REAL-TIME PCR INSTRUMENT SET-UP AND RESULT ANALYSIS

1. CFX96™ Real-time PCR Detection System (CFX Manager™ Software)

1.1. Real-time PCR Instrument Set-up

Note: CFX96™ Real-time PCR Detection System (Bio-Rad) experiment setup can be divided into three steps: Protocol Setup, Plate Setup, and Start run.

A. Protocol Setup

1) In the main menu, select **File** → **New** → **Protocol** to open **Protocol Editor**.

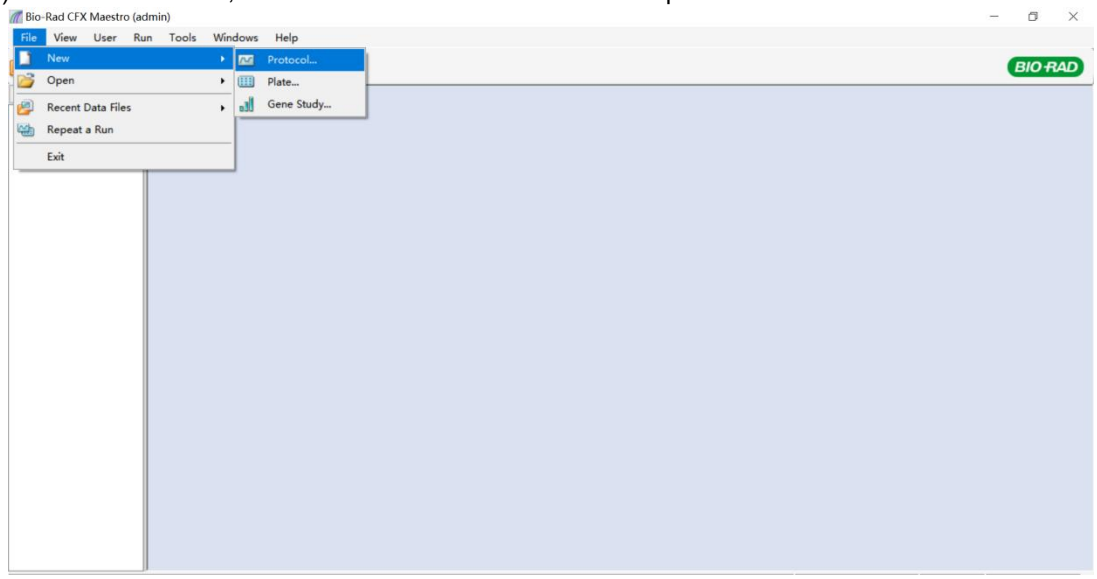


Fig. 1. **Protocol Setup.** Create a new protocol or load an existing protocol for the run

2) In **Protocol Editor**, define the thermal profile as follows:

Step	No. of cycles	Temperature	Duration
1	1	50°C	2 min
2	1	95°C	2 min
3	5	95°C	5 sec
		70°C	20 sec
4	45	95°C	5 sec
5*		67°C*	10 sec
6		76°C	20 sec
7	1	95°C	10 sec
8	1	40°C	1 min
9*	1	**Melting curve 40°C ~ 90°C (Increment:0.5°C)**	

