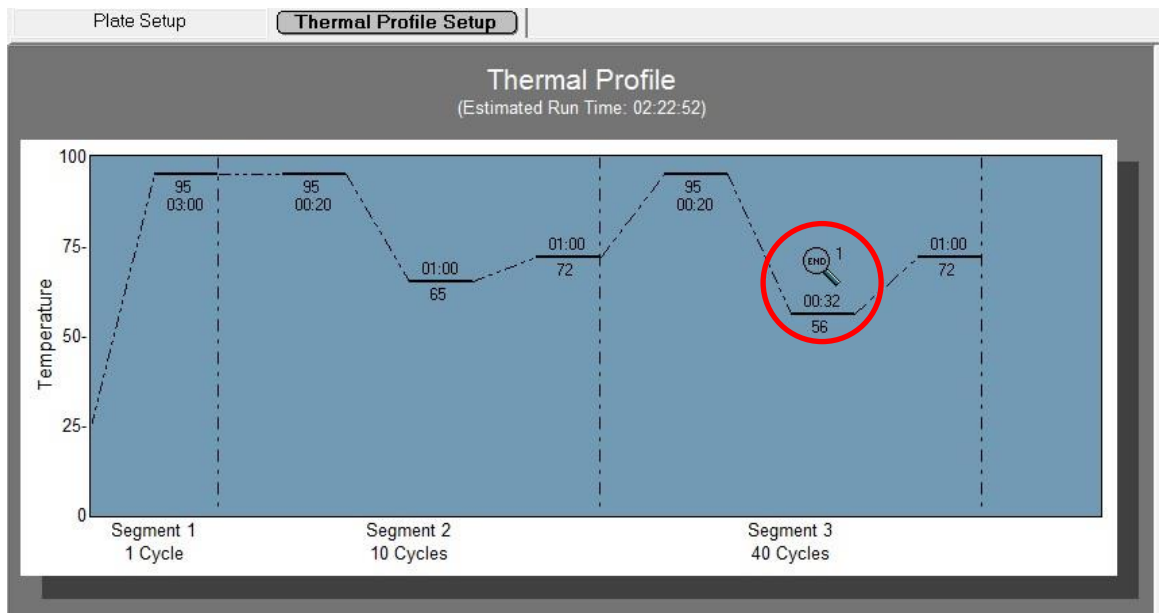
### 3. Choose Segment 3, double click on Add plateau with Ramp to add two steps.



