TB-MDR Multiplex PCR Detection Kit（Lyophilized）

Use Manual

Cat. No.: RH027T/RH028T

Label	Description	Label	Description
	Contains sufficient for <N> tests		In vitro diagnostic
	Batch number		Consult instruction before use
	Date of manufacturing		European Anthorized Representative
	Date by which the device should be used		Temperature limitation
	Name and address of Manufacturer		Reference Number

【PRODUCT NAME】

TB-MDR Multiplex PCR Detection Kit（Lyophilized）

【SIZE】

24test/kit(8-well strip version,RH028T); 50test/kit(bottle version, RH027T)

【INTENDED USE】

This product is intended for the qualitative detection of TB(Tuberculosis) and multi drug resistance (Rifampin-resistance and Isoniazid-resistance mutations) in Mycobacterium tuberculosis from cultured Mycobacterium tuberculosis specimens, Sputum, Pharyngeal swab, BALF or fresh tissue samples. The kit employs multiplex real-time PCR and melting curve analysis method that permits simultaneous amplification and detection of 18 mutations of rpoB gene (codons 507~533) to determine the resistance of Rifampin(RIF), 18 mutations of resistance of Isoniazid(INH) covering ahpC promoter region (positions -44~-30 and -15~4), inhA promoter region (positions -17~-8), katG codon 315, and Internal Control(IC).

18 Isoniazid (INH) resistance-causing mutations:

[katG S315I(ATC), S315N(AAC), S315T(ACC), S315T(ACA), S315R(AGA), S315R(AGG), inhA promoter -15(T), -8(A), -8(C); ahpC promoter region -39(T), -32G(A), -30(T),-15(T), -12(T), -10(T), -10(G), -10(A), -6(A)].

18 Rifampicin (RIF) resistance-causing mutations

[rpoB L511P(CCG), Q513K(AAA), Q513L(CTA), Q513P(CCA), 3 amino acid-deletion in 513~516, D516V(GTC), D516Y(TAC), S522L(TTG), S522Q(CAG), H526C(TGC), H526D(GAC), H526L(CTC), H526N(AAC), H526R(CGC), H526Y(TAC), S531L(TTG), S531W(TGG), L533P(CCG)].

【PRINCIPLE OF DETECTION】

This kit is based on TaqMan PCR and the Probe-based Melting Curve Analysis. The CY5 channel of TB-MDR Mix B is to detect the TB target gene. The melting curve for the hybridization product of probe and the mentioned sequence is achieved by calculating the derivative between fluorescence value measured and the temperature, then the melting point (Tm) is obtained and the mutation information of the sequences is concluded accordingly. Where, exact match of target

sequence and probe raise highest Tm; incomplete match, for instance, point mutation, insertion or deletion, would grow decreased Tm than that of exact match. The decreased degree is related to the type of point mutation, base number of insertion or deletion as well as the mutation location. The internal control is used in the kit for quality control from the beginning of sample collection to avoid false negative results.

【PRODUCT CONTENTS】

Component	Volume	Package		Ingredients
TB-MDR Mix A (Lyophilized)	15μl×24	3×0.2ml or 0.1ml 8 well-strip tube	Strip version	Contains primers and fluorescent probes, dNTPs, $MgCl_2^+$,Taq DNA polymerase
	750uL	1×bottle	Bottle version	
TB-MDR Mix B (Lyophilized)	15μl×24	3×0.2ml or 0.1ml 8 well-strip tube	Strip version	Contains primers and fluorescent probes, dNTPs, $MgCl_2^+$,Taq DNA polymerase
	750uL	1×bottle	Bottle version	
Dissolving solution	1200μl	1×1.5ml tube		/
Negative Control	200μl	1×1.5ml tube		0.9%NaCl
Positive Control (Lyophilized)	40μl×2	2×0.2ml tube (Lyophilized)		Plasmid containing genes of TB and wild type of all mutation sites and IC gene specific fragments

Note:

1. The components in the kits of different batch numbers are not interchangeable.
2. Prepare your own experimental consumables: tubes, micropipettes, etc. of applicable specifications.

【STORAGE & SHELF LIFE】

Storage condition： 2~30℃. And the kit is stable for 12 months. All the components including the Dissolving solution and Negative control can be also stored at room temperature(2~30℃). The remaining part of lyopilized PCR Mix and Positive control after dissolving should be stored at -20℃ and should be used within 3 months.

【INSTRUMENTS】

Real-time fluorescent PCR instrument with four fluorescent channels(FAM, HEX, ROX, CY5) and with function of melting curve analysis, such as Roche LC480/COBAS Z480, Bio-Rad CFX-96, SLAN96p and other real-time fluorescent PCR instruments.