***Plate read at Step 5 and 9.** Fluorescence is detected at 67°C and Melting curve.

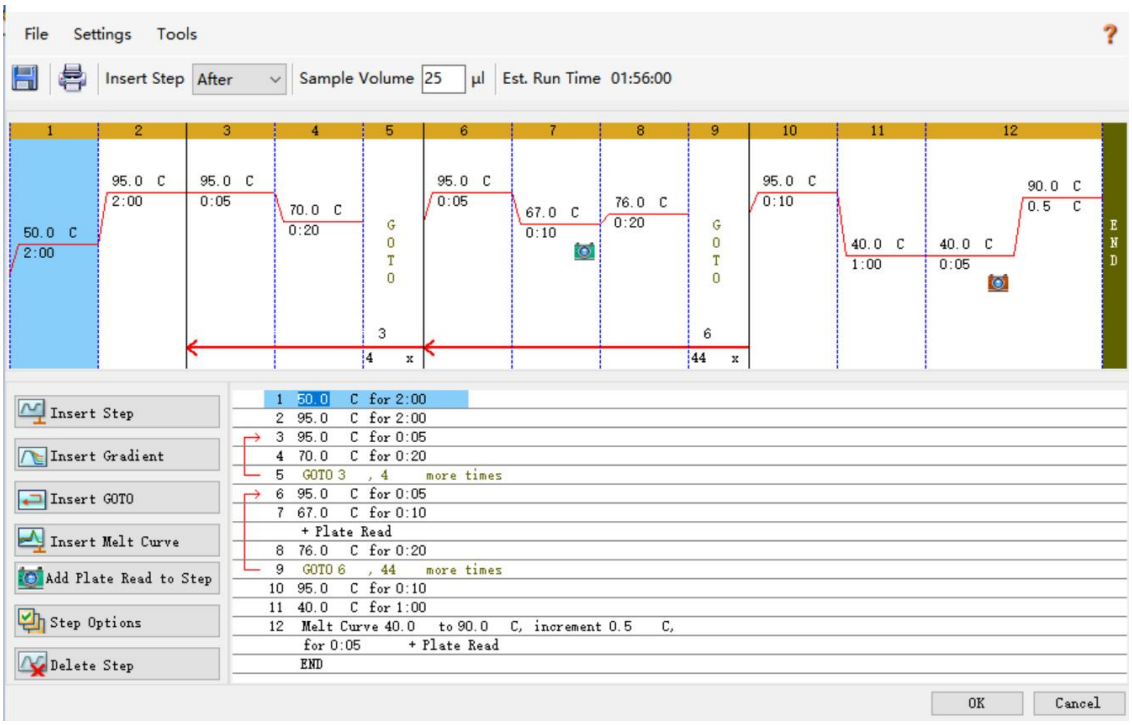


Fig. 2. **Protocol Editor**

3) Click the box next to **Sample Volume** to directly input **25 µL**.

3) Click **OK** and save the protocol to open the **Experiment Setup** window.

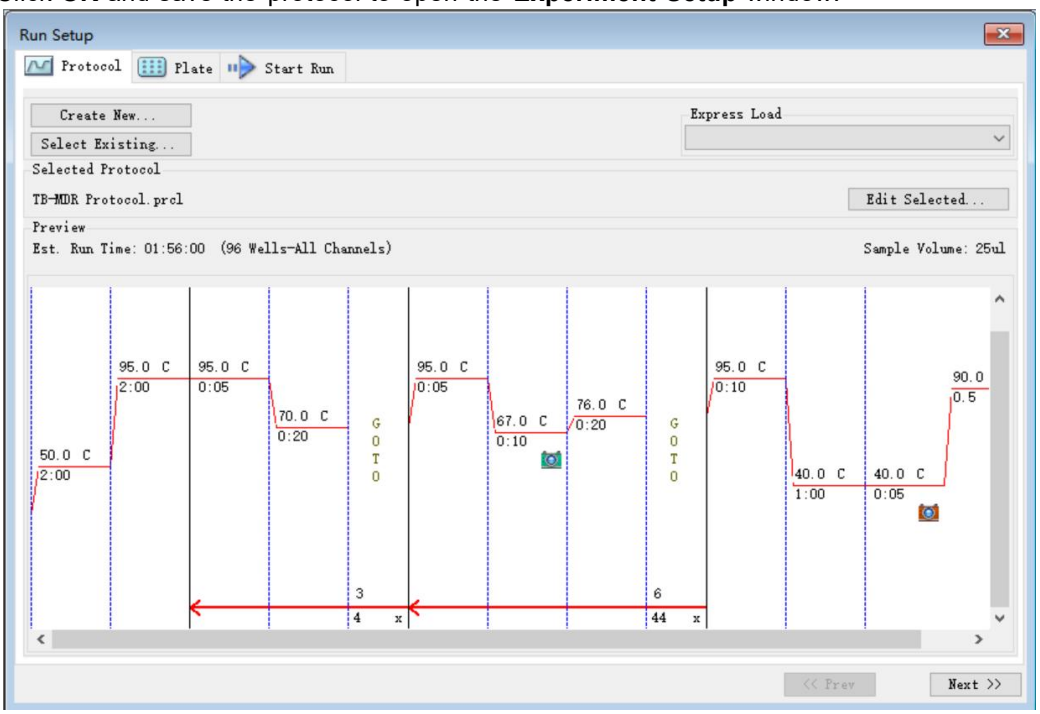


Fig. 3. **Experiment Setup: Protocol**

B. Plate Setup

1) From **Plate** tab in **Experiment Setup**, click **Create New** to open **Plate Editor** window.