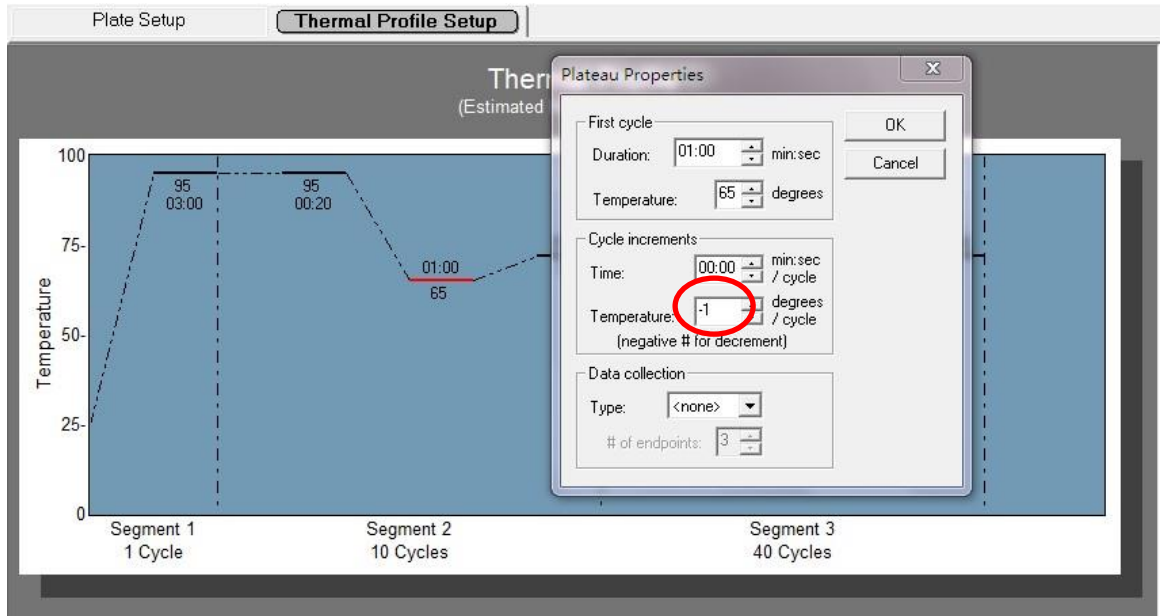
#### 4. Program modification

	Thermal cycle	Cycles
Segment 1	95°C 3 min	1
Segment 2	95°C 20 sec	10
	65°C 1min	
	72°C 1min	
Segment 3	95°C 20 sec	40
	56°C 32 sec	
	72°C 1min	

#### 5. Move the data acquisition lens to 56°C 32 sec.



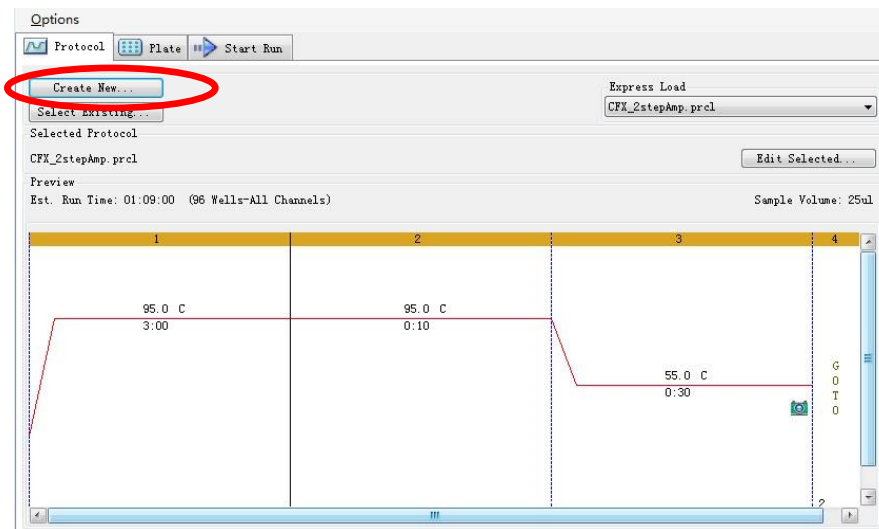
6. Add Touchdown program. Choose 65°C 1\_min and double click, within the pop-up window, type in -1 at Temperature inside Cycle increments, click on OK.



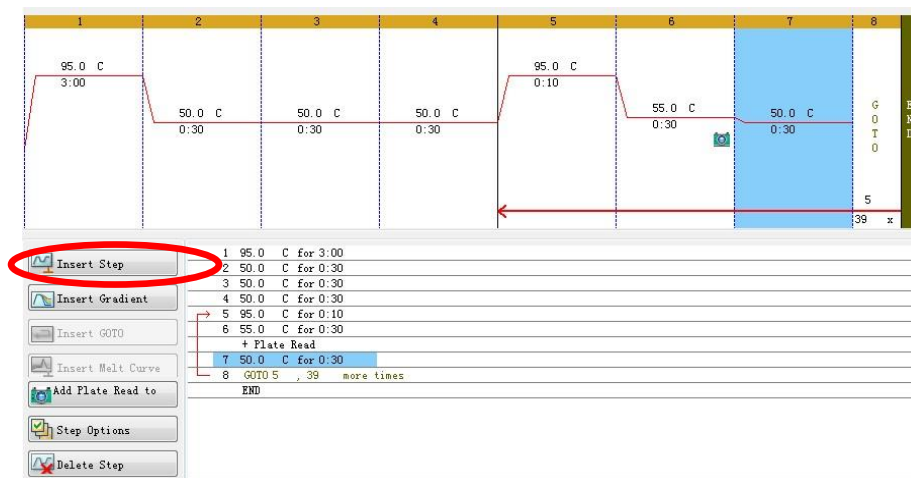
7. Save and Run.

