For Professional Use Only

Anyplex™

MTB/NTM
Real-time Detection (V2.0)

(Cat. No. TB7200X)

Multiplex Real-time PCR System for identification of *Mycobacterium tuberculosis* and non-tuberculosis mycobacteria.

For use with

1. Microlab NIMBUS IVD and Microlab STARlet IVD

2. Seegene NIMBUS and Seegene STARlet

For use with

1. CFX96™ Real-time PCR Detection System (CFX Manager™ Software-IVD v1.6)

2. CFX96™ Dx System (CFX Manager™ Dx Software v3.1)

3. 7500 Real-time PCR System, Software v2.0.5 (Life Technologies)

CE Σ 100

IVD **For in vitro diagnostic use only**

Seegene Inc.,

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Not available in the U.S.

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NOTICES

- For in vitro diagnostic use only.
- Reliability of the results depends on adequate specimen collection, storage, transport, and processing procedure.
- **This product is only for use with Microlab NIMBUS IVD, Microlab STARlet IVD, Seegene NIMBUS and Seegene STARlet maximum 5 separate runs.**
- **This test has been validated for the following specimen types: sputum, culture, fresh tissue, and bronchial washing.** This test has not been validated for any other types of specimens.
- **Store DNA samples at ≤-20°C until use and keep on ice during use.**
- Sensitivity of the assay may decrease if samples are repeatedly frozen/thawed or stored for a longer period of time.
- Workflow in the laboratory should proceed in a unidirectional manner.
- Always wear disposable gloves in each area and change them before entering different areas. Change gloves immediately if contaminated or treat them with DNA decontaminating reagent.
- Dedicate supplies and equipment to separate working areas and do not move them from one area to another.
- Do not pipette by mouth.
- Do not eat, drink, or smoke in laboratory work areas. Wear disposable powder-free gloves, laboratory coats, and eye protections when handling specimens and reagents. Wash hands thoroughly after handling specimens and test reagents.
- Avoid contamination of reagents when removing aliquots from reagent tubes. The use of sterile disposable pipette tips is recommended.
- Do not pool reagents from different lots or from different tubes of the same lot.
- Do not use a product after its expiry date.
- All disposable items are for one time use. Do not reuse.
- Use screw-capped tubes and prevent any potential splashing or cross-contamination of specimens during preparation.
- Please be careful not to contaminate reagents with extracted nucleic acids, PCR products, and Positive Control. To prevent contamination of the reagents, use of filter tips is recommended.
- Use separated and segregated working areas for each experiment.

- To avoid contamination of working areas with amplified products, open PCR reaction tubes or strips only at designated working areas after amplification.
- Store positive materials separated from the kit's reagents.
- Laboratory safety procedures (refer to Biosafety in Microbiological and Biomedical Laboratories & CLSI Documents) must be taken when handling specimens. Thoroughly clean and disinfect all work surfaces with 0.5% sodium hypochlorite (in deionized or distilled water). Product components (product residuals, packaging) can be considered as laboratory waste. Dispose of unused reagents and waste in accordance with applicable federal, state, and local regulations.
- Expiry date is 12 months at ≤ -20℃ from the date of manufacture. Please refer to label for final expiry date.
- Seegene NIMBUS and Seegene STARlet are the same equipment as the Microlab NIMBUS IVD and Microlab STARlet IVD, although the manufacturer is different. Since there are no hardware changes on the device, the test results are the same.
- The brand name of "CFX96™ Real-time PCR Detection System-IVD" is changed to "CFX96™ Dx System". Since there are no hardware changes to the systems, it is expected to obtain the same results from both systems.
- "CFX Manager™ Dx Software v3.1" is an upgrade version of "CFX Manager™ Software-IVD v1.6". The upgraded software includes enhancements to the "Run" menu. These enhancements do not impact the results of data analysis; therefore, results will be the same.
- This kit is intended to aid in the differential diagnosis of target pathogen infections; *Mycobacterium tuberculosis* and non-tuberculosis mycobacteria.

INTENDED USE

Anyplex™ MTB/NTM Real-time Detection (V2.0) is a qualitative *in vitro* test for detection of *Mycobacterium tuberculosis* (MTB) and non-tuberculosis mycobacteria (NTM) from sputum, culture (solid culture and liquid culture), fresh tissue, and bronchial washing of symptomatic patients.

Anyplex™ MTB/NTM Real-time Detection (V2.0) does not differentiate among members of the *M. tuberculosis* complex, i.e., *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum, M. microti, M. canetti,* and *M. pinnipedii.*

PRINCIPLES AND PROCEDURE OVERVIEW

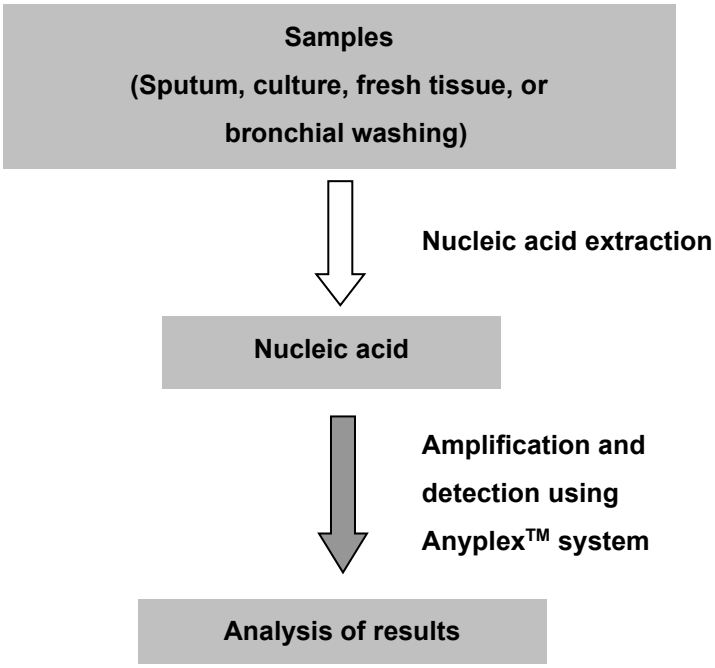
1. Principles

The polymerase chain reaction (PCR) DNA amplification technique is highly sensitive and specific. Seegene developed a proprietary oligo technology called “Dual Priming Oligonucleotide (DPO™)”, which provides freedom in primer design and PCR optimization and maximizes PCR specificity and sensitivity by fundamentally blocking non-specific priming. Anyplex™ MTB/NTM Real-time Detection (V2.0) is based on two major processes: nucleic acid extraction and PCR amplification of target nucleic acid using DPO primers with real-time PCR machine. Anyplex™ MTB/NTM Real-time Detection (V2.0) is a multiplex real time PCR assay that permits amplification of target nucleic acids of *M. tuberculosis* (MTB), mycobacteria and Internal Control (IC).

The Internal Control is added to Anyplex™ MTB/NTM Real-time Detection (V2.0) to identify processed specimens containing substances that may interfere with PCR amplification. The Internal Control is a DNA plasmid. These features are selected to ensure equivalent amplification of the Internal Control, MTB and mycobacteria target DNA. The Internal Control is introduced into each amplification reaction and co-amplified with target DNA from the clinical specimen.

To prevent amplification product acting as potential contaminants, Uracil-DNA glycosylase (UDG)-dUTP system is employed in Anyplex™ MTB/NTM Real-time Detection (V2.0). The natural function of UDG is to prevent mutagenesis by eliminating uracil from DNA molecules by cleaving *N*-glycosylic bond and initialting base-excision repair (BER) pathway. Therefore, UDG systems are used to control cross-contamination of samples with amplicons.

2. Procedure Overview



BACKGROUND INFORMATION

The rapid and accurate diagnosis of symptomatic patients is the cornerstone of global strategies for tuberculosis (TB) control. The advantages and limitations of each available TB diagnostic method are evident and no test is yet available that meets target specificity. Furthermore, the quality of the test results with existing methods is dependent on the availability of sufficient human and financial resources, training of laboratory personnel and monitoring of performance.

Active tuberculosis is currently diagnosed using an assortment of techniques, symptoms, and clinical signs. These techniques include the X-ray, microscopy (after AFB staining), culture, and molecular diagnostics. The current gold standard is culture. Although effective implementation of current techniques can substantially reduce the time needed, the physician planning treatment for a suspected tuberculosis patient vitally needs this diagnostic information. In the past, delays in obtaining this information were thought to be acceptable. But this is no longer true. The rapid progress of the disease in HIV-infected individuals and the continued emergence of drug-resistant diseases underscore the need for new rapid and sensitive diagnostics.

REAGENTS

The reagents contained in one kit are sufficient for 100 reactions.

Order information (REF **TB7200X**)

Anyplex™ MTB/NTM Real-time Detection (V2.0)			
Symbol	**Contents**	**Volume**	**Description**
PRIMER	10X MTB/NTM OM	200 µL	Oligo Mix (OM): ➢ Amplification and detection reagents ➢ Template of Internal Control
PREMIX	EM3	1,000 µL	➢ DNA polymerase ➢ Uracil-DNA glycosylase (UDG) ➢ Buffer containing dNTPs
CONTROL +	MTB/NTM PC	50 µL	Positive Control (PC): ➢ Mixture of pathogen and IC clones
WATER	RNase-free Water	1,000 µL	Ultrapure quality, PCR-grade
DNA ES	DNA Extraction Solution	10 mL X 2	Reagent for bacterial DNA extraction
	User manual		

STORAGE AND HANDLING

All components of Anyplex™ MTB/NTM Real-time Detection (V2.0) should be stored at ≤ -20°C. All components are stable under recommended storage conditions until the expiry date stated on the label. The performance of kit components is not affected for up to 5 freezing and thawing. If the reagents are to be used only intermittently, they should be stored in aliquots.

MATERIALS REQUIRED BUT NOT PROVIDED

- NALC-NaOH (0.5% NALC, 2% NaOH, 1.47% trisodium citrate) or 4% NaOH (1N NaOH)
- 1X PBS solution
- Disposable powder free gloves (latex or nitrile)
- Pipettes (adjustable) and sterile pipette tips
- 1.5mL microcentrifuge tubes
- Ice maker
- Heat block
- Desktop centrifuge
- Vortex mixer
- Clean bench
- CFX96™ Real-time PCR Detection System (Bio-Rad)
- CFX96™ Dx System (Bio-Rad)
- Low-Profile 0.2 mL 8-Tube Strips without Caps (white color, Cat. No. TLS0851, Bio-Rad)
- Optical Flat 8-Cap Strips (Cat. No. TCS0803, Bio-Rad)
- EU 0.1 mL, Thin-wall 8-tube Strip, white (Cat. No. B77009; BIOplastics)
- EU 8-single attachable optical wide indented cap-strip (Cat. No. B79501; BIOplastics)
- 7500 Real-time PCR System, Software v2.0.5 (Life Technologies)
- MicroAmp® Optical 8-Tube Strip (0.2 mL) (Part No. 4316567, Life Technologies)
- MicroAmp® Optical 8-Cap Strip (Part No. 4323032, Life Technologies)

PROTOCOL

1. Specimen Collection, Storage, and Transport

Note: All samples should be treated as potentially infectious materials. Only those sample materials are permitted, which are collected, stored and transported attending strictly the following rules and instructions.

Note: To ensure high sample quality, specimens should be transported as fast as possible. The specimens should be transported at indicated temperatures.

A. Specimen Collection

Sputum

- Give clear instructions to patients when collecting sputum specimens. Patients must collect samples either outside in the open air or away from other people. Patients should not collect samples in confined spaces such as toilets.
- Rinse mouth with water before collecting sputum. Patients should cough deeply to expectorate sputum directly into the container.
- A sputum sample must have a volume of 3~5 mL.

Solid culture (Ogawa)

- Samples may be tested as soon as growth is visible and during the subsequent 60 days of incubation.
- Colony can be collected with a disposable plastic loop or needle. Avoid collecting any of the Ogawa media along with the cell.

Liquid culture (MGIT)

- Mycobacterial growth indicator tubes (MGITs) are examined daily with a UV lamp for bright orange fluorescence at the bottom of the tube reflected at the meniscus. With the positive signal of fluorescence, pipette a 0.5 mL of sample from the bottom of the tube.

Fresh tissue

- Any tissue to be tested must be collected aseptically into a sterile container without fixatives or preservatives. Do not place tissue specimen for culture into formalin.
- If the specimen dries, add sterile saline to keep moist. Keep refrigerated until transport.

Bronchial washing

- Bronchial washing should be aseptically collected in a sterile container by the physician using aspiration techniques or surgical procedures.

B. Specimen Storage and Transport

Specimen	Storage and Transport		Note
	Temp.	Duration*	
Sputum Solid culture (Ogawa) Liquid culture (MGIT) Fresh tissue Bronchial washing	2~8°C	3 days	- Performance may be affected by prolonged storage of specimens. - Specimens should also adhere to local and national instructions for transport of pathogenic material.

* Duration: The time period from the specimen collection to the final test (includes transport and storage of specimens in prior to test).

2. Pretreatment of Specimens

Sputum

1) For Manual Nucleic Acid Extraction

- Add same volume of NALC-NaOH (0.5% NALC, 2% NaOH, and 1.47% trisodium citrate) to the specimen in the sputum container and vortex for 1 minute.

Note: 4% NaOH (1 N NaOH) can be used instead of NALC-NaOH.

- Incubate for 15 minutes at room temperature.
- Transfer 1.5 mL to a new sterile tube and centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes.
- Discard supernatant, add 1 mL of 1X PBS solution, and mix well.
- Centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes and discard supernatant with a pipette.
- Add 1 mL of 1X PBS solution and mix well.
- Centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes and discard supernatant with a pipette.

2) For Automated Nucleic Acid Extraction System

- Add same volume of NALC-NaOH (0.5% NALC, 2% NaOH, and 1.47% trisodium citrate) to the specimen in the sputum container and vortex for 1 minute.
- Incubate for 15 minutes at room temperature.
- Transfer 1 mL to a new sterile tube and centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes.
- Discard supernatant, add 300 µL Lysis Buffer of Universal kit to the pellet, and mix well.
- Lock the tube cap using a cap-lock and boil for 5 minutes on heat block.

Fresh tissue

- Cut or grind tissue specimen in a sterile vessel.
- Suspend in 1 mL of 1X PBS solution.
- Centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes and discard supernatant with a pipette.

Bronchial washing

- Without adding NALC-NaOH, centrifuge 1.5 mL of specimen at 15,000 x *g* (13,000 rpm) for 5 minutes.
- Discard supernatant, add 1 mL of 1X PBS solution, and mix well.
- Centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes and discard supernatant with a pipette.

3. Nucleic Acid Extraction

A. Manual Nucleic Acid Extraction Kits

DNA Extraction Solution is included in Anyplex™ MTB/NTM Real-time Detection (V2.0) kit.

All specimens except culture samples

- (Optional) Add 1 mL of sterile water to the prepared sediment, centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes, and discard supernatant with a pipette.
- Add 100 µL of DNA Extraction Solution to the sediment and vortex for 30 seconds.
- Lock the tube cap using a cap-lock and boil at 100°C for 20 minutes on heat block.
- Centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes.
- Use 5 µL of supernatant as PCR template.

Solid culture (Ogawa)

- Suspend a colony in 200 µL of DNA Extraction Solution in a 1.5 mL microcentrifuge tube.
- Vortex for 30 seconds.
- Lock the tube cap using a cap-lock and boil at 100°C for 20 minutes on heat block.
- Centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes.
- Use 5 µL of supernatant as PCR template.