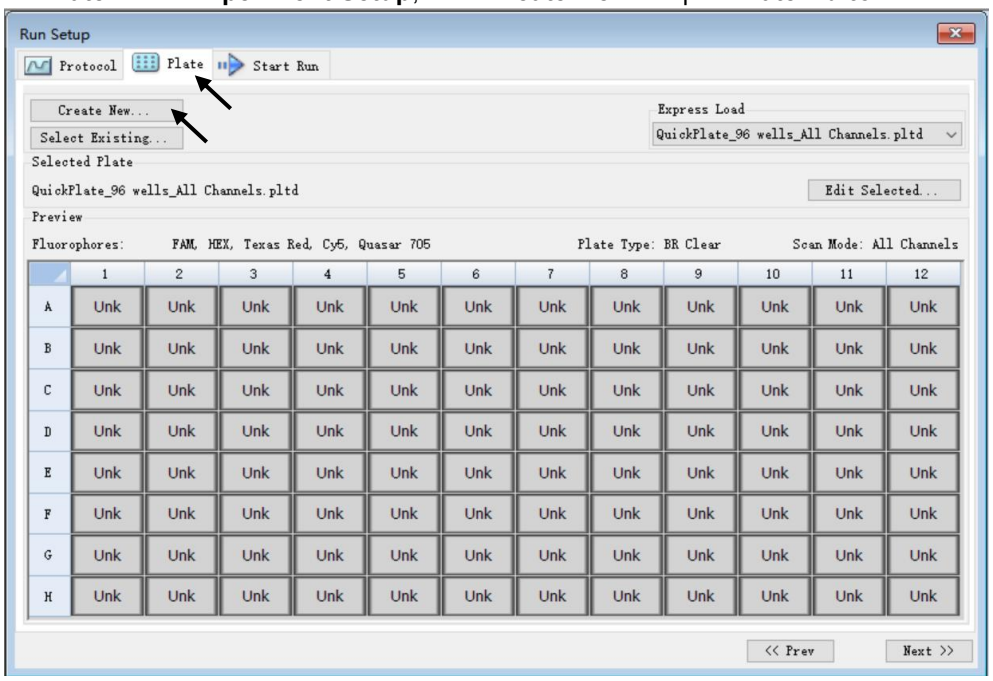


Fig. 4. **Plate Editor.** Create a new plate

2) Click **Select Fluorophores** to indicate the fluorophores (**FAM**, **HEX**, **ROX**, and **CY5**) that will be used and click **OK**.