## qPCR program set-up for Bio-Rad CFX96

### 1. Click on Create New



### 2. Choose 1, click on Insert Step three times to add three steps; choose 6, add one step

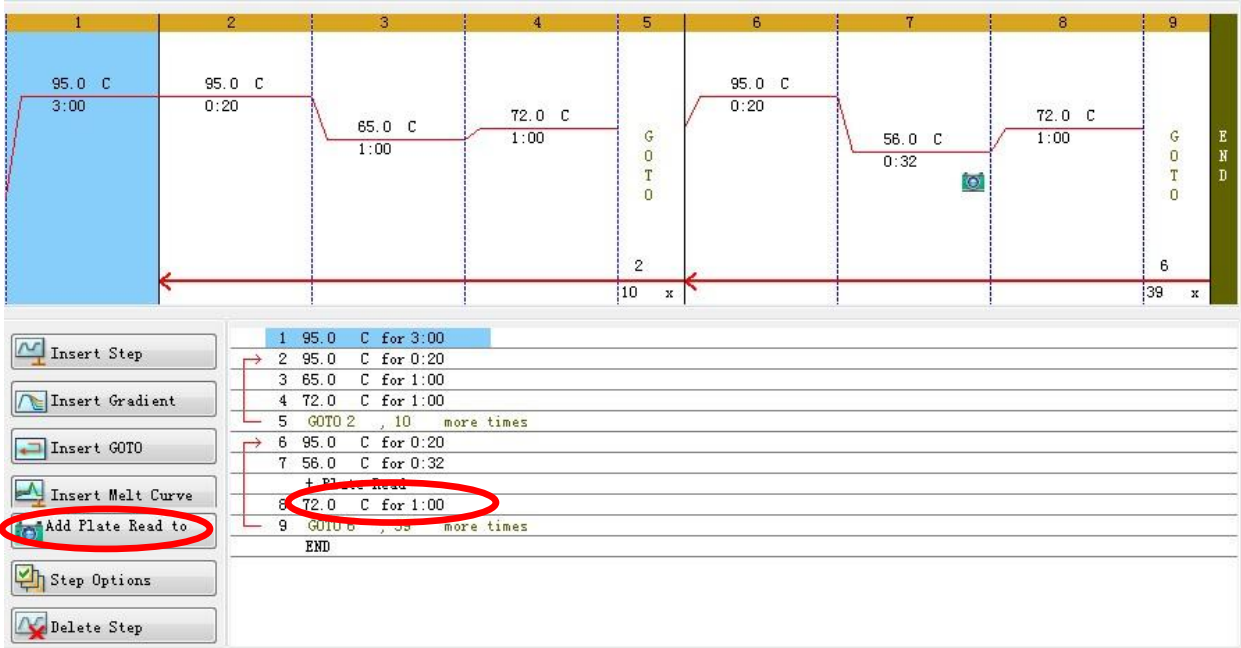


### 3. Change temperature and time:

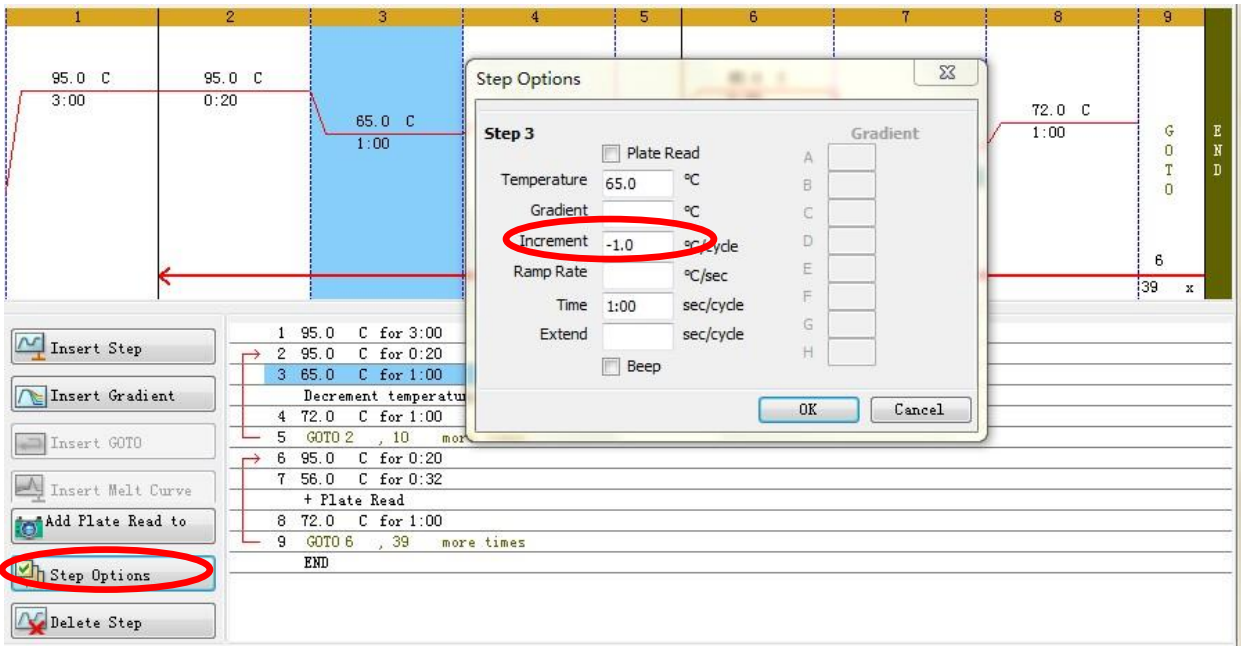
95°C 3 min, 9 more times (95 °C 20s, 65 °C 1min, 72 °C 1min)

39 more times (95 °C 20s, 56 °C 32s, 72 °C 1min)

Choose 4, click on Insert GOTO, type in 2, 9 more times behind GOTO.



4. Add Touchdown: choose step 3, click on Step Options, type in -1 at Increment in the pop-out window, click on OK.



5. Make sure the data acquisition camera is placed at 56°C, 32sec.

6. Save and run.



## qPCR program set-up for Rotor-Gene series instrument

1. Log into Rotor-Gene Series Software, choose “Advanced”, select “Empty Run” and then click on “NEW” (Figure 1).

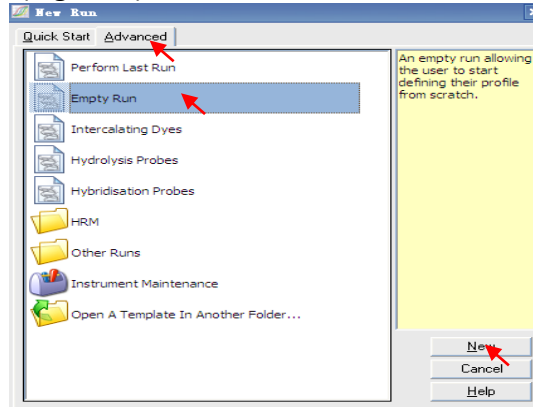


Figure 1

2. Choose “Rotor Type” according to the type of the instrument. Select the “Locking Ring Attached” to make sure the ring has been put on the rotor, then click on “Next” (Figure 2).