Liquid culture (MGIT)

- Transfer 0.5 mL of the culture at the bottom to a 1.5 mL microcentrifuge tube.
- Centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes.
- Discard supernatant and add 200 µL of DNA Extraction Solution to the pellet.
- Vortex for 30 seconds.
- Lock the tube cap using a cap-lock and boil at 100°C for 20 minutes on heat block.
- Centrifuge at 15,000 x *g* (13,000 rpm) for 5 minutes.
- Use 5 µL of supernatant as PCR template.

B. Automated Nucleic Acid Extraction System

Note: Microlab NIMBUS IVD, Microlab STARlet IVD, Seegene NIMBUS and Seegene STARlet have been validated for only sputum.

Note: Please use the recommended specimen and elution volumes as indicated below. For all others, refer to the manufacturer's protocol.

B-1. Microlab NIMBUS IVD

Note: See Microlab NIMBUS IVD operation manual.

Automated Extraction System	Manufacturer	Cat. No.	Recommended Vol.
Microlab NIMBUS IVD	Hamilton	65415-02*	-
STARMag 96 X 4 Universal Cartridge Kit	Seegene	744300.4. UC384	Specimen: 300 µL Elution: 100 µL

* If you would like to purchase this product from Seegene Inc., please use this catalog number.

B-2. Microlab STARlet IVD

Note: See Microlab STARlet IVD operation manual.

Automated Extraction System	Manufacturer	Cat. No.	Recommended Vol.
Microlab STARlet IVD	Hamilton	173000-075*	-
STARMag 96 X 4 Universal Cartridge Kit	Seegene	744300.4. UC384	Specimen: 300 µL Elution: 100 µL

* If you would like to purchase this product from Seegene Inc., please use this catalog number.

B-3. Seegene NIMBUS

Note: See **Seegene NIMBUS** operation manual.

Automated Extraction System	Manufacturer	Cat. No.	Recommended Vol.
Seegene NIMBUS	Seegene	65415-03	-
STARMag 96 X 4 Universal Cartridge Kit	Seegene	744300.4. UC384	Specimen: 300 µL Elution: 100 µL

B-4. Seegene STARlet

Note: See **Seegene STARlet** operation manual.

Automated Extraction System	Manufacturer	Cat. No.	Recommended Vol.
Seegene STARlet	Seegene	67930-03	-
STARMag 96 X 4 Universal Cartridge Kit	Seegene	744300.4. UC384	Specimen: 300 µL Elution: 100 µL

4. Preparation for Real-time PCR

Note: The correct tubes and caps must be used (see MATERIALS REQUIRED BUT NOT PROVIDED).

Note: Aerosol-resistant filter tips and tight gloves must be used when preparing PCR reactions. Use extreme care to prevent cross-contamination.

Note: Completely thaw all reagents on ice.

Note: Briefly centrifuge the reagent tubes to remove droplets from the inside of the cap.

Note: The steps A~D are automatically processed on Microlab NIMBUS IVD, Microlab STARlet IVD, Seegene NIMBUS and Seegene STARlet. Refer to each operation manual.

A. Prepare PCR Mastermix.

2 µL	10X MTB/NTM OM
3 µL	RNase-free Water
10 µL	EM3
15 µL	Total volume of PCR Mastermix

Note: Calculate the necessary volume of each reagent needed based on the number of reactions (samples + controls).

B. Mix by inverting the tube 5 times or quick vortex, and briefly centrifuge.

C. Aliquot 15 µL of the PCR Mastermix into 0.2-mL PCR tubes.

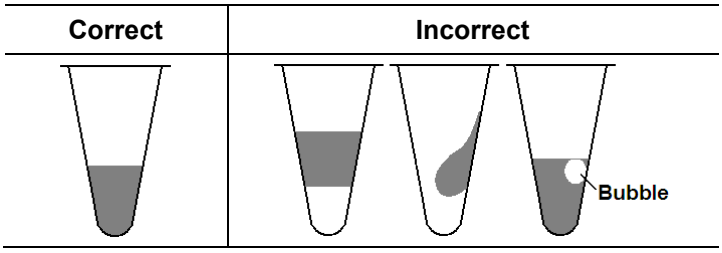
D. Add 5 µL of each sample's nucleic acids into the tube containing PCR Mastermix.

15 µL	PCR Mastermix
5 µL	Sample's nucleic acid
20 µL	Total volume of reaction

E. Close the cap, and briefly centrifuge the PCR tubes.

F. Verify that the liquid containing all PCR components is at the bottom of each PCR tube. If not, centrifuge again at a higher rpm for a longer time.

Note: It is recommended to centrifuge PCR tubes before PCR to eliminate air bubbles and collect all residual liquids at the bottom of tubes.



Note: Use a new sterile pipette tip for each sample.

Note: For **Negative Control (NC)**, use 5 µL of RNase-free Water instead of sample's nucleic acid.

Note: For **Positive Control (PC)**, use 5 µL of MTB/NTM PC instead of sample's nucleic acid.

Note: Please be careful not to cross contaminate the PCR Mastermix and samples with Positive Control.

Note: Do not label the cap of the reaction tubes as fluorescence is detected from the top of the cap.

REAL-TIME PCR INSTRUMENT SET UP AND RESULTS ANALYSIS

1. CFX96™ Real-time PCR Detection System (CFX Manager™ Software-IVD v1.6)

1.1. Real-time PCR Instrument set up

Note: CFX96™ Real-time PCR Detection System (Bio-Rad) experiment setup is 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.**

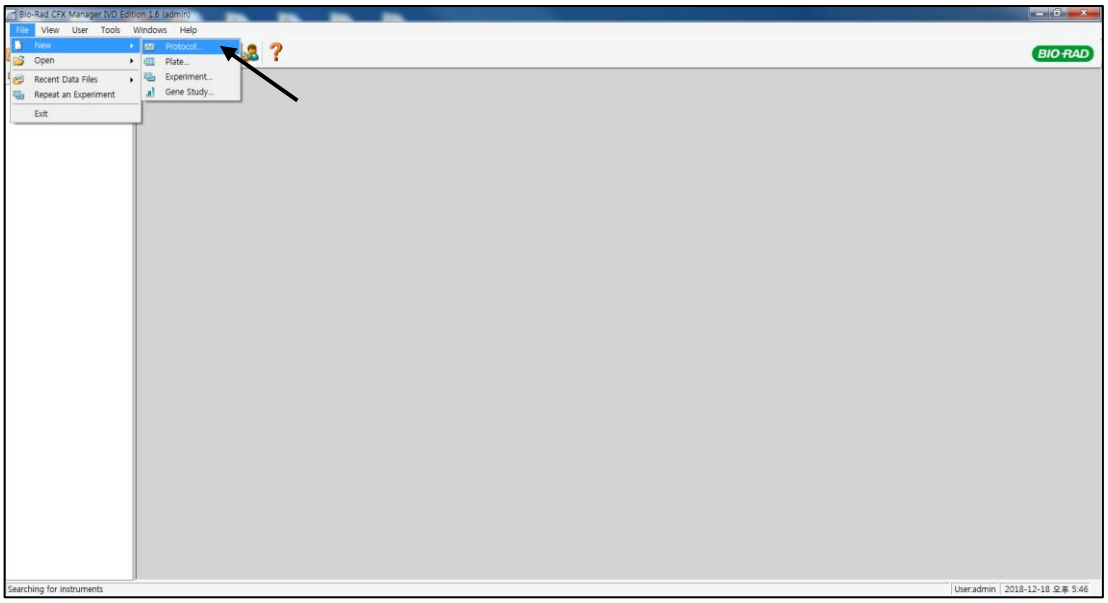


Fig. 1. **Protocol Setup**. Create a new protocol or load an existing protocol for the run

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

Step	No. of cycles	Temperature	Duration
1	1	95°C	15 min
2	45	95°C	30 sec
3*		60°C	1 min
4	GOTO Step 2, 44 more times		

*** Plate Read at Step 3.** Fluorescence is detected at 60°C.

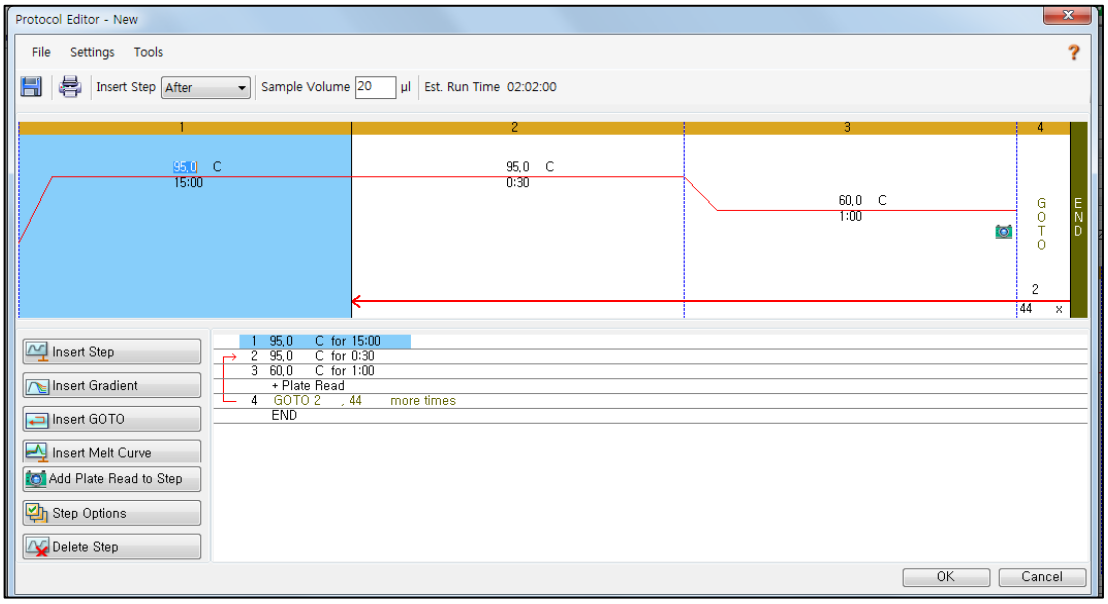


Fig. 2. **Protocol Editor**

3) Click the box next to **Sample Volume** to directly input 20 µL.

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

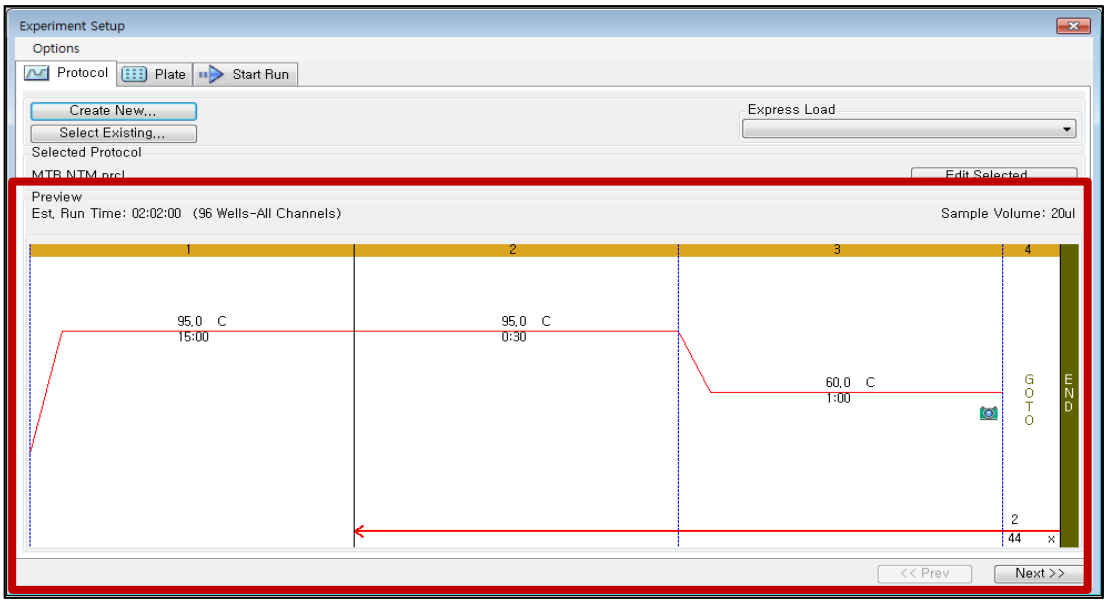


Fig. 3. **Experiment Setup: Protocol**

B. Plate Setup

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

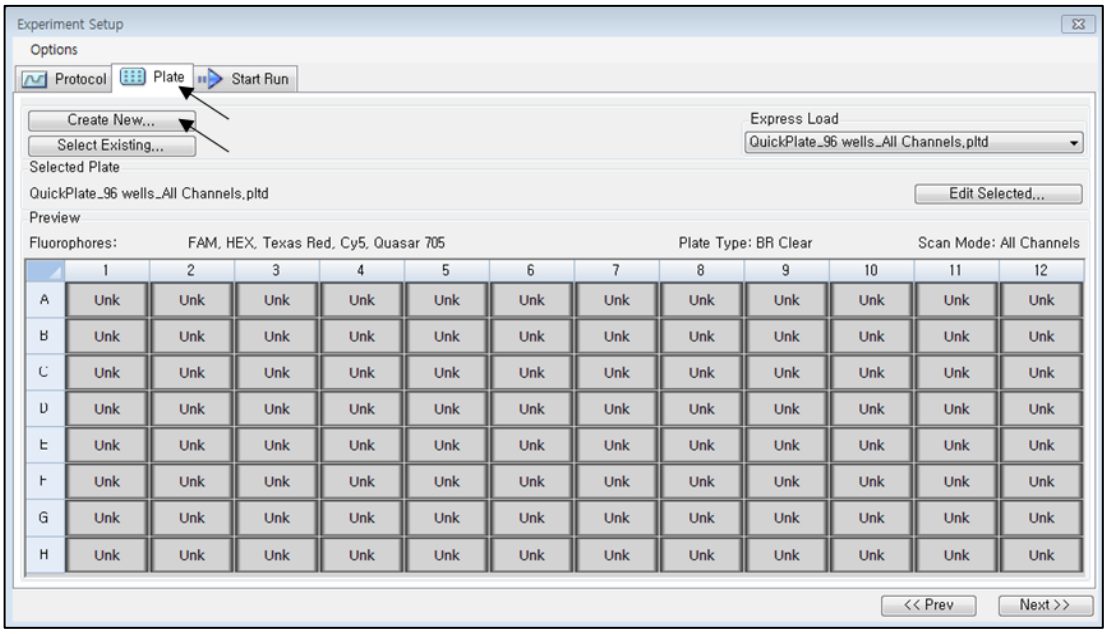


Fig. 4. **Plate Editor**. Create a new plate

2) Click **Select Fluorophores** to indicate the fluorophores (**FAM, Cal Red 610** and **Quasar 670**) that will be used and click **OK**.