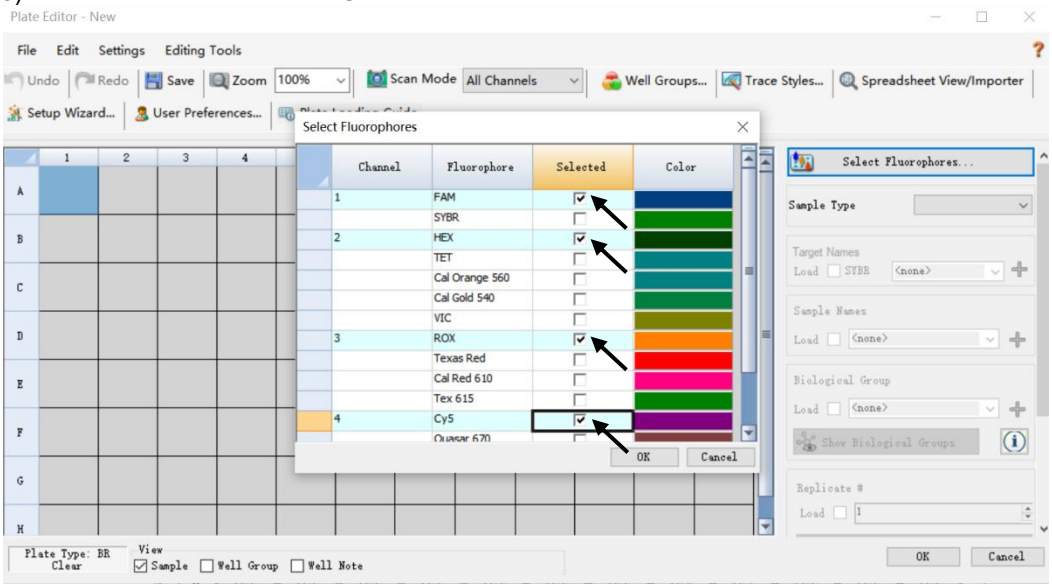


Fig. 5. **Select Fluorophores (FAM, HEX, ROX,** and **CY5)**

3) Select the wells where the PCR tube will be placed and select its sample type from the **Sample Type** drop-down menu.

- **Unknown**: Clinical samples
- **Negative Control**
- **Positive Control (**Wild-type Control**)**

4) Click on the appropriate checkboxes (**FAM**, **HEX**, **ROX**, and **CY5**) to specify the fluorophores to be detected in the selected wells.

5) Type in **Sample Name** and press enter key.

6) In **Settings** of the **Plate Editor** main menu, choose **Plate Size (96wells)** and **Plate Type (BR White)**.

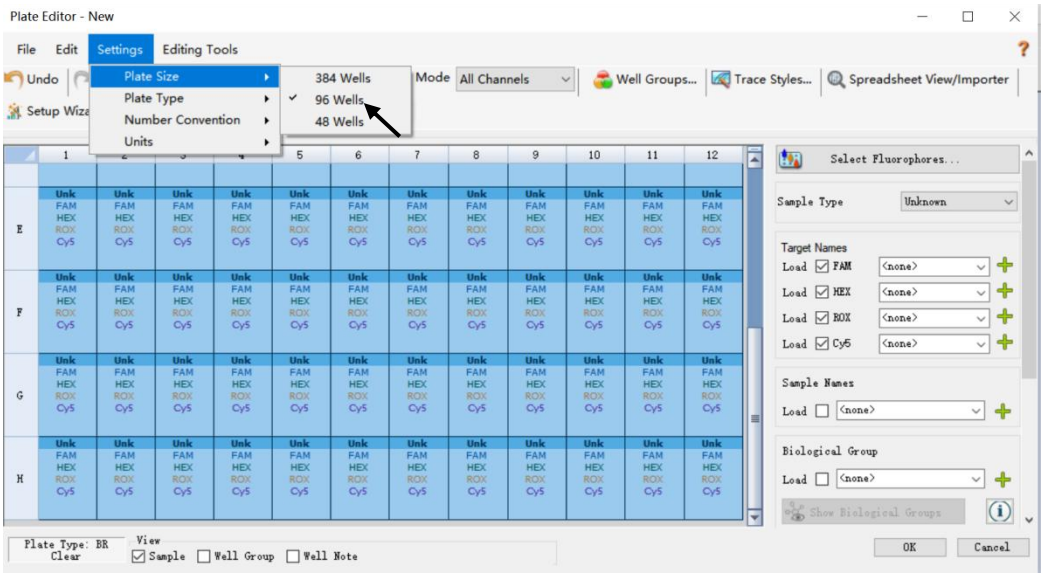


Fig. 6. **Plate Setup**

7) Click **OK** to save the new plate.

8) You will be returned to the **Experiment Setup** window.