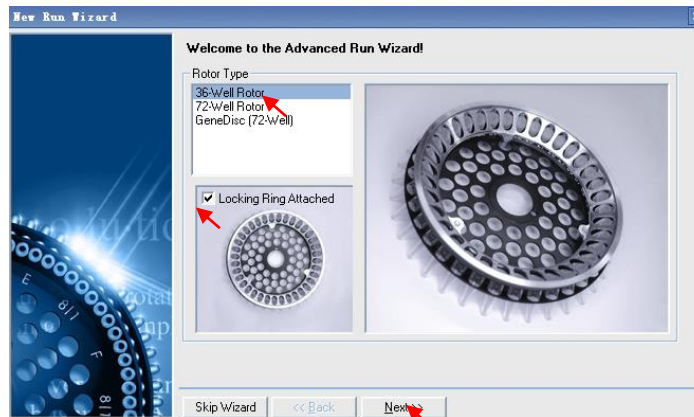


Figure 2

3. Type in the name of the “Operator”, fill in the “Reaction Volume” as 25  $\mu$ L, and then click on “Next” (Figure 3).

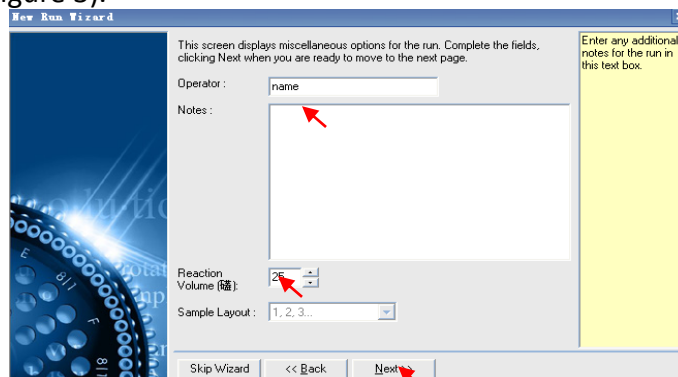


Figure 3

4. Click on “Edit Gain” to modify the gain values of the Green, Yellow, Orange and Red channels (Figure 4). Gain value 5 is recommended for each channel. Click on “OK” to complete the gain set-up (Figure 5).

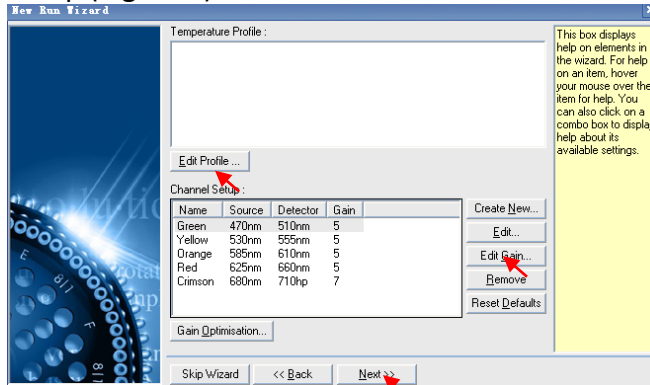


Figure 4

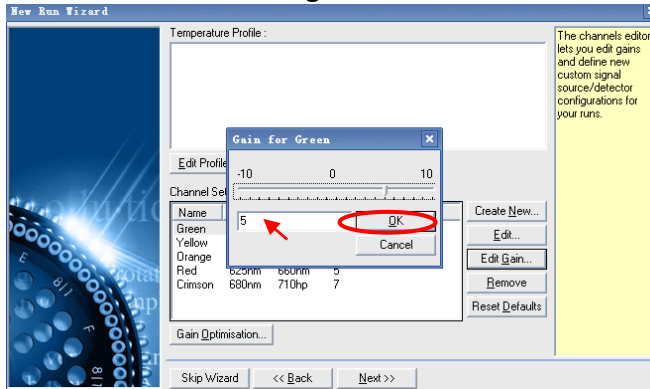


Figure 5

5. Choose “Edit Profile” as shown in Figure 4. Click on “Insert after” to insert the three thermal stages: “Hold”, “Cycling” and “Cycling2”.

a. For the hold stage, select “Hold”, click on “Hold Temperature” to set the temperature as 95 degrees, click on “Hold Time” to set the time as 3 mins (Figure 6).