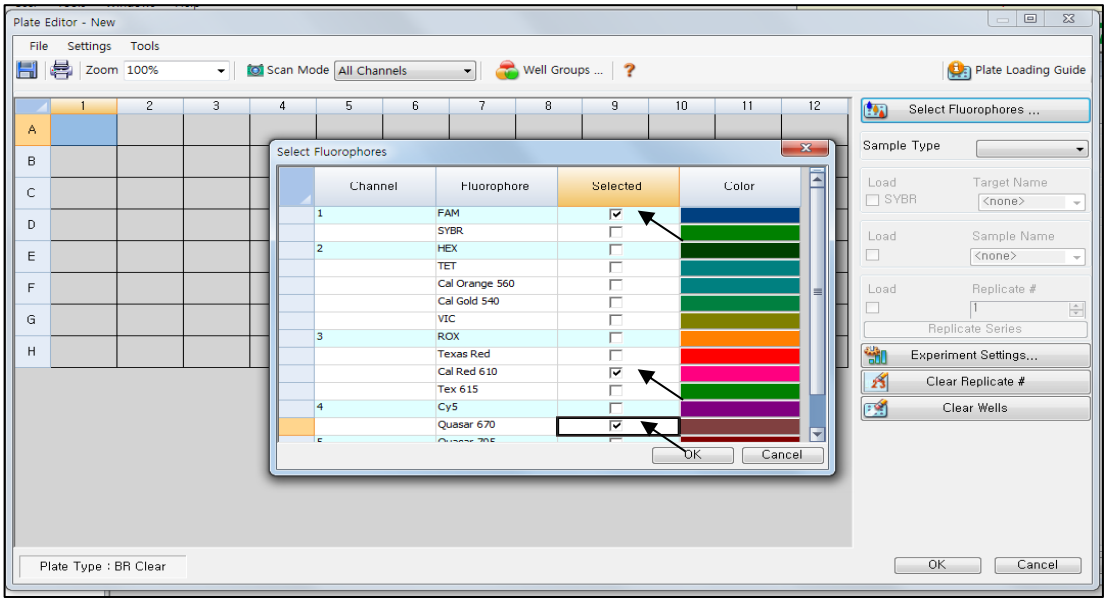


Fig. 5. **Select Fluorophores** (**FAM**, **Cal Red 610** and **Quasar 670**)

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**

4) Click on the appropriate checkboxes (**FAM, Cal Red 610** and **Quasar 670**) 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 (96 wells)** and **Plate Type (BR White)**.

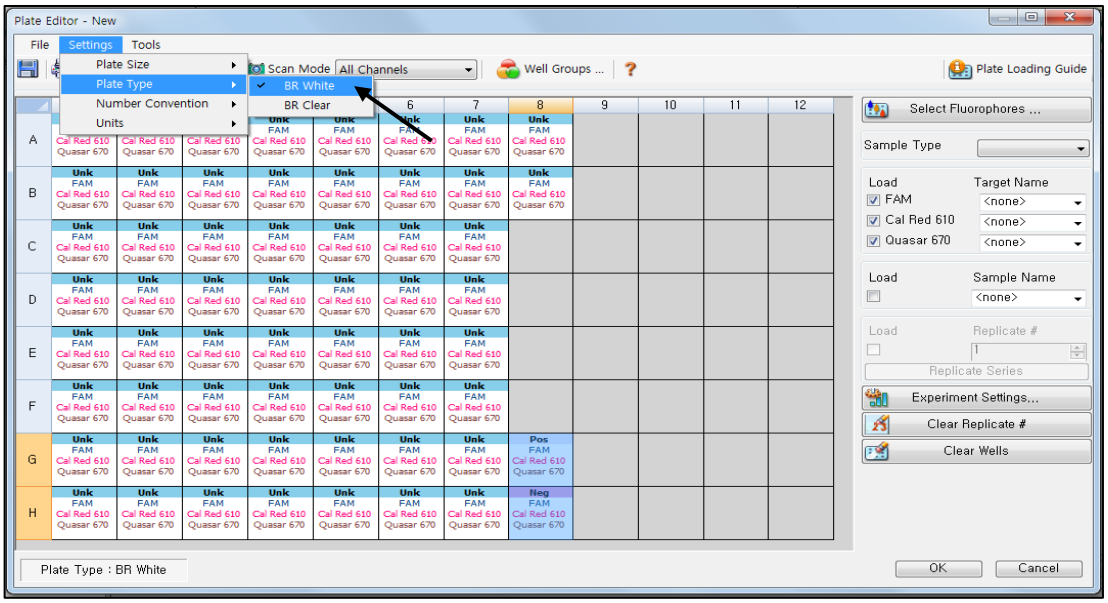


Fig. 6. **Plate Setup**

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

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

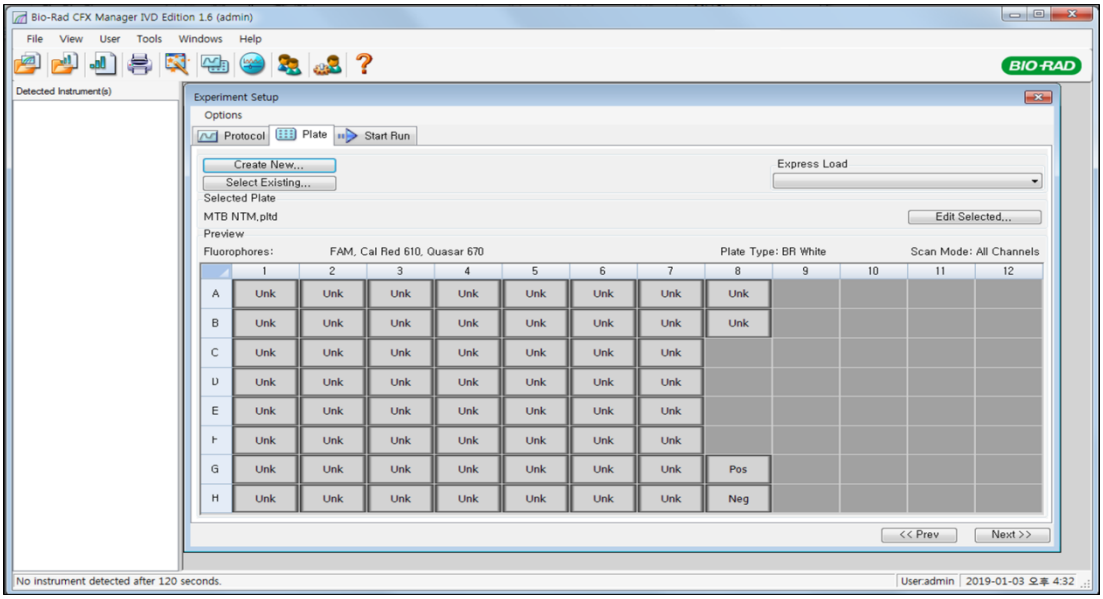


Fig. 7. **Experiment Setup: Plate**

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.

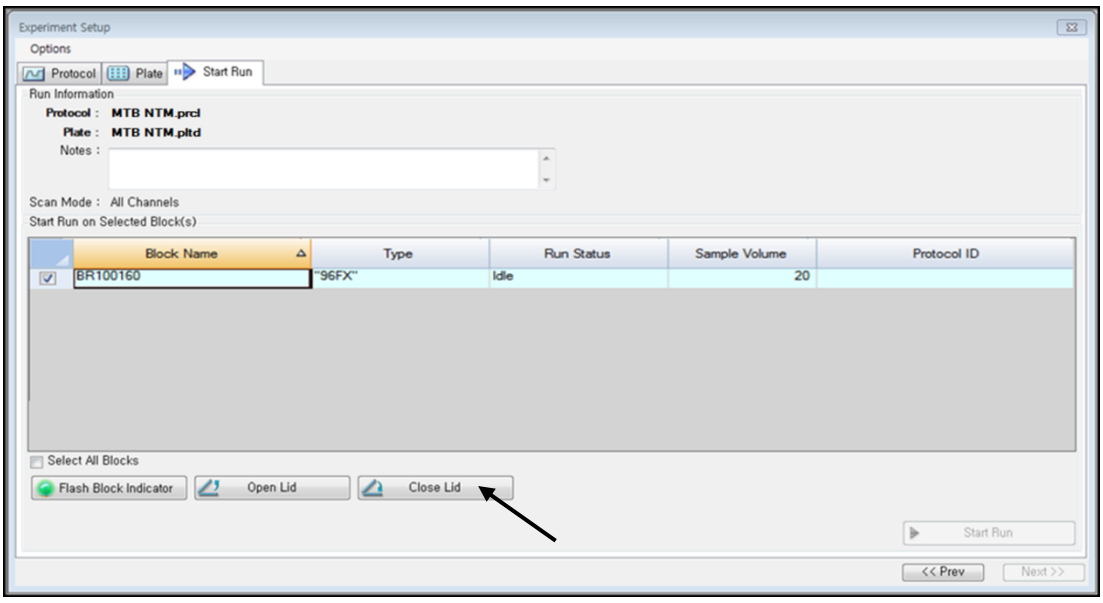


Fig. 8. **Close Lid**

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 then the run will start.

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

A. Pre-settings

Note: Check the setting of **Single Threshold** and **Baseline Subtracted Curve Fit**.

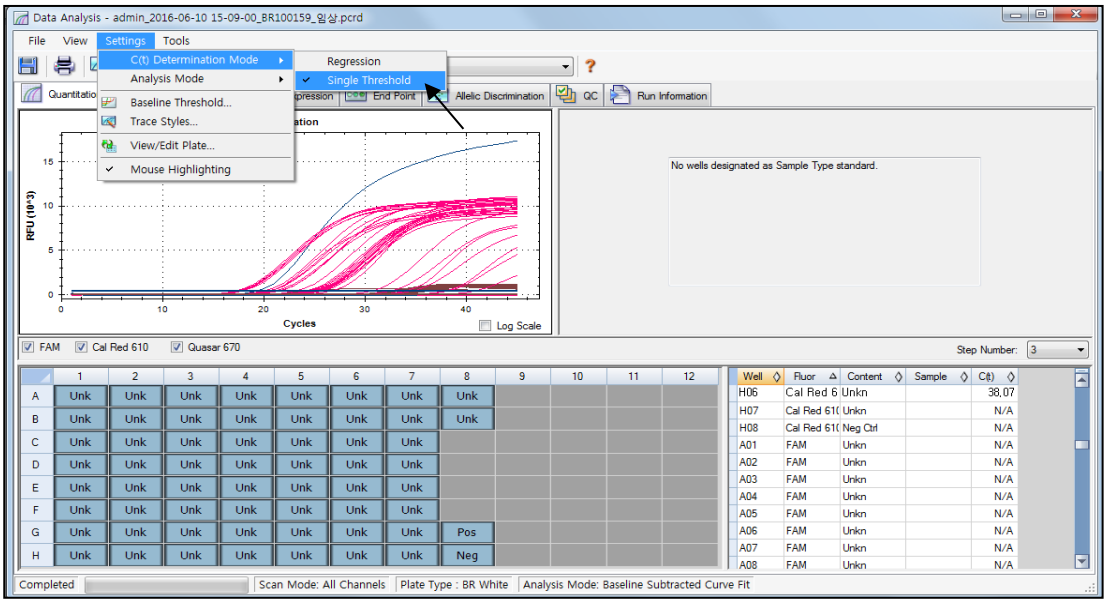


Fig. 9. **Single Threshold**

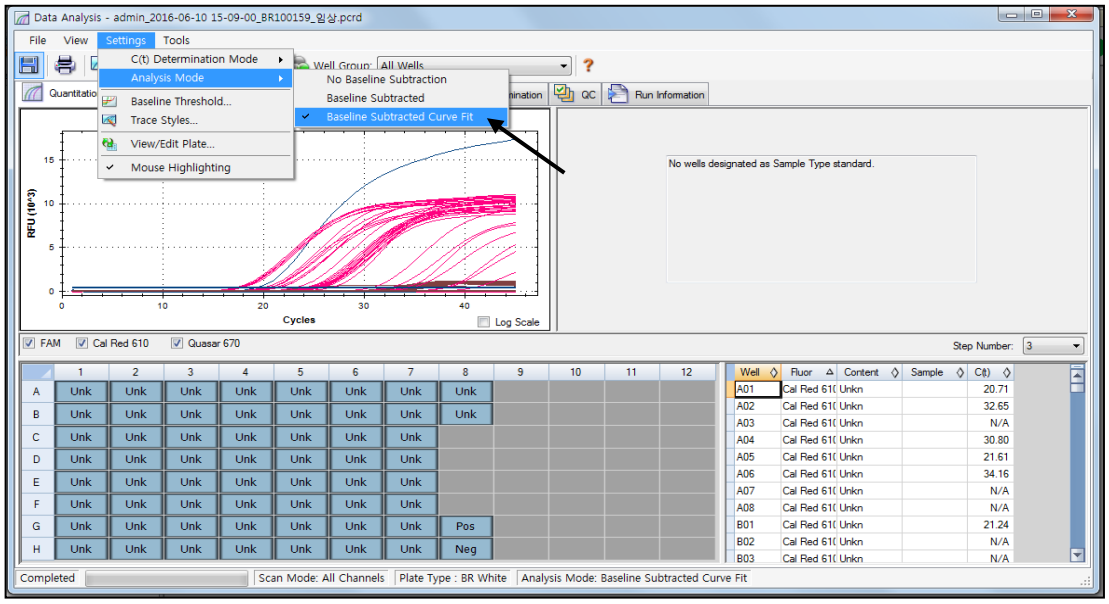


Fig. 10. **Baseline Subtracted Curve Fit**

B. Baseline Threshold

B-1. Baseline Threshold for Bio-Rad tube

Note: Set the Single Threshold of Baseline Threshold.

Note: For the setting of Baseline Threshold, you must select the **FAM**, **Cal Red 610**, and **Quasar 670** signal **separately**.

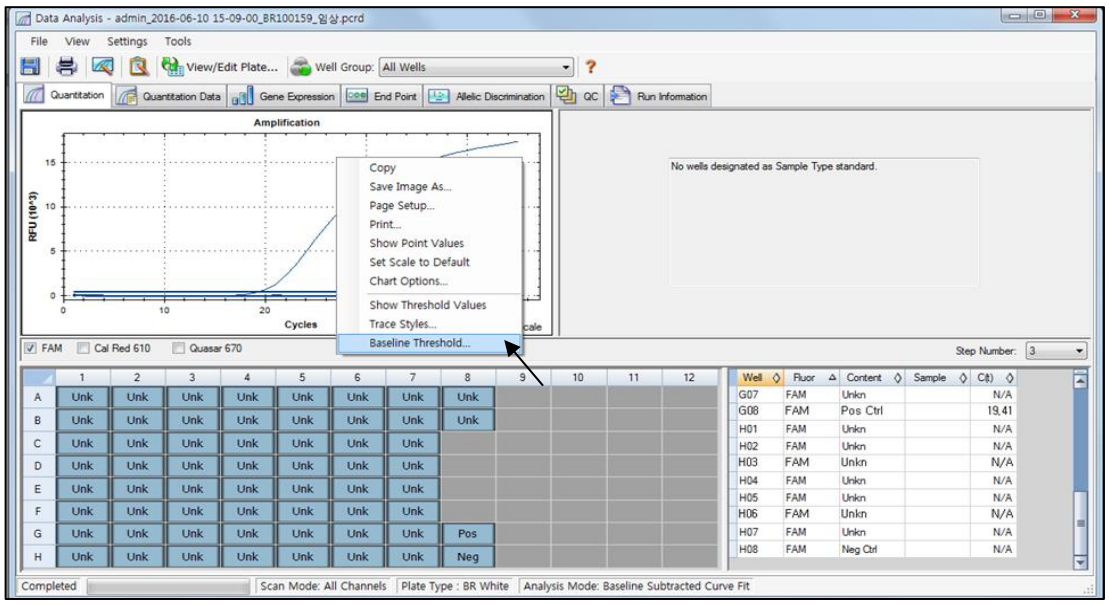


Fig. 11. **Checking of Baseline Threshold.** Click the right button of mouse on graph

Note: Set the **Baseline Cycles** as **Auto Calculated**.