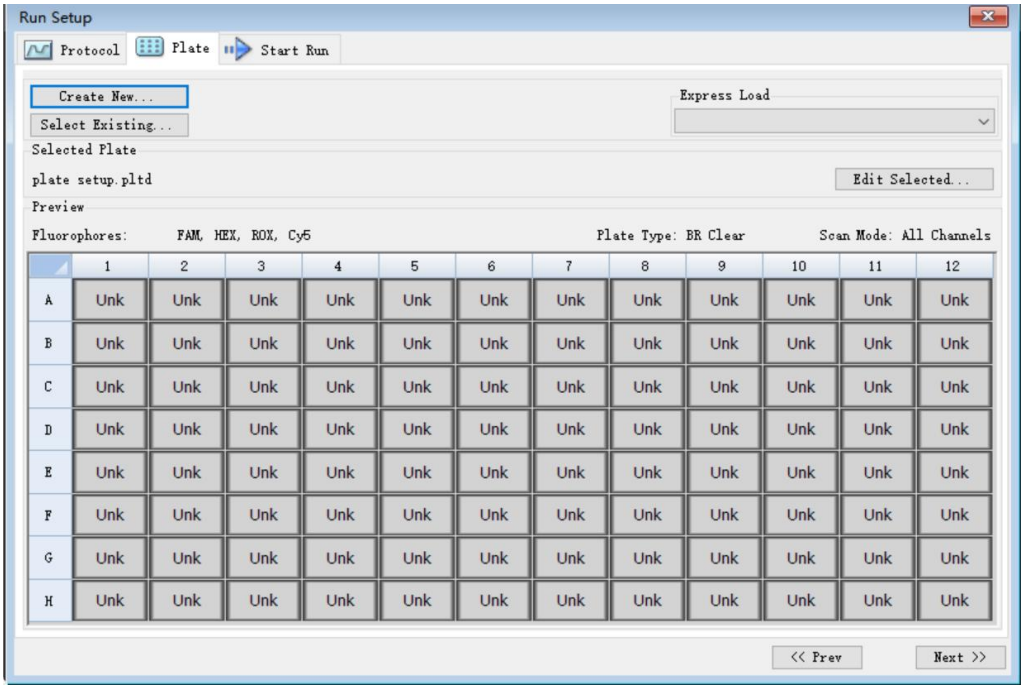


Fig. 7. **Experiment Setup: Plate**

9) Click **Next** to Start Run.

C. Start Run

1) From **Start Run** tab in **Experiment Setup**, click **Close Lid** to close the instrument lid.

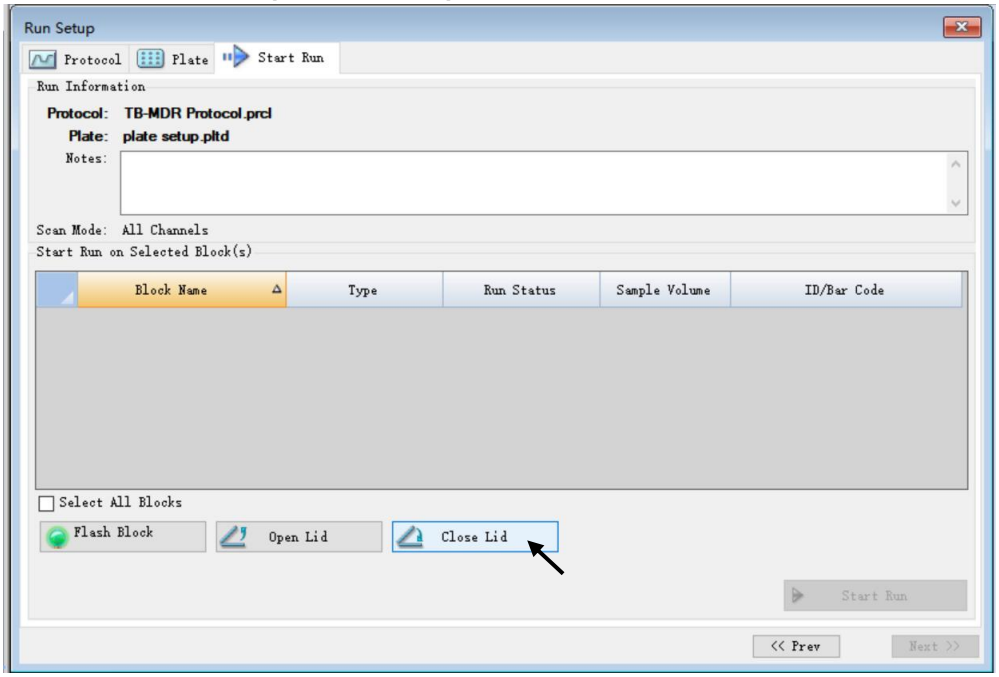


Fig. 8. **Close Lid**

2) Click **Start Run**.