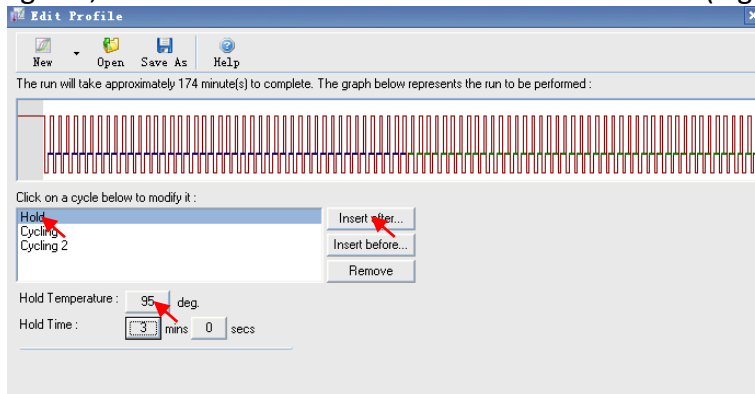


Figure 6

b. For the cycling stage, first choose “Cycling”, click on “this cycle repeats times” and set as 10 times, then click on “OK”(Figure 7).

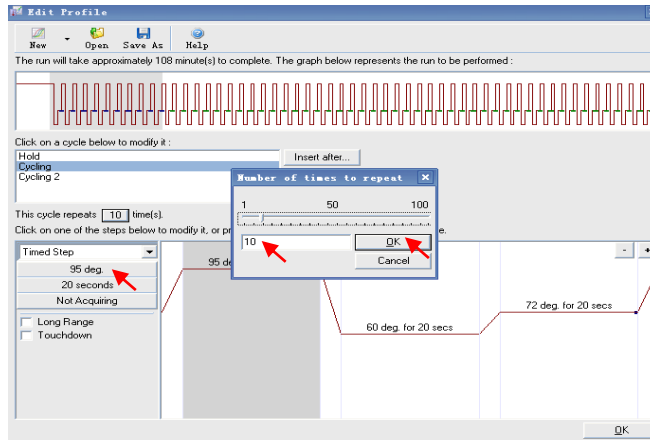


Figure 7

c. Choose the first step of cycling, click on buttons on the left and set the temperature as 95 degrees, time for 20 seconds, choose “not acquiring” for this step, and then click on “OK” (Figure 8).

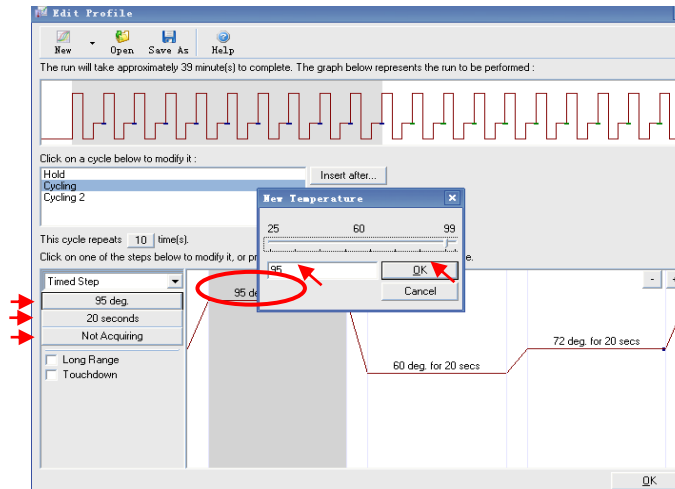


Figure 8

d. Choose the second step of “cycling”. Set the temperature as 60 degrees, set the time as 60 seconds, choose “not acquiring” and click on the “touchdown”. In the “Cycles to touch down”, set the “Decreases temperature” by 1 degree for each cycle and 10 cycles, click on “OK” to complete the set-up of touchdown (Figure 9).