Note: Set the Single Threshold value for each fluorophore as follows:

Analyte	Fluorophore	Threshold value
MTB	FAM	500
Mycobacteria	Cal Red 610	500
Internal Control	Quasar 670	60

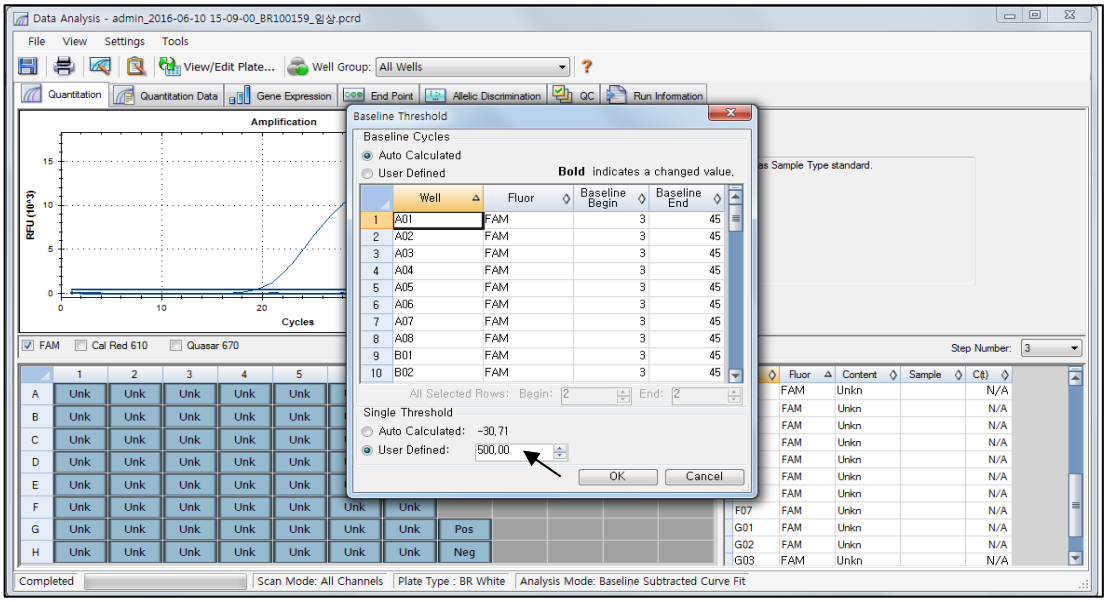


Fig. 12. **Setting of Single Threshold value**. Click User Defined button and change the Single Threshold value

B-2. Baseline Threshold for BIOplastics tube

Note: Set the Single Threshold of Baseline Threshold.

Note: For the setting of Baseline Threshold, you must select the **FAM**, **Cal Red 610**, and **Quasar 670** signal **separately**.

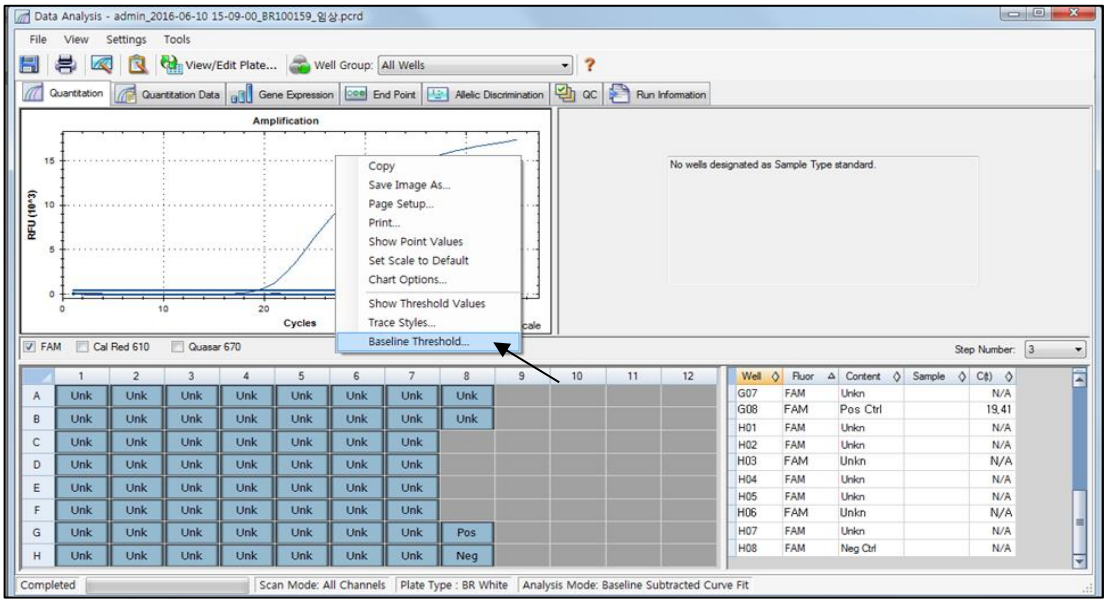


Fig. 13. **Checking of Baseline Threshold.** Click the right button of mouse on graph

Note: Set the Baseline Cycles as follows: **Begin: 3, End: 10**

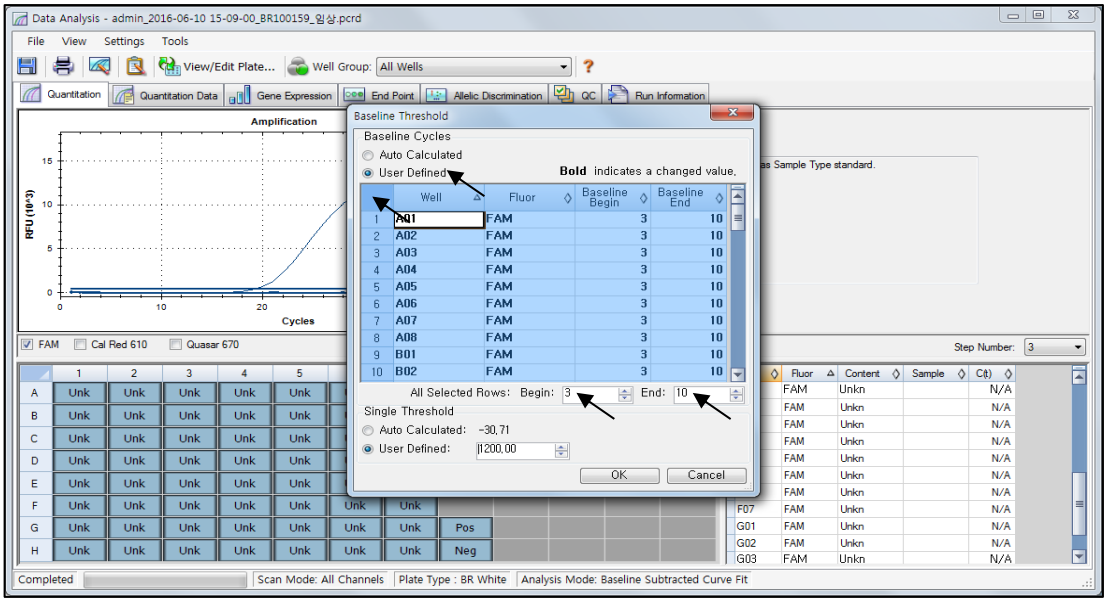


Fig. 14. **Setting of Baseline Cycles**. Click User Defined button and change the Baseline Cycles

Note: Set the Single Threshold value for each fluorophore as follows:

Analyte	Fluorophore	Threshold value
MTB	FAM	1200
Mycobacteria	Cal Red 610	1000
Internal Control	Quasar 670	100

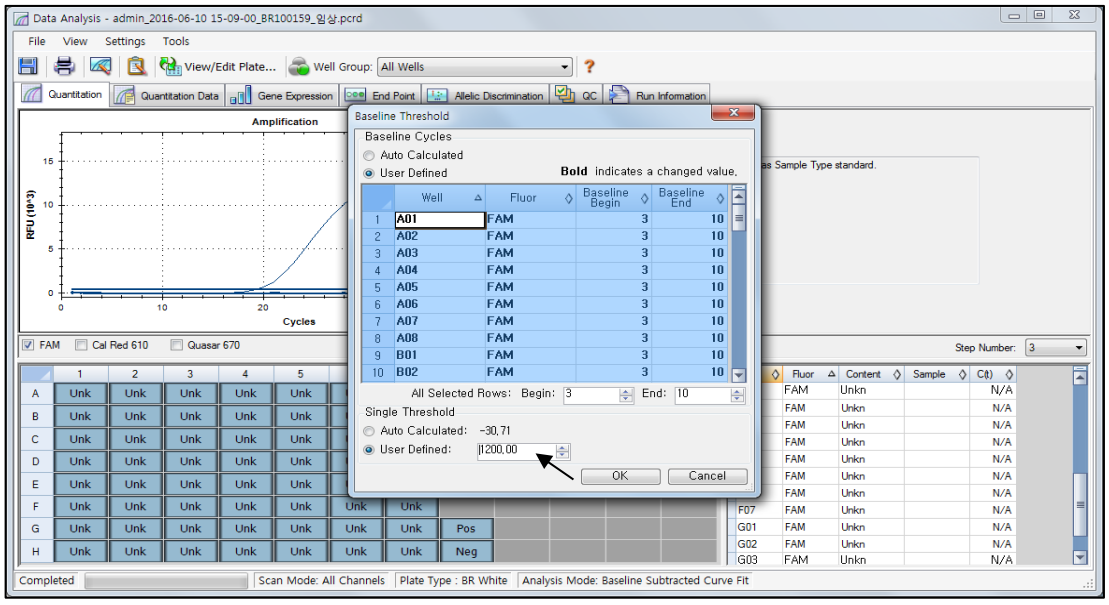


Fig. 15. **Setting of Single Threshold value**. Click User Defined button and change the Single Threshold value

2. CFX96™ Dx System (CFX Manager™ Dx Software v3.1)

2.1. Real-time PCR Instrument set up

Note: CFX96™ Dx System (Bio-Rad) experiment setup is 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.**

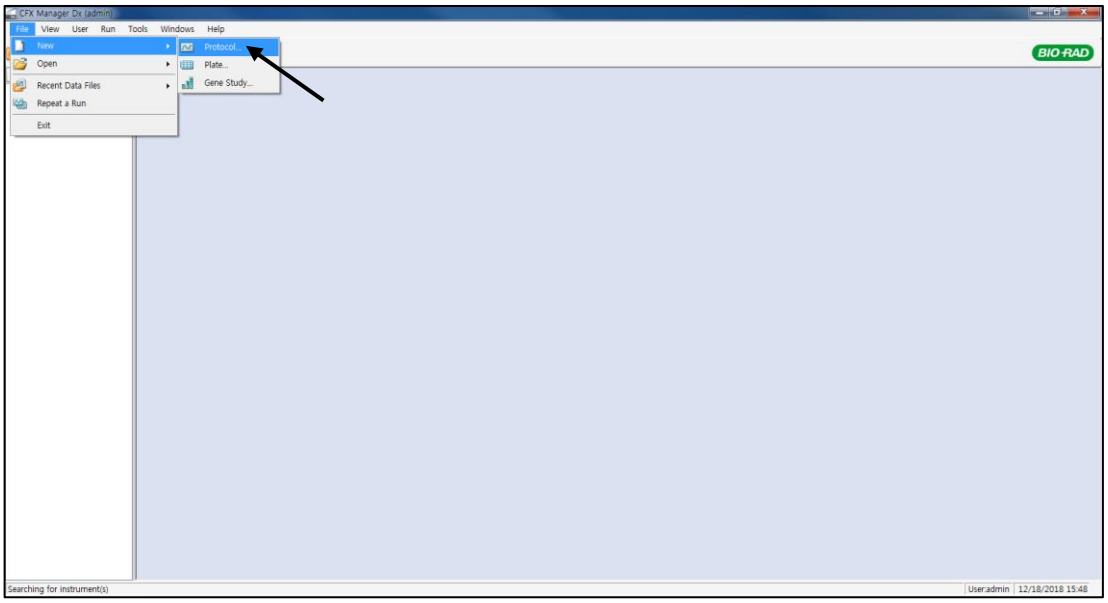


Fig. 1. **Protocol Setup**. Create a new protocol or load an existing protocol for the run

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

Step	No. of cycles	Temperature	Duration
1	1	95°C	15 min
2	45	95°C	30 sec
3*		60°C	1 min
4	GOTO Step 2, 44 more times		

*** Plate Read at Step 3.** Fluorescence is detected at 60°C.

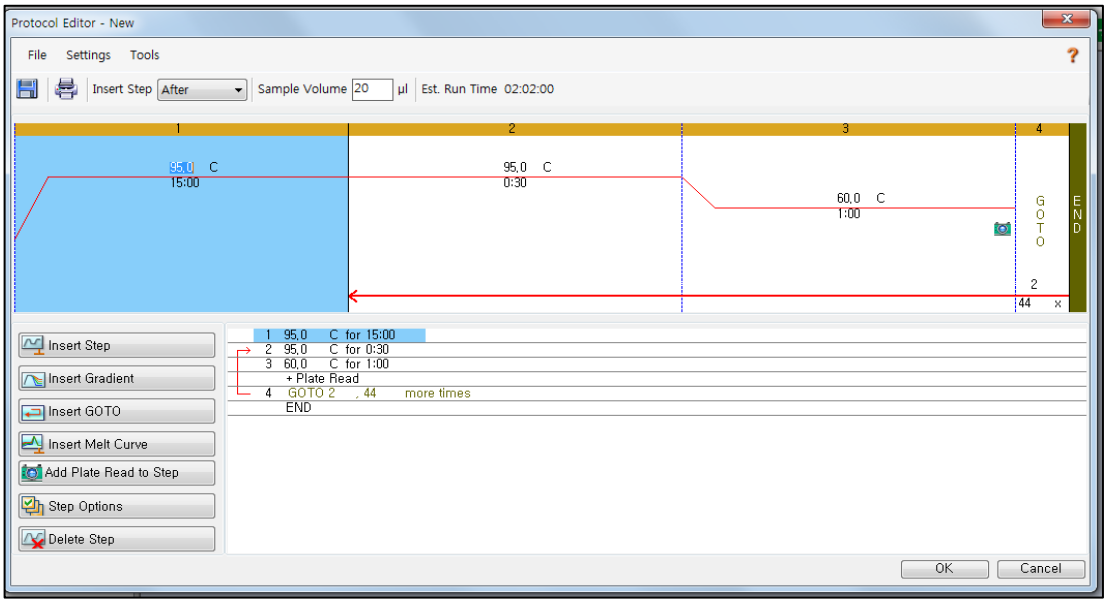


Fig. 2. **Protocol Editor**

3) Click the box next to **Sample Volume** to directly input 20 µL.

4) Click **OK** and save the protocol to open **Run Setup** window.