3) Store the run file either in My Documents or in a designated folder. Input the file name, click **SAVE**, and the run will start.

1.2. Data Analysis

A. Create folders for data export

1) To save data for all of amplification curve detection step from the result file, create one folder.
2) Folder name may be as desired by user.

B. Pre-settings for Data Analysis in CFX Manager™

1) After the test, click the Quantitation and Melt Curve tab to confirm and analyze the results.

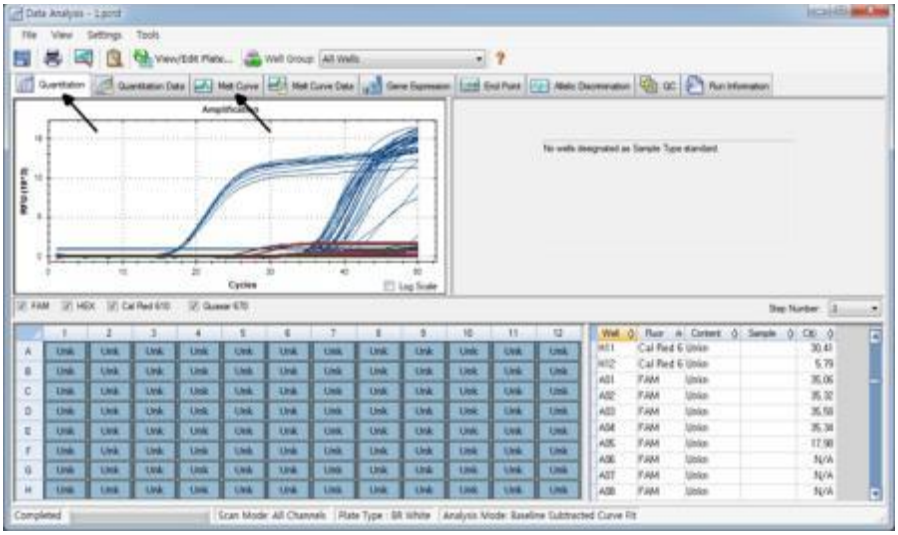
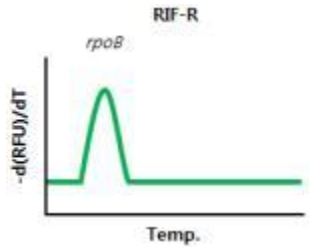
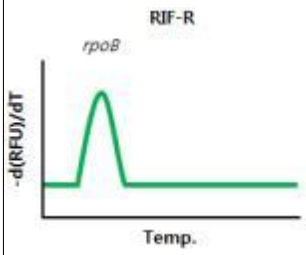
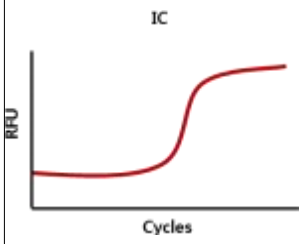
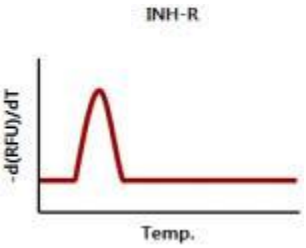
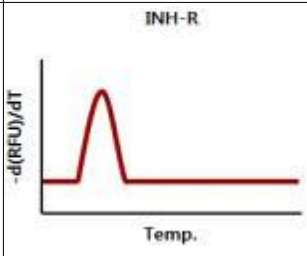
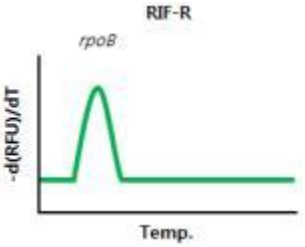
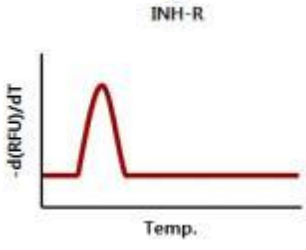
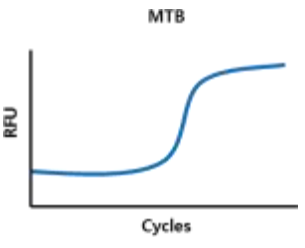