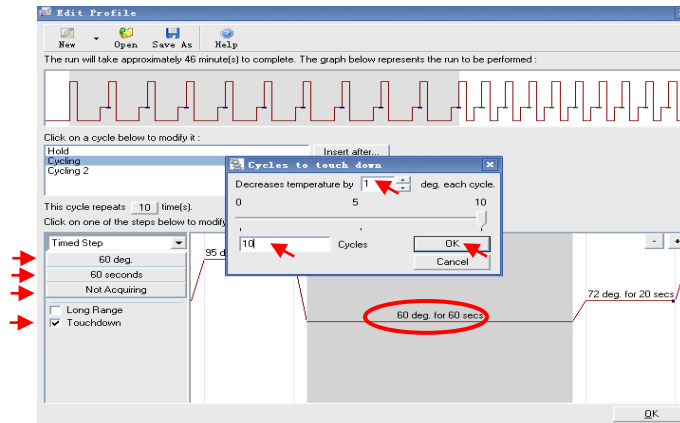


Figure 9

e. Choose the third step of cycling, set the temperature as 72 degrees, set the time as 60 seconds and choose “not acquiring”. Click on “OK” to complete the set up of the cycling stage (Figure 10).

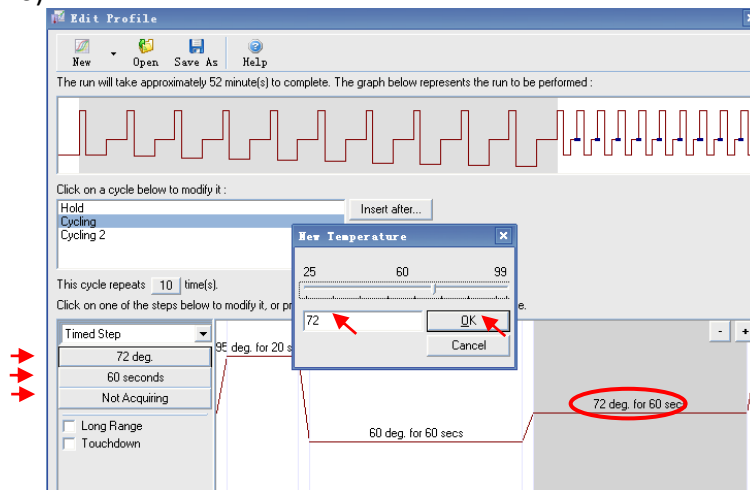


Figure 10

6. For the stage of “Cycling2”, choose “Cycling2”, set the temperature and time as below for each step: 95 degrees for 20 secs; 56 degrees for 32 secs and 72 degrees for 60 secs. Make sure “not acquiring” is selected for the first (95 deg.) and the third step (72 deg.). Data is acquired at the second step (56 deg.)