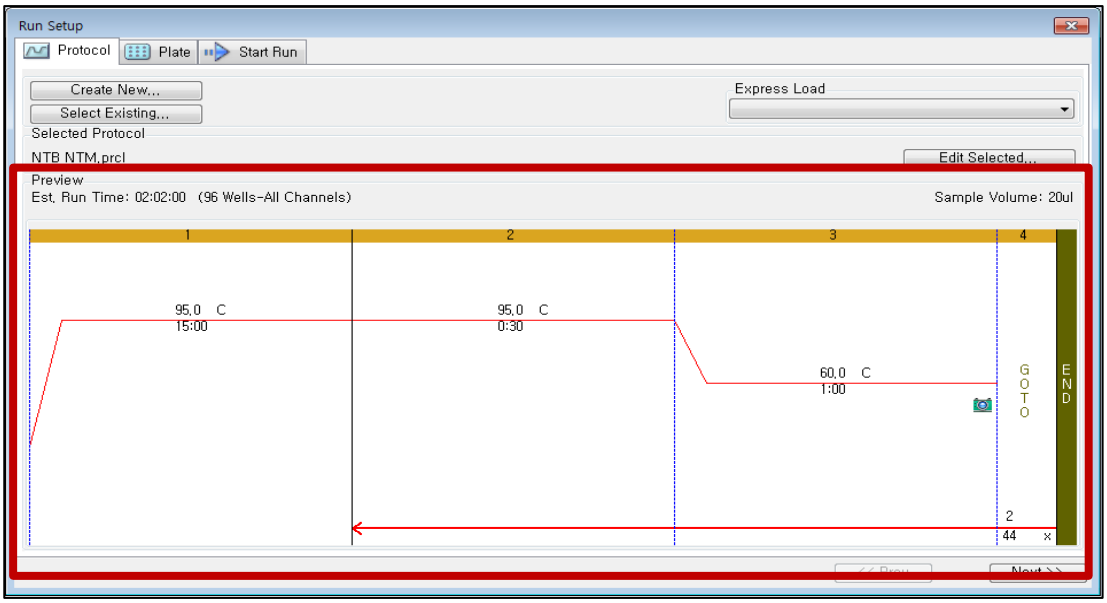


Fig. 3. **Run Setup: Protocol**

B. Plate Setup

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

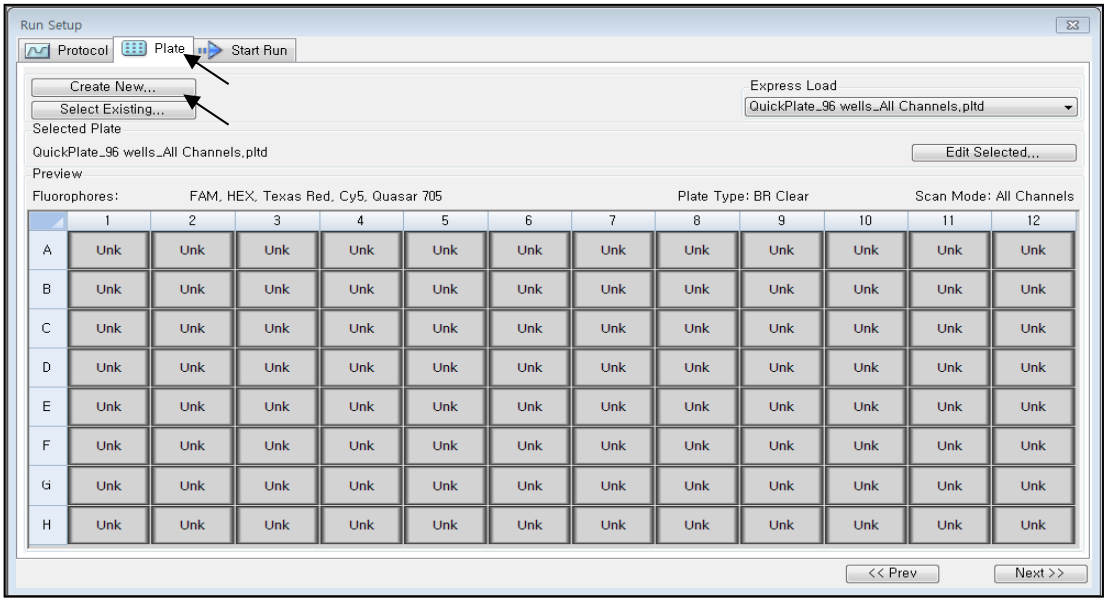


Fig. 4. **Plate Editor**. Create a new plate

2) Click **Select Fluorophores** to indicate the fluorophores (**FAM, Cal Red 610** and **Quasar 670**) that will be used and click **OK**.

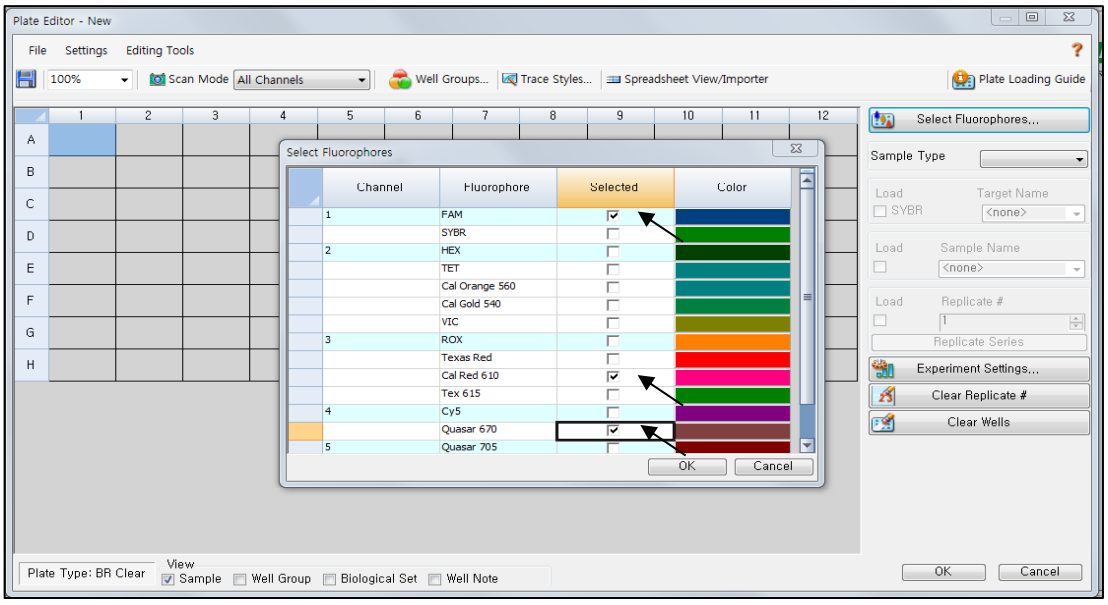


Fig. 5. **Select Fluorophores** (**FAM**, **Cal Red 610** and **Quasar 670**)

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**

4) Click on the appropriate checkboxes (**FAM, Cal Red 610** and **Quasar 670**) 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 (96 wells)** and **Plate Type (BR White)**.

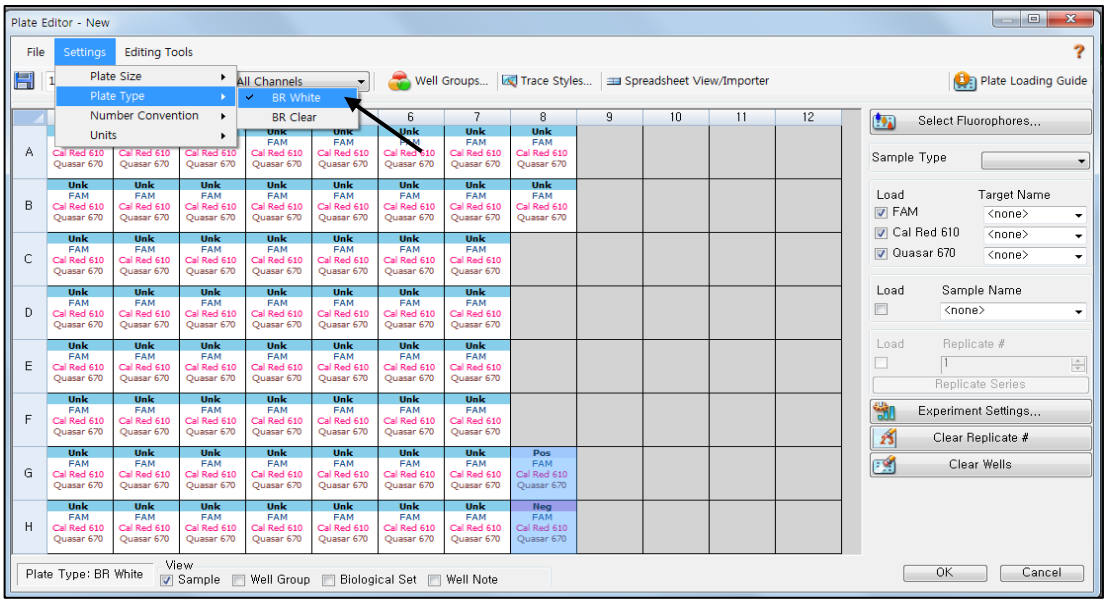


Fig. 6. **Plate Setup**

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

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

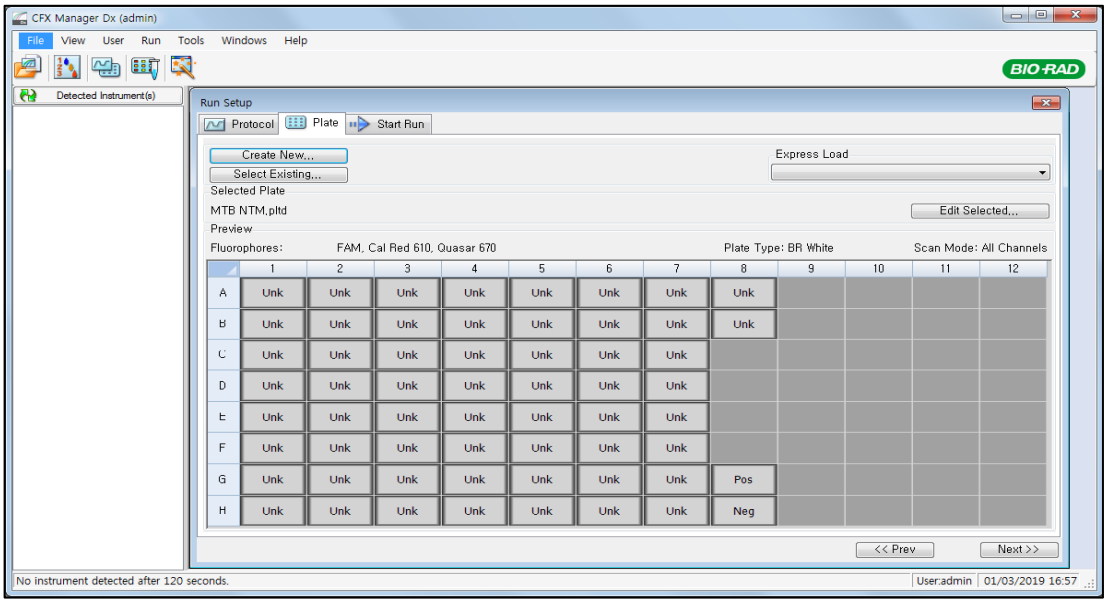


Fig. 7. **Run Setup: Plate**

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

C. Start Run

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

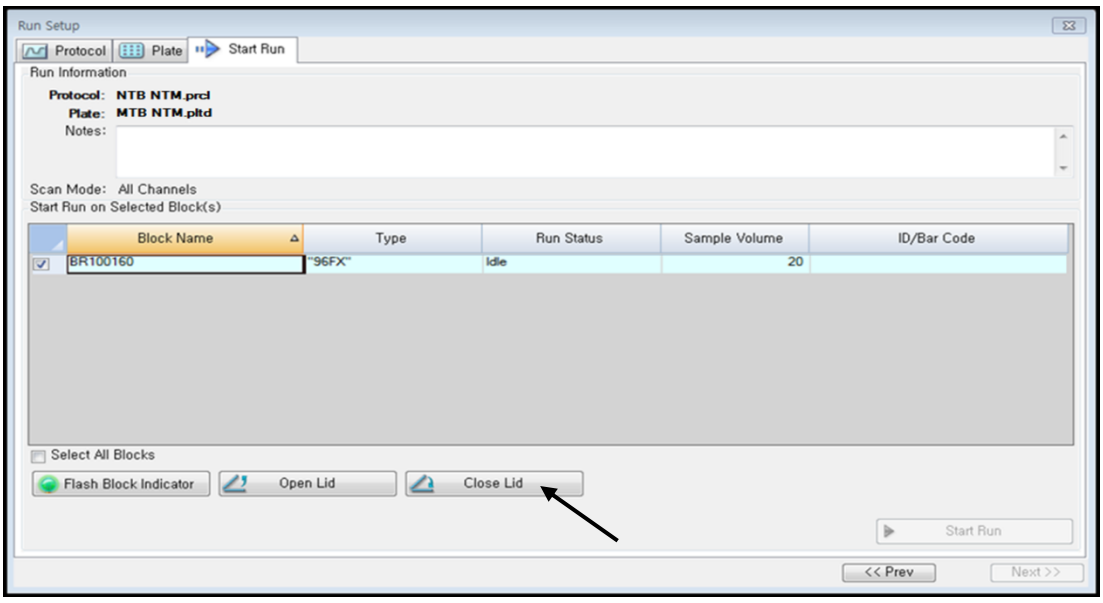


Fig. 8. **Close Lid**

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 then the run will start.

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

A. Pre-settings

Note: Check the setting of **Single Threshold** and **Baseline Subtracted Curve Fit**.

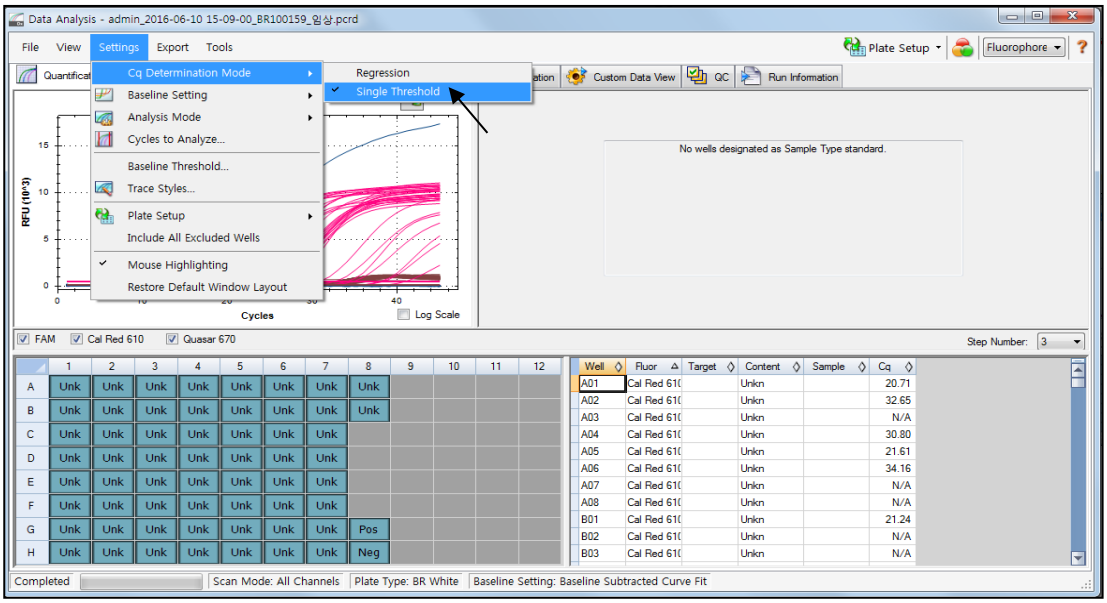


Fig. 9. **Single Threshold**

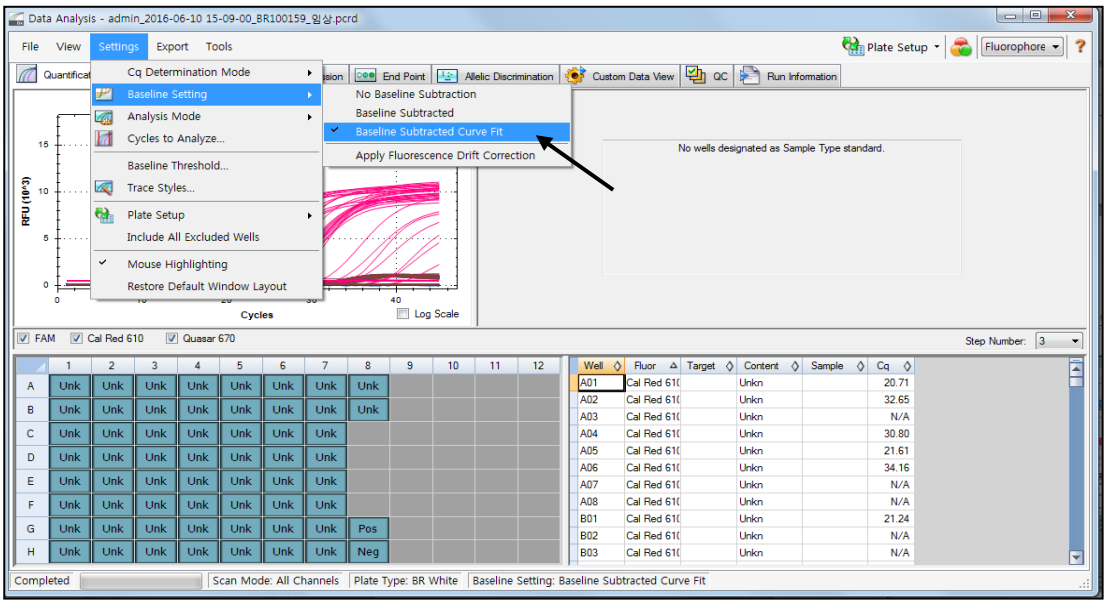


Fig. 10. **Baseline Subtracted Curve Fit**

B. Baseline Threshold

B-1. Baseline Threshold for Bio-Rad tube

Note: Set the Single Threshold of Baseline Threshold.