Fig. 9. **Amplification curve and Melt Curve results**

RESULTS ANALYSIS

1. Analysis Summary Guide

Fluorophore	TB-MDR Mix A	TB-MDR Mix B
FAM		
HEX		
ROX		
CY5		

2. Interpretation of Results

2.1. MTB Detection Analysis

C_t value	Result
≤ 38	Detected (+)
> 38 or N/A	Not detected (-)

Case	Result		Interpretation
	MTB (CY5 of Mix B)	IC (HEX of Mix A)	
1	+	+	MTB detected
2	-	+	MTB not detected
3	+	-	MTB detected, IC Invalid[1)]
4	-	-	Invalid[1)]

2.2. MDR Detection Analysis

Result of MDR analysis is dependent on result of MTB detection in following:

A. In case of 'MTB detected'

Case	Result			Interpretation
	IC (HEX of Mix A)	RIF-R (FAM of Mix A, FAM of Mix B, ROX of Mix B)	INH-R (ROX of Mix A, CY5 of Mix A, HEX of Mix B)	
1	+	+	+	INH-R & RIF-R detected
2		+	-	RIF-R detected
3		-	+	INH-R detected
4		-	-	MTB detected
5	-	+/-	+/-	Invalid[1)]

B. In case of 'MTB not detected'

Case	Result			Interpretation
	IC (HEX of Mix A)	RIF-R (FAM of Mix A, FAM of Mix B, ROX of Mix B)	INH-R (ROX of Mix A, CY5 of Mix A, HEX of Mix B)	
1	+	+	+	Invalid[2)]
2		+	-	Invalid[2)]
3		-	+	Invalid[2)]
4		-	-	-
5	-	+/-	+/-	Invalid[1)]

C. In case of MTB detection is 'Invalid'

Case	Result			Interpretation
	IC (HEX of Mix A)	RIF-R (FAM of Mix A, FAM of Mix B, ROX of Mix B)	INH-R (ROX of Mix A, CY5 of Mix A, HEX of Mix B)	
1	+	+	+	Invalid[3)]
2		+	-	
3		-	+	
4		-	-	
5	-	+/-	+/-	

Note:

Whether a specimen is a mutant or not is determined by △Tm between the specimen and the positive control.

For each channel, mutant is determined by comparing the difference in Tm values (△Tm) between the peak of the specimen and that of the positive control:

- Wild peak(sensitive): △Tm ＜1.5℃
- Mutant peak(resistant): △Tm ≥1.5℃

2.3 Supplementary explanation about Invalid

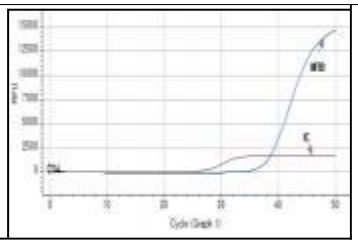
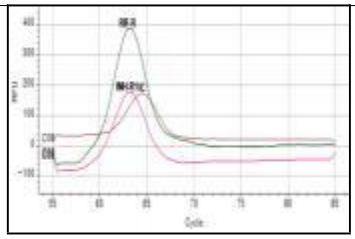
Invalid[1)] Repeat the test from nucleic acid extraction using another aliquot of the original specimen. If the same result is shown in re-extracted nucleic acid, please dilute the specimen (1/10~1/100) in RNase-free Water and repeat the extraction and PCR.

Invalid[2)] MTB is not detected but drug-resistance is detected. Repeat the test from nucleic acid extraction using another aliquot of the original specimen. If the same result is shown, refer to the results of other diagnostic methods.

Invalid[3)] MTB test result is valid. To confirm the result of drug-resistance, repeat the test from nucleic acid extraction using another aliquot of the original specimen. If the same result is shown in re-extracted nucleic acid, please dilute the specimen (1/10~1/100) in RNase-free Water and repeat the extraction and PCR.