【SAMPLING AND PRETREATMENT】

(1) Pharyngeal swab: Wipe bilateral pharyngeal tonsils and posterior pharyngeal wall at the same time with a plastic rod swab with polypropylene fiber head, immerse the swab head into a tube filled with normal saline or VTM solution(Cat No.:VTM001,manufactured by CHKBiotech), discard the tail, and tighten the tube cap. Vertex and use 200uL of the sample for nucleic acid extraction

(2) Sputum: After rinsing with clean water in the morning, inhale deeply and cough up the second sputum deep in the respiratory tract, and spit it in a clean sputum container. The sample shall not be less than 1ml, and mouthwash and food residues shall not be mixed in sputum.

Add same volume of 4% NaOH to the collected sputum sample and vertex shortly, digest it for 30min, take 1mL of the digested sample to a 1.5mL centrifuge tube and centrifuge at 13000rpm for 5 min, discard the supernatant, add 1mL saline or 1 × PBS solution into the tube, centrifuge at 13000rpm for 5 min, discard the supernatant, add 500uL saline or 1 × PBS solution into the tube,vertex and use 200uL of the sample for nucleic acid

extraction.

(3) Broncho-alveolar lavage fluid(BALF): Correctly collect broncho-alveolar lavage fluid. Take 1mL of the collected sample to a 1.5mL centrifuge tube and centrifuge at 13000rpm for 5 min, discard the supernatant, add 500uL saline or 1×PBS solution into the tube, vertex and use 200uL of the sample for nucleic acid extraction.

(4) Cultured Mycobacterium tuberculosis specimens: Collect culture with inoculating loop and then suspend in 500μL of normal saline or 1×PBS solution; for Mycobacterium tuberculosis cultured on liquid medium, collect 1mL culture and centrifuge at 13000rpm for 5 min, then suspend bacterial pellet in 500μL of normal saline or 1×PBS solution, vertex and use 200uL of the sample for nucleic acid extraction.

(5) Fresh tissue: Cut a fresh tissue specimen of 50 mg and grind it in a sterile vessel. Suspend in 500μL of saline or 1×PBS solution,vertex and use 200uL of the sample for nucleic acid extraction.

The sample can be used for testing immediately, or stored at 2-8°C for no more than 3 days, stored at -20°C for six months, and at -70°C for 2 years. The samples should be transported in cold chain box to avoid repeated freezing and thawing and in accordance with biosafety regulations.

【PROTOCOL】

1. DNA extraction of samples

Use the appropriate nucleic acid extraction kit and follow the extraction kit manual to extract nucleic acid from the specimen. Nucleic Acid Extraction Kit (Magnetic Beads)(Cat No.:EX004) of CHKBiotech is recommended.

After DNA extraction, the nucleic acid template should be added to the PCR reaction tube within 30 minutes, or store them at 2℃~8℃ temporarily(no more than 24hours). For longer storage should be at -20℃±5℃(no more than one month).

2. Reagent preparation

a) Lyophilized in bottle version kit:Add **750uL** dissolving solution to the bottle to dissolve the TB-MDR Mix A and TB-MDR Mix B. Divide **15uL** of the dissolved reagent into each PCR reaction tube.

b) Lyophilized 8-well strip version kit: Add **15uL** dissolving solution to each tube well to resolve the TB-MDR Mix A and TB-MDR Mix B.

c) Positive Control :Add **40uL** dissolving solution to one tube well of positive control to resolve it. Shock and centrifuge them at low speed.

The dissolved reagent and positive control can be temporarily stored at 4℃(no more than one hour) for later use.

*Notes:When using the lyophilized in bottle version kit, after dissolving the reagent can be stored at -20℃(no more than 3 months) and repeated freeze -thaw should be less than 4 times.

3. Template Addition

Add 5uL negative control, 5uL positive control, and **5uL** nucleic acid template of each treated specimen to each PCR reaction tube. Vertex and centrifuge at low speed. Then, place them into the real-time PCR machine.

4. PCR Amplification

Recommended program setup

	steps	temperature(℃)	time	cycles
1	UNG function	50	2min	1
2	Pre-degeneration	95	2min	1
3	Degeneration	95	5s	5
	Annealing and extension	70	20s	
4	Degeneration	95	5s	45

	Annealing	67	10s	
	Extension	76	20s	
5	Melting curve program	95	10s	1
		40	1min	
		90	40~90℃ Constantly collect the fluorescence signal	

- Note: *1 During amplification steps choose to collect FAM, HEX, ROX and CY5 channel fluorescence in the step of "67℃"; During the melting curve step choose to constantly collect fluorescence signal for all channels.