Note: For the setting of Baseline Threshold, you must select the **FAM**, **Cal Red 610**, and **Quasar 670** signal **separately**.

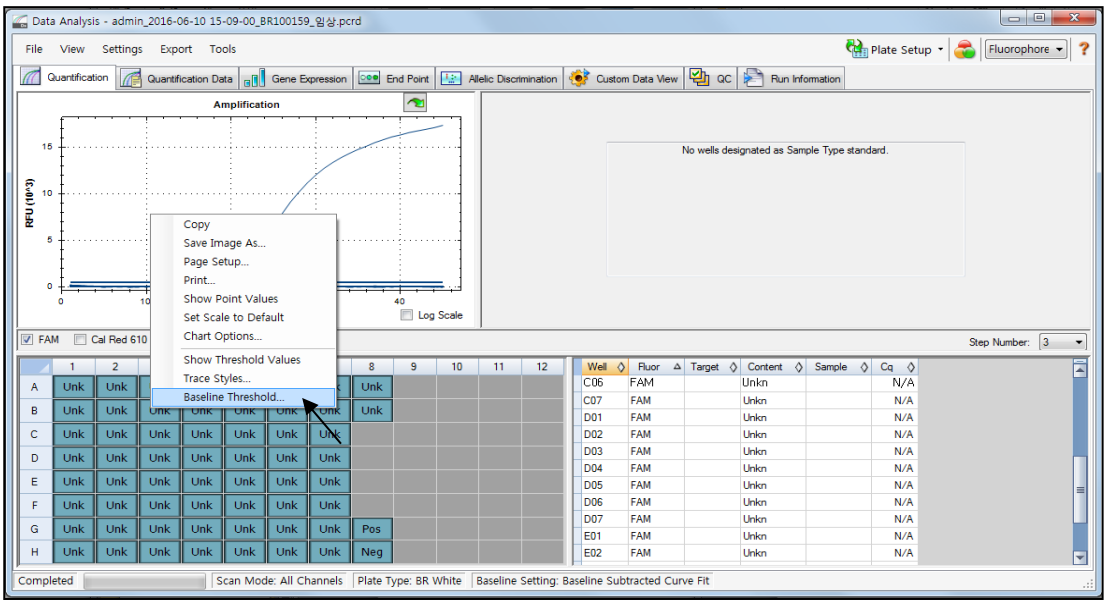


Fig. 11. **Checking of Baseline Threshold.** Click the right button of mouse on graph

Note: Set the **Baseline Cycles** as **Auto Calculated**.

Note: Set the Single Threshold value for each fluorophore as follows:

Analyte	Fluorophore	Threshold value
MTB	FAM	500
Mycobacteria	Cal Red 610	500
Internal Control	Quasar 670	60

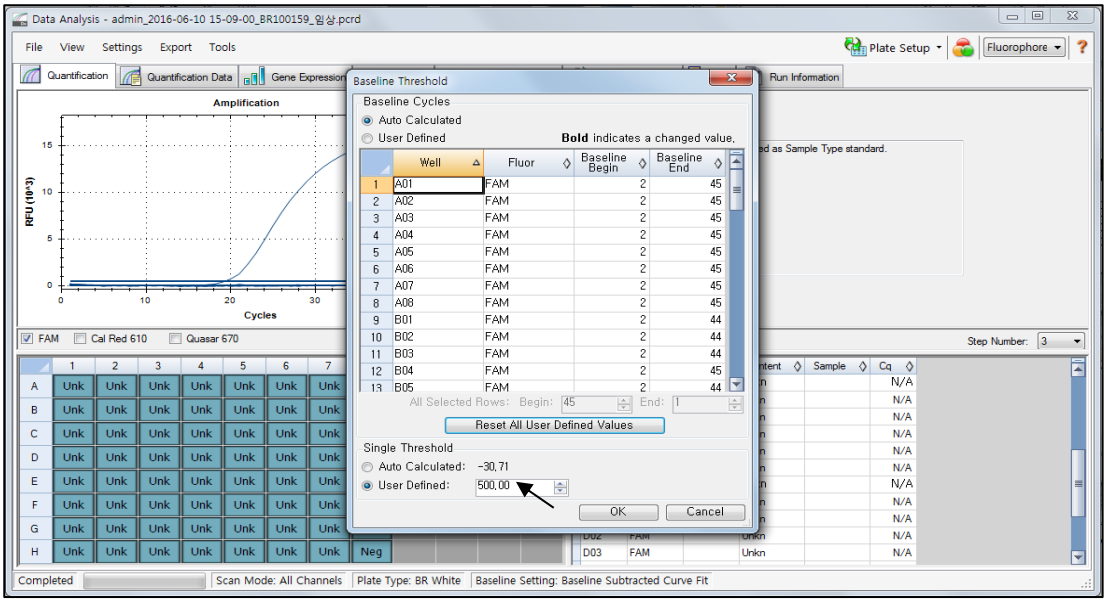


Fig. 12. **Setting of Single Threshold value**. Click User Defined button and change the Single Threshold value

B-2. Baseline Threshold for BIOplastics tube

Note: Set the Single Threshold of Baseline Threshold.

Note: For the setting of Baseline Threshold, you must select the **FAM**, **Cal Red 610**, and **Quasar 670** signal **separately**.

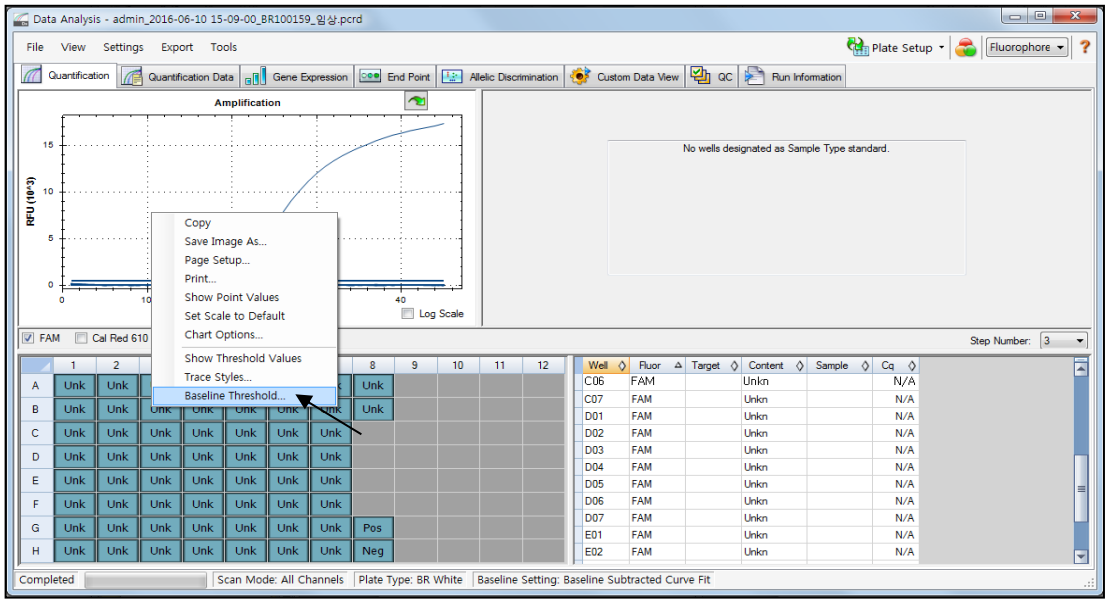


Fig. 13. **Checking of Baseline Threshold.** Click the right button of mouse on graph

Note: Set the Baseline Cycles as follows: **Begin: 3, End: 10**

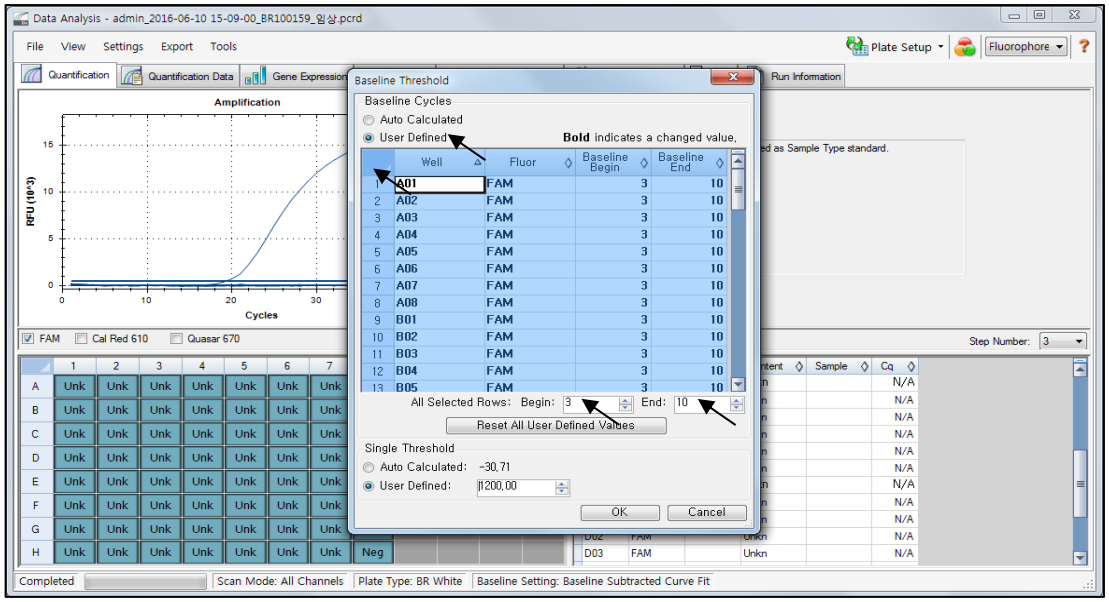


Fig. 14. **Setting of Baseline Cycles**. Click User Defined button and change the Baseline Cycles

Note: Set the Single Threshold value for each fluorophore as follows:

Analyte	Fluorophore	Threshold value
MTB	FAM	1200
Mycobacteria	Cal Red 610	1000
Internal Control	Quasar 670	100

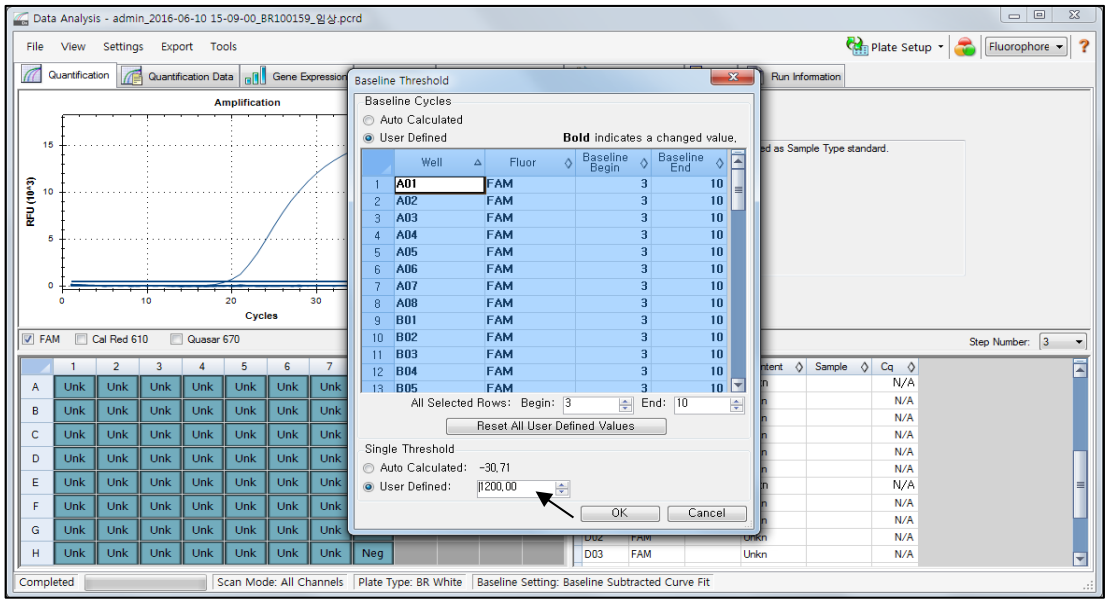


Fig. 15. **Setting of Single Threshold value**. Click User Defined button and change the Single Threshold value

3. 7500 Real-time PCR System, Software v2.0.5 (Life Technologies)

3.1. Real-time PCR Instrument set up

Note: Instructions provided for the 7500 Real-time PCR Instrument, **Software v2.0.5**.

Note: The 7500 Real-time PCR System (Life Technologies) experiment setup program for the detection of MTB, mycobacteria and Internal control can be divided into following steps: Experiment Properties Set Up, Plate Set Up, and Run Method Set Up

A. Experiment Properties Set Up

1) In the program home, select **Advanced Setup** icon.