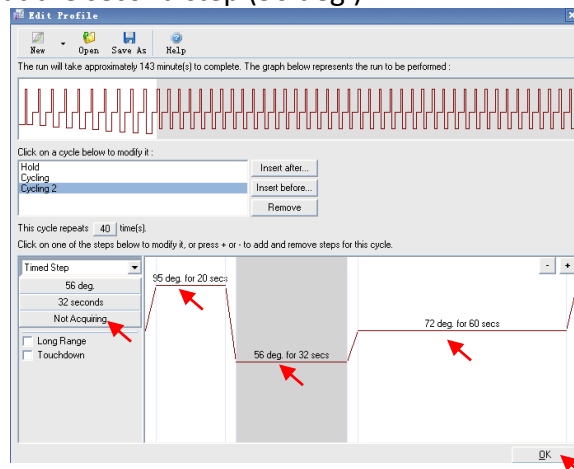


Figure 11

Click on the “Not Acquiring” button. In the “Acquisition”, use the > to shift the Green, Orange, Red and Yellow channels in the “Available Channels” into the “Acquiring Channels”, click on “OK” to complete the set-up of this part (Figure 12). Then click on “OK” as shown in Figure 9.

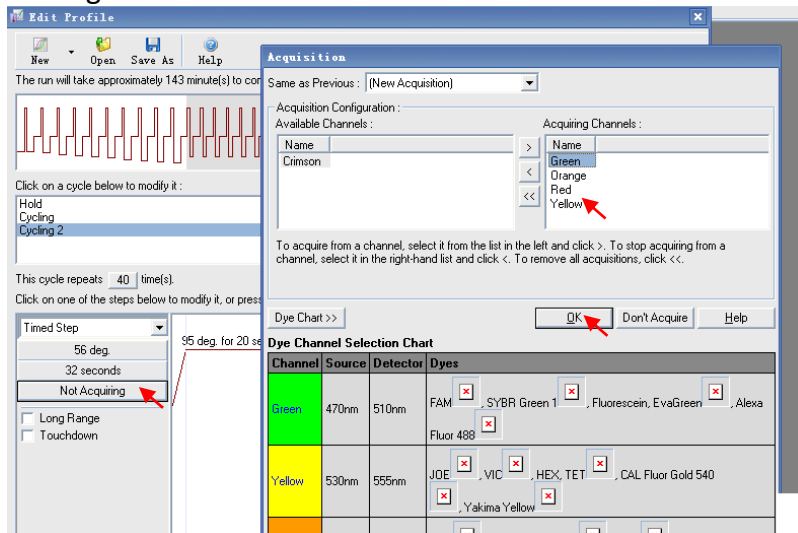


Figure 12

7. Click on “Next” (Figure 13) and “Start Run” (Figure 14) to save the file into the computer and finish the set-up.

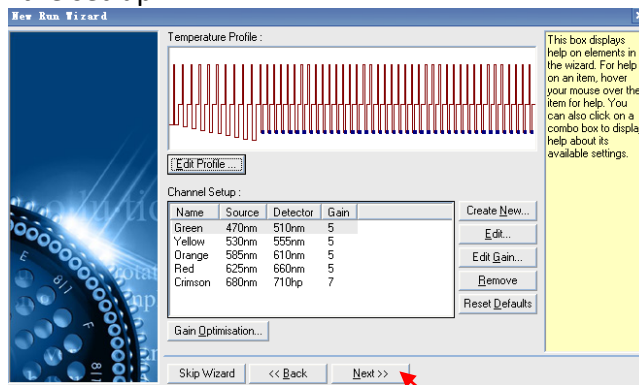


Figure 13

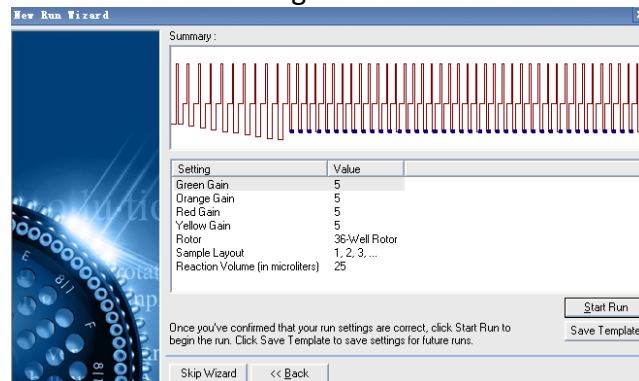


Figure 14