  *2 Different PCR instrument may have different melting curve setting parameters, normally the increment for temperature increasing during melting curve step can be set around 0.03~0.5℃. For Bio-Rad CFX-96 the increment can be set as 0.5℃, for SLAN96P it can be set at 0.04℃.

5. Quality control

Negative and positive controls provide quality control should be tested for each run of test. The results are valid if all of the following conditions are met. Otherwise, the test is invalid. In this case, the instrument, reagent, amplification conditions, etc. should be checked for errors and the experiment should be repeated.

1) For SLAN96P instrument：

Product quality control	Quality control requirements			
	FAM channel	HEX channel	ROX channel	CY5 channel
TB-MDR Mix A	72.4℃±1.5℃	Ct≤35 (IC)	71.5℃±1.5℃	71.8℃±1.5℃
TB-MDR Mix B	72.1℃±1.5℃	75.7℃±1.5℃	76.3℃±1.5℃	Ct≤35 (TB)

2）For CFX-96 instrument：

Product quality control	Quality control requirements			
	FAM channel	HEX channel	ROX channel	CY5 channel
TB-MDR Mix A	71.0℃±1.5℃	Ct≤35 (IC)	71℃±1.5℃	72.5℃±1.5℃
TB-MDR Mix B	72℃±1.5℃	75.5℃±1.5℃	76℃±1.5℃	Ct≤35 (TB)

6. Result Analysis

6.1 TB Result Analysis:

Whether a specimen is TB positive or not is determined by the Ct value of CY5 channel in TB-MDR Mix B reaction. The cut-off values of TB positive is Ct≤38.

Channel		Interpretation of result
CY5 Channel of TB-MDR Mix B (TB)	HEX Channel of TB-MDR Mix A (IC)	
Ct≤38	Ct≤35	TB Positive
Undet or Ct>38	Ct≤35	TB Negative
Any	Undet or Ct>35	Invalid, Re-sampling an test again

6.2 MDR Result Analysis:

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

a. For each run, the Tm value of positive control of all channels should be within the range of reference values according quality control requirement; there should be no amplification signal observed for negative control and this indicates that there is no contamination during DNA extraction or detection.

b. All melting curve peaks in all 6 channels (FAM, ROX, CY5 channels of TB-MDR Mix A and FAM, HEX, ROX channels of TB-MDR Mix B) must be searched within the suggested peak range of Tm, and

the mutant peak should be obviously different with the curve of positive control and negative control.

Tube	Channel	Suggested searching peak range
TB-MDR Mix A	FAM (RIF)	58-75℃
	ROX (INH)	64-74℃
	CY5 (INH)	63-75℃
TB-MDR Mix B	FAM (RIF)	58-75℃
	HEX (INH)	65-78℃
	ROX (RIF)	66-78℃

c. 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℃

6.3 Result interpretation:

-**Wild-type (sensitive to RIF and INH)**: all peaks in all 6 channels(FAM, ROX, CY5 channels of TB-MDR Mix A and FAM, HEX, ROX channels of TB-MDR Mix B) are wild peaks;

- **RIF mutant type (resistant to RIF)**: at least one mutant peak appears in FAM channel of TB-MDR Mix A or FAM, ROX channels of TB-MDR Mix B;

- **INH mutant type (resistant to INH)**: at least one mutant peak appears in ROX, CY5 channels of TB-MDR Mix A or HEX channel of TB-MDR Mix B;

- **MDR type (resistant to both RIF and INH)**: both characteristic mutant peaks raise for RIF and INH resistant;

- **Not detected**: For a low concentration MTB positive sample, if the Ct value of TB(CY5 channel of Mix B) is >30, there is possibility that both the wild peaks and the mutant peaks of RIF do not appear, or both the wild peaks and the mutant peaks of INH do not appear because of the low concentration. Then it should be reported as "**RIF not detected**" or "**INH not detected**".

- **Invalid result**: For a MTB positive sample with the Ct value of TB(CY5 channel of Mix B)≤30, if both the wild peaks and the mutant peaks of RIF do not appear, or both the wild peaks and the mutant peaks of INH do not appear, the result is invalid and re-sampling and re-testing are required.

The possible reasons are: (1) improper sampling; (2) lower loading amount than LOD of the kit; (3) misoperation of extaction or PCR test process; (4) invalid kit used.