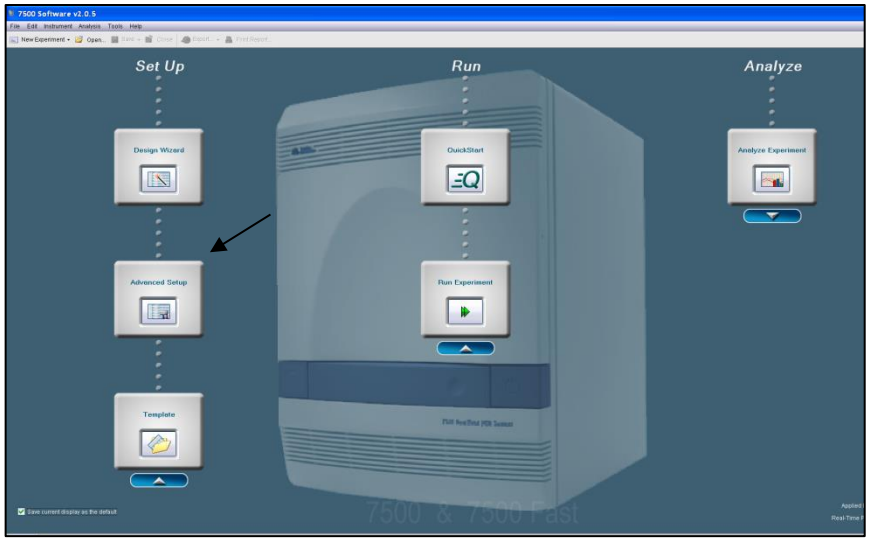


Fig. 1. **Create new document**

2) Set **Experiment Properties** of the new experiment in **Experiment Menu**.

- **Experiment Name** : Enter an experiment name.
- **Instrument Type** : 7500 (96 Wells)
- **Type of experiment to set up** : Quantitation - Standard Curve
- **Reagents to detection** : TaqMan® Reagents
- **Run Mode** : Standard

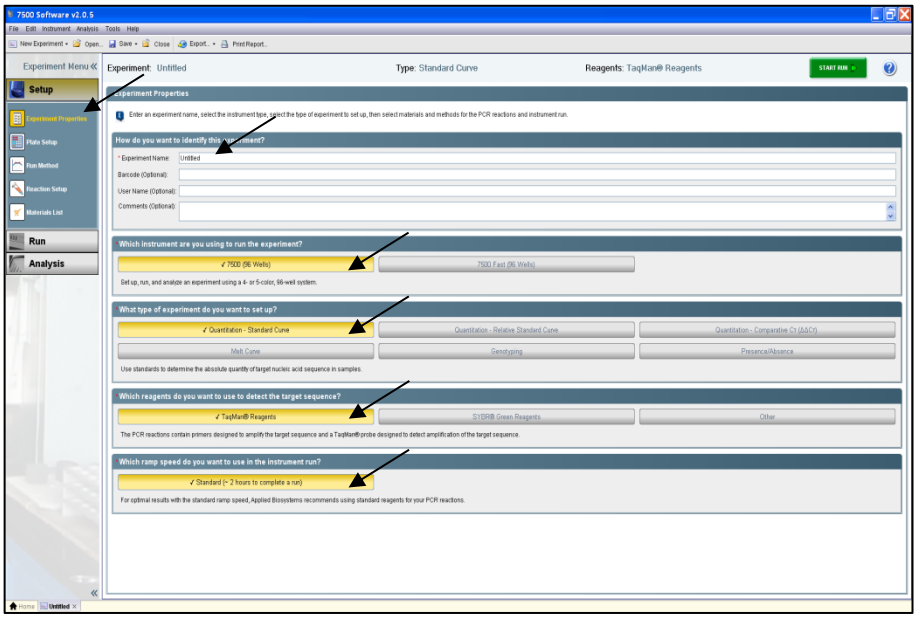


Fig. 2. **Set Properties of the New Experiment**

B. Plate Set Up

1) In **Experiment Menu**, click **Plate Setup**.
2) Define Targets: Click **Add New Target** as needed.
3) Enter an each **Target Name**.
4) Select **Reporter** and **Quencher** for the targets as follows:

Target Name	Reporter	Quencher
MTB	FAM	None
Mycobacteria	ROX	
Internal Control	CY5	

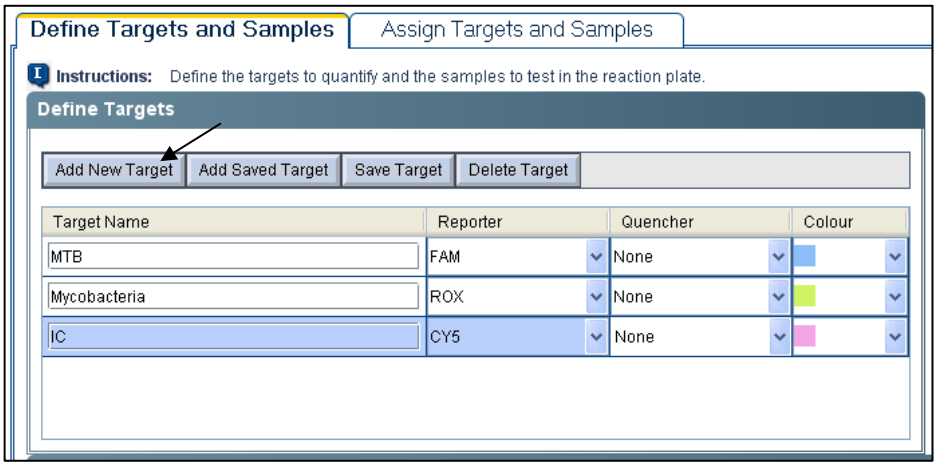


Fig. 3. **Select detector**

5) In **Assign Targets and Samples** tab of **Plate Setup**, select the **wells** you want to load.

6) Assign Targets to the selected wells.

7) Select the dye to use as the **passive reference** to '**None**'.

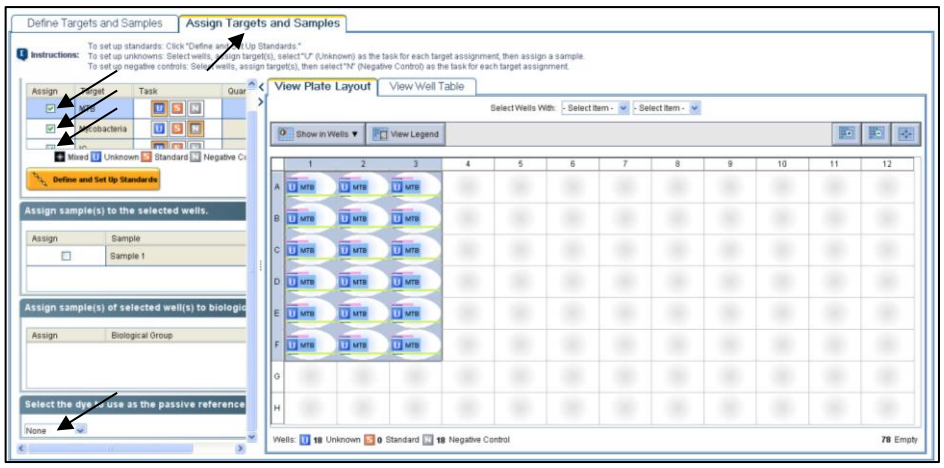


Fig. 4. **Assign Targets**

C. Run Method Set Up

1) In **Experiment Menu**, click **Run Method**.

2) Input a reaction volume: **20** (μL).

3) Set a thermal profile as follows:

	Step	No. of cycles	Temperature	Duration
Holding Stage	1	1	95°C	15 min
Cycling Stage	1	45	95°C	30 sec
	2		60°C	1 min

Note: Click **Data Collection On** at Step 2 of Cycling Stage. Reporter is detected at 60°C.

Note: Uncheck **Enable AutoDelta**.

4) Click **Start Run** button.

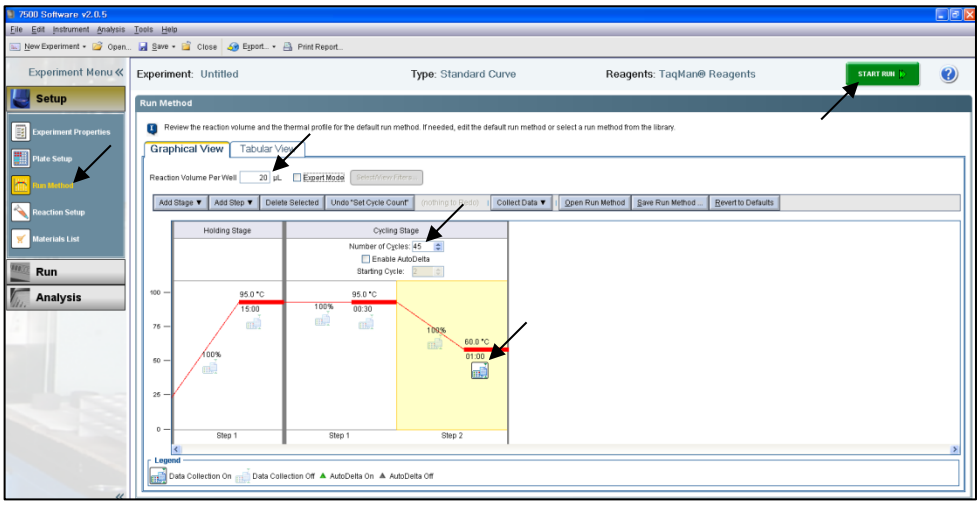


Fig. 5. **Set up Run Method**

3.2 Pre-settings for Data Analysis

A. Pre-settings

1) In **Analysis** of the **Experiment Menu**, click **Amplification Plot**.
2) Select **Plot Type** (ΔRn vs Cycle) and **Graph Type** (Linear) in **Plot Settings** tab.
3) Set the threshold value for each target in **Options** tab.
4) Refer to the threshold value for each target as follows:

Analyte	Reporter	Threshold Value
MTB	**FAM**	**5.0e+004**
Mycobacteria	**ROX**	**5.0e+004**
Internal Control	**CY5**	**5.0e+003**

Note: For the setting of threshold value, you must analyze the **FAM** signal, **ROX** signal, and **CY5** signal **separately**.

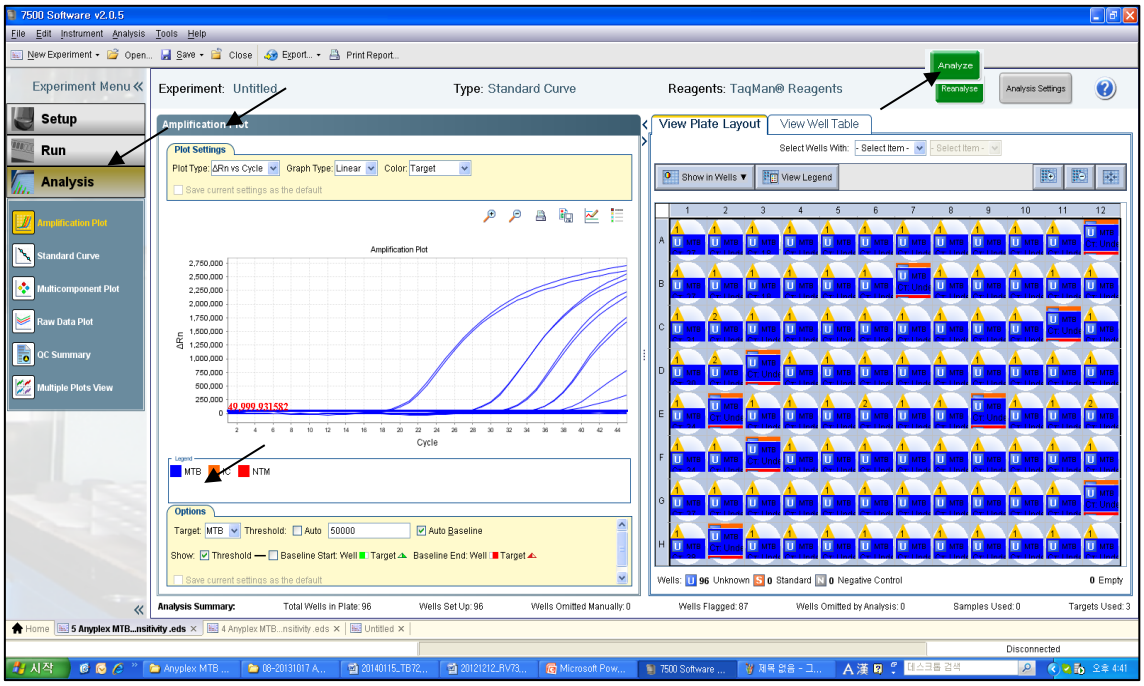


Fig. 6. **Analysis settings**

5) Select **Auto Baseline**.

6) Click **Analyze** button.

RESULTS

1. CFX96™ Real-time PCR Detection System (Bio-Rad) / CFX96™ Dx System (Bio-Rad)

1.1. Interpretation of Results

Analyte	Fluorophore	Sample / Negative Control		Positive Control	
		Ct value	Result	Ct value	Result
MTB	FAM	< 40	Positive **(+)**	< 25	Valid
		≥ 40 N/A	Negative **(-)**	≥ 25 N/A	Invalid
Mycobacteria	Cal Red 610	< 38	Positive **(+)**	< 25	Valid
		≥ 38 N/A	Negative **(-)**	≥ 25 N/A	Invalid
Internal Control	Quasar 670	< 35	Positive **(+)**	< 25	Valid
		≥ 35 N/A	Negative **(-)**	≥ 25 N/A	Invalid

	Result			Interpretation	
	Quasar 670 (IC)	FAM (MTB)	Cal Red 610 (Mycobacteria)		
Case 1	+	+	+	MTB : Detected Mycobacteria : Detected	MTB
				Ct value of Mycobacteria < Ct value of MTB	MTB&NTM co-infection
Case 2		+	-	MTB : Detected Mycobacteria : Not detected	MTB
Case 3		-	+	MTB : Not detected Mycobacteria : Detected	NTM
Case 4		-	-	MTB : Not detected Mycobacteria : Not detected	Negative
Case 5	-	+	+	MTB : Detected Mycobacteria : Detected	MTB*
				Ct value of Mycobacteria < Ct value of MTB	MTB&NTM co-infection*
Case 6		+	-	MTB : Detected Mycobacteria : Not detected	MTB*
Case 7		-	+	MTB : Not detected Mycobacteria : Detected	NTM*
Case 8		-	-	MTB : Not detected Mycobacteria : Not detected	Invalid **

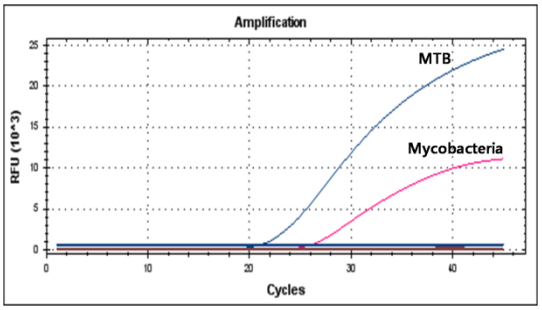
* PCR reaction has been inhibited. The result is still valid.

** For invalid specimen results, pathogen nucleic acid, if present, would not be detectable. Process another aliquot of the original specimen and repeat the test. Inhibitors are often labile and specimens initially inhibitory may not be inhibited when repeated. If the original specimen is not available, 10~100 fold dilution of the template nucleic acid in distilled water may improve the PCR result.