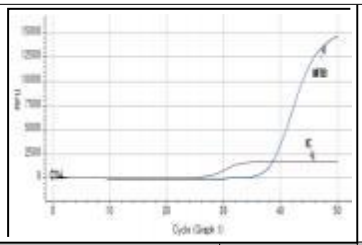
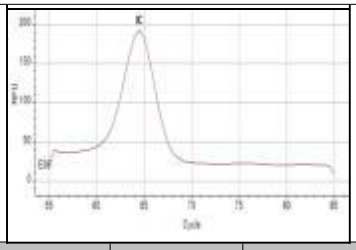
3. Application to Clinical Samples

Sample 1

MTB detection	MDR detection
	

Name	FAM	HEX	ROX	CY5	Interpretation
TB-MDR Mix A	RIF-R	IC	INH-R	INH-R	MTB(+), RIF-R, INH-R
	+	26.47	-	-	
TB-MDR Mix B	RIF-R	INH-R	RIF-R	MTB	
	-	+	-	37.71	

Sample 2

MTB detection	MDR detection
	

Name	FAM	HEX	ROX	CY5	Interpretation
TB-MDR Mix A	RIF-R	IC	INH-R	INH-R	MTB(+), RIF-S, INH-S
	-	28.09	-	-	
TB-MDR Mix B	RIF-R	INH-R	RIF-R	MTB	
	-	-	-	36.9	

Note:

Whether a specimen is a mutant or not is determined by △Tm between the specimen and the positive control.

For each channel, mutant is determined by comparing the difference in Tm values (△Tm) between the peak of the specimen and that of the positive control:

- Wild peak(sensitive): △Tm ＜1.5℃
- Mutant peak(resistant): △Tm ≥1.5℃

RIF-R: drug resistance to rifampicin; **INH-R:** drug resistance to isoniazide

RIF-S: drug sensitive to rifampicin; **INH-S:** drug sensitive to isoniazide

TROUBLESHOOTINGS

TB-MDR Multiplex PCR Detection		
OBSERVATION	**PROBABLE CAUSE**	**SOLUTION**
No signal	The fluorophores for data analysis do not comply with the protocol	Select the correct fluorophores for data analysis and export the data again. There is no need to repeat the test in this case.
	Incorrect setting of real-time thermal cycler	Please check the thermal cycling conditions and repeat the test under the correct settings.
	Incorrect storage or past expiry date of the test kit	Please check the storage conditions (See page 11) and the expiry date (refer to label) of the test kit and use a new kit if necessary.
	Nucleic acid extraction failure	No signal including IC may indicate loss of nucleic acid during extraction. Make sure that you use recommended extraction method. If due to inhibitors, re-extract the original specimen or dilute the specimen (1/10~1/100) in RNase-free Water and repeat the test from extraction step.
No Internal Control signal	High load of specimen's nucleic acid	If target pathogen signal is observed but not IC, then IC amplification may have been inhibited by high titer of target pathogen. In order to confirm IC signal, dilute the specimen (1/10~1/100) in RNase-free Water and repeat the test from extraction step.
	Presence of inhibitor	Please dilute the specimen (1/10~1/100) in RNase-free Water and repeat the test from extraction step.
Putative false positive or target signals observed in Negative Control	Contamination	Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol. Only use filter tips throughout the procedure and change tips between tubes. Repeat entire procedure from nucleic acid extraction to detection with a new set of reagents.

TB-MDR Multiplex PCR Detection		
OBSERVATION	**PROBABLE CAUSE**	**SOLUTION**
Putative false negative or no signal observed in Positive Control	Error in specimen collection	Please check the specimen collection method, and re-collect specimen.
	Incorrect storage of the specimen	Please re-collect the specimen and repeat the entire procedure. Ensure that the specimen is stored as recommended.
	Error in nucleic acid extraction	Please check the nucleic acid extraction procedure as well as nucleic acid concentration, and re-extract the nucleic acid.
	Error in adding nucleic acid to corresponding PCR tubes	Check the sample numbers of tubes containing nucleic acid and make sure to add nucleic acid into the correct PCR tubes and carefully repeat the test if necessary.
	Presence of inhibitor	Please dilute (1/10~1/100) the template nucleic acid with RNase-free Water and repeat the test with the diluted nucleic acid.