	Channel	Interpretation of results (Wild type)		Interpretation of results (Mutant type)	
TB-MDR Mix A	FAM	△Tm<1.5℃	all 6 channels △Tm<1.5℃ indicate sensitive to RIF and INH	△Tm≥1.5℃	△Tm ≥1.5℃ indicate resistant to RIF
	ROX	△Tm<1.5℃		△Tm≥1.5℃	△Tm ≥1.5℃ indicate resistant to INH
	CY5	△Tm<1.5℃		△Tm≥1.5℃	△Tm ≥1.5℃ indicate resistant to INH
TB-MDR Mix B	FAM	△Tm<1.5℃		△Tm≥1.5℃	△Tm ≥1.5℃ indicate resistant to RIF
	HEX	△Tm<1.5℃		△Tm≥1.5℃	△Tm ≥1.5℃ indicate resistant to INH
	ROX	△Tm<1.5℃		△Tm≥1.5℃	△Tm ≥1.5℃ indicate resistant to RIF
TB-MDR Mix A	HEX	Ct≤35	Internal control is valid[*1]	Ct≤35	Internal control is valid[*1]

*1 If the internal control (HEX channel of TB-MDR Mix A) Ct is Undet or Ct>35, the test result is invalid, and re-sampling and re-testing are required.

【CUT-OFF VALUE OR REFERENCE INTERVAL】

The judgment Cut-off value for positive TB is Ct≤38(CY5 of TB-MDR Mix B).
The judgment Cut-off value for positive mutation types(RIF or INH resistance) are all △Tm≥1.5℃.

【WARNINGS】

1. Contamination of the laboratory environment and reagents, or cross-contamination during specimen processing may lead to false positive results.
2. When reagents are transported, stored, or manipulated incorrectly, they may even lead to false negative results, resulting in a decrease in the detection effect.

【ASSAY LIMITATIONS】

1. The positive result of the test kit indicate whether there is mutation of nucleic acid sequences rather than amino acid sequences, and there is still rare possibility that silent mutations(wild type) may be reported as mutant type theoretically.
2. For the heterozygous specimens, if the mutant ratio is lower than a certain degree, the melting curve will be consistent with the positive control (wild type). That may lead to false negative results.
3. Although the target sequences detected by this kit are the conserved gene regions of Mycobacterium tuberculosis(IS6110 gene), theoretically, the missed detection of TB with rare mutations in the conserved regions can not be completely avoided.
4. The kit only detects isoniazid resistance caused by rpoB (codons 507~533), ahpC promoter region (positions -44~-30 and -15~4), inhA promoter region (positions -17~-8), and katG (codon 315). Rifampin and isoniazid resistance caused by other genetic alleles or other mechanism could not be reported by this kit.

【PERFORMANCE SPECIFICATIONS】

Limit of detection(LOD):500 copies / mL or 5 copies/reaction.
Precision: Within the scope of the test kit, the precision reference product were repeated 10 times, and the test results were consistent.
Cross-reactivity: No cross-reactivity with other related pathogens including Influenza A virus (H1N1, H3N2, Flu-A), Influenza B virus, Streptococcus pneumoniae, Mycoplasma pneumoniae, Escherichia coli, Candida albicans, Hepatitis B virus , parainfluenza virus (I, II and III) and Metapneumovirus.
Sensitivity/Specificity: For clinical sensitivity and specificity evaluation, 50 Pharyngeal swab samples, 55 Sputum samples, 32 Broncho-alveolar lavage fluid samples, 20 Cultured Mycobacterium tuberculosis samples and 10 fresh tissue samples were tested.

Table 1. Sensitivity/Specificity/Total consistent rate

Test reagent	Control reagent		Total
	Positive	Negative	
Positive	134	3	137
Negative	1	29	30
Total	135	32	167
Sensitivity		99.26%	
Specificity		90.6%	
Total consistent rate		97.6%	

【ATTENTIONS】

1) This kit is only used for in vitro auxiliary diagnosis; the clinical diagnosis and treatment of patients should be comprehensively considered in conjunction with their symptoms/signs, medical history, other laboratory tests and treatment responses;
2) Please read the instructions of this kit carefully before the experiment, and strictly follow the operation steps;
3) Unreasonable sample collection, transfer, storage and processing procedures may lead to erroneous test results; if the sample processing process is not controlled, cross-contamination may occur, and false positive results may occur;
4) Steps such as sample processing must be carried out in a biological safety cabinet or other protective facilities.

5) Laboratory personnel must undergo professional training. The experiment process should be carried out in different areas (reagent preparation area, sample preparation area, amplification and product analysis area), and dedicated instruments and equipment should be used in each stage of the experimental operation, and supplies in each area and stage must not be used interchangeably; personnel flow and air flow in each area There should be strict requirements to avoid cross-contamination to the greatest extent.

【General Information】

Manufacturer: Shanghai Chuangkun Biotech Inc.

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【European Anthorized Representative】

EC REP Name: MedUnion S.L.

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【Date of Approval and Revision of Instructions】 2023-12-8 V5.2