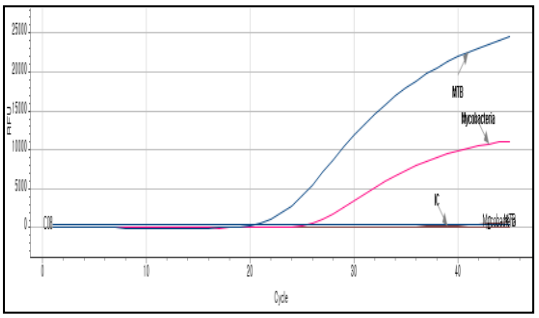
1.2. Application to Clinical Samples

Sample1

CFX96™



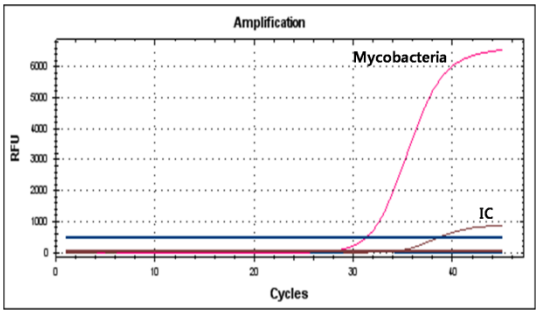
Seegene Viewer



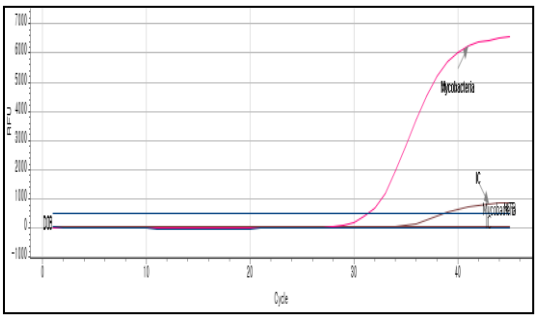
Fluorophore	Ct value	Result
FAM	20.81	MTB
Cal Red 610	25.84	
Quasar 670	33.60	

Sample2

CFX96™



Seegene Viewer



Fluorophore	Ct value	Result
FAM	N/A	NTM
Cal Red 610	31.33	
Quasar 670	34.38	

2. 7500 Real-time PCR System (Life Technologies)

2.1. Interpretation of Results

Analyte	Reporter	Sample / Negative Control		Positive Control	
		Ct value	Result	Ct value	Result
MTB	FAM	< 40	Positive **(+)**	< 25	Valid
		≥ 40 Undet.	Negative **(-)**	≥ 25 Undet.	Invalid
Mycobacteria	ROX	< 38	Positive **(+)**	< 25	Valid
		≥ 38 Undet.	Negative **(-)**	≥ 25 Undet.	Invalid
Internal Control	CY5	< 35	Positive **(+)**	< 25	Valid
		≥ 35 Undet.	Negative **(-)**	≥ 25 Undet.	Invalid

	Result			Interpretation	
	CY5 (IC)	FAM (MTB)	ROX (Mycobacteria)		
Case 1	+	+	+	MTB : Detected Mycobacteria : Detected	MTB
				Ct value of Mycobacteria < Ct value of MTB	MTB&NTM co-infection
Case 2		+	-	MTB : Detected Mycobacteria : Not detected	MTB
Case 3		-	+	MTB : Not detected Mycobacteria : Detected	NTM
Case 4		-	-	MTB : Not detected Mycobacteria : Not detected	Negative
Case 5	-	+	+	MTB : Detected Mycobacteria : Detected	MTB*
				Ct value of Mycobacteria < Ct value of MTB	MTB&NTM co-infection*
Case 6		+	-	MTB : Detected Mycobacteria : Not detected	MTB*
Case 7		-	+	MTB : Not detected Mycobacteria : Detected	NTM*
Case 8		-	-	MTB : Not detected Mycobacteria : Not detected	Invalid **

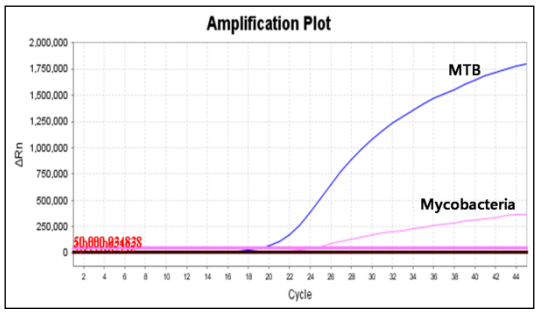
* PCR reaction has been inhibited. The result is still valid

** For invalid specimen results, pathogen nucleic acid, if present, would not be detectable. Process another aliquot of the original specimen and repeat the test. Inhibitors are often labile and specimens initially inhibitory may not be inhibited when repeated. If the original specimen is not available, 10~100 fold dilution of the template nucleic acid in distilled water may improve the PCR result.

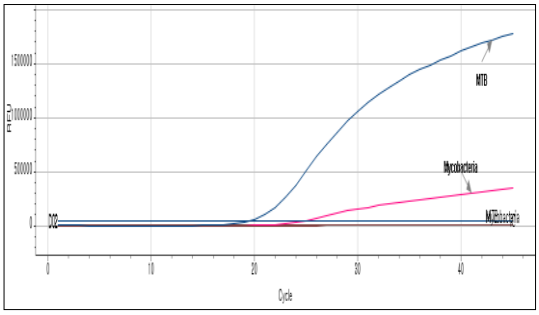
2.2. Application to Clinical Samples

Sample1

7500 Real-time PCR System



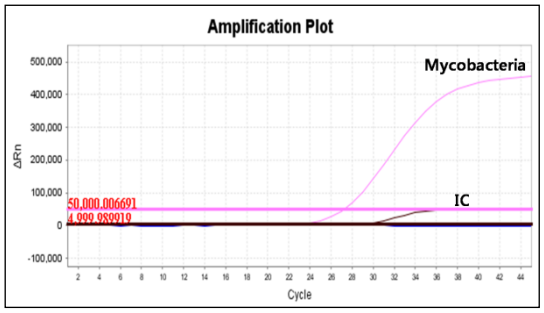
Seegene Viewer



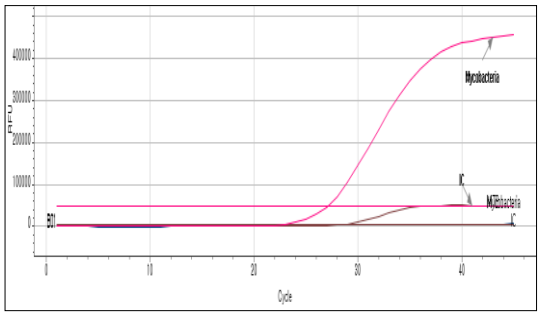
Fluorophore	Ct value	Result
FAM	19.66	MTB
ROX	25.14	
CY5	40.04	

Sample2

7500 Real-time PCR System



Seegene Viewer



Fluorophore	Ct value	Result
FAM	N/A	NTM
ROX	27.14	
CY5	28.54	

TROUBLESHOOTINGS

Anyplex™ MTB/NTM Real-time Detection (V2.0)		
OBSERVATION	**PROBABLE CAUSES**	**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 expiration date (refer to label) of the test kit, and use a new kit if possible.
No Internal Control signal	High load of specimen's nucleic acid	Please dilute (1/10~1/100) the template nucleic acid with RNase-free Water and repeat the test with the diluted nucleic acid.
	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.
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 and throughout the procedure and change tips between tubes. Repeat entire procedure from nucleic acid extraction with the new set of reagents.
Putative false negative or no signal observed in Positive Control	Error in specimen collection	Please check the specimen collection method, and re-collect the specimen.
	Incorrect storage of specimen	Please check the thermal cycling conditions and repeat the test under the correct settings.
	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.
	Incorrect PCR mixture	Confirm that all components are added to the PCR mixture. (Sensitivity is compromised with pre-composed premix,) All reagents must be homogenized and spun down before use.
	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.

PERFORMANCE

1. Specificity

The high specificity of Anyplex™ MTB/NTM Real-time Detection (V2.0) is ensured by the oligos designed specifically for the targets of interest under the set reaction conditions. Anyplex™ MTB/NTM Real-time Detection (V2.0) was tested for cross-reactivity to 49 different pathogens, and PCR amplification and detection was only identified in the specified targets.

Organism	Source No.	Test result†		
		IC	Analyte	
			MTB	Mycobacteria
Mycobacterium tuberculosis	Korean isolation	+	+	+
Mycobacterium abscessus	ATCC 19977D-5	+	-	+
Mycobacterium avium (Chester)	ATCC 700735	+	-	+
Mycobacterium asiaticum	KCTC 9503	+	-	+
Mycobacterium avium subsp. avium	ATCC 25291	+	-	+
Mycobacterium bovis BCG	Korean isolation	+	+	+
Mycobacterium chelonae	KCTC 9505	+	-	+
Mycobacterium fallax	KCTC 9508	+	-	+
Mycobacterium fortuitum	KCTC 1122	+	-	+
Mycobacterium inracellulare	KCTC 9514	+	-	+
Mycobacterium kansasii	KCTC 9515	+	-	+
Mycobacterium neoaurum	KCTC 19096	+	-	+
Mycobacterium paraseoulense	KCTC 19145	+	-	+
Mycobacterium phlei	KCTC 9087	+	-	+
Mycobacterium seoulense	KCTC 19146	+	-	+
Mycobacterium smegmatis	KCTC 9108	+	-	+
Mycobacterium szulgai	KCTC 9520	+	-	+
Mycobacterium terrae	KCTC 9614	+	-	+
Mycobacterium gordonae	KCTC 9513	+	-	+
Mycobacterium vaccae	KCTC 19087	+	-	+
Mycobacterium mucogenicum	KCTC 19088	+	-	+
Mycobacterium massiliense	KCTC 19086	+	-	+

Organism	Source No.	Test result†		
		IC	Analyte	
			MTB	Mycobacteria
Nocardia asteroides	KCTC 9956	+	-	-
Nocardia brasiliensis	KCTC 9136	+	-	-
Nocardia farcinica	KCTC 9958	+	-	-
Nocardia otitidiscaviarum	KCTC 9960	+	-	-
Corynebacterium aquaticum	KCTC 9098	+	-	-
Corynebacterium diphtheriae	KCTC 3075	+	-	-
Corynebacterium flavescens	KCTC 3414	+	-	-
Corynebacterium glutamicum	KCTC 1854	+	-	-
Rhodococcus equi	KCTC 1298	+	-	-
Gordonia bronchialis	KCTC 3076	+	-	-
Gordonia sputi	KCTC 3436	+	-	-
Gordonia rubripertincta	ATCC 27864	+	-	-
Streptococcus pneumoniae	KCTC 3932	+	-	-
Staphylococcus aureus	KCCM 40881	+	-	-
Haemophilus parainfluenzae	KCTC 5485	+	-	-
Bordetella pertussis	ATCC BAA-589D	+	-	-
Mycoplasma pneumoniae	ATCC 29342	+	-	-
Pseudomonas aeruginosa	KCTC 1636	+	-	-
Haemophilus ducreyi	ATCC 700724D-5	+	-	-
Candida albicans	ATCC 10231D-5	+	-	-
Rothia dentocariosa	KCTC 19319	+	-	-
Dietzia sp.	KCTC 19232	+	-	-
Arthrobacter oxydans	KCTC 3383	+	-	-
Enterococcus faecalis	KCTC 2011	+	-	-
Enterococcus faecium	KCTC 2022	+	-	-
Parvopolyspora pallida	KCTC 9188	+	-	-
Human gDNA	Biochain D1234275	+	-	-

† To prove reproducibility of the results, the experiment was repeated three times.

2. Sensitivity

In order to determine the sensitivity of Anyplex™ MTB/NTM Real-time Detection (V2.0), the test was performed with the genomic DNAs at the concentrations from 10^4 to 10^0 copies/reaction. Detection limit for MTB and Mycobacteria was 100 copies/reaction. However, in the case of MTB sample that has multiple copy number of IS6110, MTB target of Anyplex™ MTB/NTM Real-time Detection (V2.0) can be detected up to 10 copies/rxn.

3. Reproducibility

Reproducibility tests were carried out at 5 different time points in the course of 5 days by 3 different experimenters. The same results were obtained in every test, confirming the reproducibility of Anyplex™ MTB/NTM Real-time Detection (V2.0).

4. Interference

This test was conducted using interfering substances composed of 8 substances in order to confirm the performance of the Anyplex™ MTB/NTM Real-time Detection (V2.0) in the presence of potential interfering substances. There was no effect on the result by adding the substances: non-specific detection or inhibition on target amplification. Based on the results, 8 interfering substances had no effect on Anyplex™ MTB/NTM Real-time Detection (V2.0).

No.	Interfering substances	Concentration
1	Mucin (bovine submaxillary gland, type I-S)	60 µg/ml
2	Mupirocin (Antibiotic, nasal ointment)	6.6 mg/ml
3	Tobramycin (Antibacterial, systemic)	4.0 µg/ml
4	Oxymetazoline (Afrin Nasal Spray)	15%(v/v)
5	Zanamivir (Anti-viral drug-Relenza)	3.3 mg/ml
6	Oseltamivir (Anti-viral drug-Tamiflu)	25 mg/ml
7	Isoniazid	50 µg/ml
8	Rifampicin	25 µg/ml

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KEY TO SYMBOLS

Key to symbols used in the manual and labels

Symbol	Explanation
IVD	In vitro diagnostic medical device
LOT	Batch code
REF	Catalogue number
	Use-by date
	Upper limit of temperature
PRIMER	Oligonucleotide mix for amplification and detection
PREMIX	PCR Master Mix or Detection Mix
WATER	RNase-free Water
CONTROL +	Positive Control (PC)
DNA ES	DNA Extraction Solution
	Consult instructions for use
	Manufacturer
	Date of manufacture
EC REP	Authorized representative in the European Community
	Caution
	Contains sufficient for <n> tests
UDI	Unique Device Identifier
rxns	Reaction barcode for automated extraction system

ORDERING INFORMATION

Cat. No.	Product	Size
Anyplex™ TB series		
TB7200Y	Anyplex™ MTB/NTM Real-time Detection (V2.0)	50 rxns
TB7200X	**Anyplex™ MTB/NTM Real-time Detection (V2.0)**	**100 rxns***
TB7202Y	Anyplex™ MTB/NTMe Real-time Detection	50 rxns
TB7202X	Anyplex™ MTB/NTMe Real-time Detection	100 rxns*
TB7203Y	Anyplex™ MTB/NTM Combi	50 rxns
TB7203X	Anyplex™ MTB/NTM Combi	100 rxns*
* For use with Microlab NIMBUS IVD, Microlab STARlet IVD, Seegene NIMBUS and Seegene STARlet only		
Allplex™ TB series		
TB10173Y	Allplex™ MTB/MDR/XDRe Detection	50 rxns
TB10174X	Allplex™ MTB/MDR/XDRe Detection	100 rxns*
TB9400Y	Allplex™ MTB/MDRe Detection	50 rxns
TB9400X	Allplex™ MTB/MDRe Detection	100 rxns*
TB9500Y	Allplex™ MTB/XDRe Detection	50 rxns
TB9500X	Allplex™ MTB/XDRe Detection	100 rxns*
* For use with Microlab NIMBUS IVD, Microlab STARlet IVD, Seegene NIMBUS and Seegene STARlet only		
Anyplex™ II TB series		
TB7500Y	Anyplex™ II MTB/MDR/XDR Detection	50 rxns
TB7301Y	Anyplex™ II MTB/MDR Detection	50 rxns
TB7302Y	Anyplex™ II MTB/XDR Detection	50 rxns
Seeplex® TB series		
TB2110Y	Seeplex® MTB Nested ACE Detection (V2.3)	50 rxns

Automated extraction systems

65415-02	Microlab NIMBUS IVD	EA
173000-075	Microlab STARlet IVD	EA
65415-03	Seegene NIMBUS	EA
67930-03	Seegene STARlet	EA
744300.4.UC384	STARMag 96 X 4 Universal Cartridge Kit	384T / 1box