RAS Lifesciences Pvt Ltd
Striving for better health

Amplisure® MTB PCR Kit

(MTB QUALITATIVE PCR Kit)

IVD

Product Insert

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INDEX **Page No.**

Introduction	4
Product Description	4
Recommended Work Areas	4
General Precautions	4
Precautions while extracting nucleic acid	4
Precautions while setting up a PCR reaction	5
Precautions for post PCR or equipment area/room	5
Precautions after completion of PCR assay	5
Usage Limitations	6
Safety Precautions	6
Storage Conditions and Product Stability	6
Symbols	7
Kit Components	8
Materials required but not supplied	8
Quality Systems	9
Sample Type/Collection/Storage/Transport	9
Sample Type	9
Sample Collection, Storage and Transport	9
Assay Procedure	**10**
DNA Extraction	10
Use of Internal Control (IC)	10
Nested PCR Protocol	10
Reaction Composition for First Cycle	10
Cycling conditions for first cycle	11
Reaction Composition for Second Cycle	11
Cycling conditions for Second cycle	12
Interpretation	12
Troubleshoot	13

Assay Characteristics **13**
Analytical Sensitivity 13
Analytical Specificity 14
Precision 15

Abbreviations **16**

References **16**

Ordering Information **16**

Introduction

Mycobacterium tuberculosis (MTB) is a major cause of tuberculosis. Tuberculosis is a disease with serious mortality rate worldwide. Identification of MTB for the diagnosis of tuberculosis is largely based on conventional approaches, which rely on acid-fast bacilli (AFB) staining and culture test. AFB staining is comfortable and rapid, but lacks sensitivity. Culture method is sensitive and specific, but is slow and extremely time consuming. The molecular methods, especially polymerase chain reaction (PCR) technique are the most promising. Amplisure® MTB PCR kit which is based on Nested PCR has increased specificity and sensitivity.

Product Description

Amplisure® MTB PCR is an *in-vitro* nucleic acid amplification test for the qualitative detection of Mycobacterium tuberculosis (MTB) complex (*Mycobacterium africanum, Mycobacterium bovis, Mycobacterium canettii, Mycobacterium microti, Mycobacterium tuberculosis*) in human clinical specimens. The kit contains all the reagents necessary for performing qualitative MTB Nested PCR. Pathogen detection by Polymerase chain reaction (PCR) is based on the amplification of specific region (rpoB region of MTB complex) of the pathogen genome. The assay principle is based on Nested PCR which allows higher specificity and sensitivity.

Recommended Work areas

Molecular Diagnostics work area includes:

a) Sample preparation area/room – for extraction of nucleic acids from clinical samples
b) Pre-PCR area/room - for setting up PCR reaction
c) PCR area/room – for performing PCR using the thermocyclers

As part of Good Laboratory Practices (GLP), it is recommended to have dedicated areas to avoid cross contamination and carry over contamination.

General Precautions

Precautions while extracting Nucleic acid

Always wear proper attire (powder free gloves, facemask and Head cap) before starting the nucleic acid extraction procedure. During preparation of samples, compliance with good laboratory practices are essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of Deoxyribonucleases (DNases) into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with DNA.

The Sample Preparation Area is dedicated to processing samples. All reagents used in the Sample Preparation Area should remain in this dedicated area at all times. Laboratory coats, pipettes, pipette tips and vortex mixer used in the Sample Preparation Area must remain in this area and not be moved to the Pre-PCR/PCR area. Discard the gloves before leaving this area. Do not bring amplified product into the Sample Preparation Area. Usage of filter tips is recommended while sample preparation and should be performed in a Biosafety cabinet.

Precautions while setting up a PCR reaction

PCR assay is sensitive and any accidental introduction of product from previous amplification reactions leads to incorrect results. Hence measures to reduce the risk of contamination in the laboratory should include physically separating the activities involved in performing PCR and complying with good laboratory practices.

It is recommended to have proper cleaning procedures to minimize the risk of cross contamination and carry over contamination (e.g. DNA OUT™, RNase OUT™, 0.1% Sodium Hypochlorite, Fumigation etc.).

It is recommended that areas should be defined in Pre-PCR room for preparation of mastermix and addition of templates. Laboratory coats and equipment used in the Pre-PCR Area must remain in this area and should not be moved to the Sample Preparation Area.

Precautions for post PCR or equipment area/room

The PCR instrument/s should be kept in a separate segregated area away from Sample preparation area and Pre-PCR area.

Precautions after completion of PCR assay

The reaction tubes should be properly discarded after the Agarose Gel Electrophoresis to avoid carry over contamination.

Usage Limitations

1. The kit and all its components are for *in-vitro* diagnostics only.
2. The product is to be used by personnel specially trained in the *in-vitro* diagnostics procedures only.
3. Follow the product insert strictly for optimal PCR results.
4. Do not use the kit beyond the expiry date mentioned on the kit box.
5. Follow the guidelines provided in product insert for sample collection, storage and transport.
6. For ideal performance, store the kit under recommended conditions only.

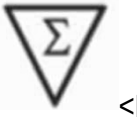
Safety Precautions

1. All patient specimens should be considered as potentially infectious and handled in a BSL2 biosafety hood with BSL3 practices.
2. Wear personal protective equipment, including gloves, head cap, face mask and lab coats when handling kit reagents/sample extraction. Wash hands thoroughly using detergents before and after performing the test.
3. Do not smoke, drink or eat in areas where kit reagents and/or human specimens are being used.
4. Dispose of unused kit reagents and human specimens as per regulatory guidelines.

Storage Conditions and Product Stability

1. All the kit reagents should be stored at -20°C. Replace all the kit components immediately at -20°C after usage.
2. Repeated thawing and freezing (more than 6 times) of all kit reagents should be avoided, as it reduces assay sensitivity. If needed, make aliquots of the kit reagents according to the volume used in the protocol prior to freezing.
3. Allow reagents to be thawed completely on Ice/4°C prior to use.
4. Kit reagents are stable through the end of the expiration date indicated on the box when stored at -20°C.

Symbols

Description of Symbol	Denotation
	*in-vitro* Diagnostic medical device
	Consult Instruction manual (Product Insert) for use
	Lot Number of the kit or components
	Catalogue number
 <N>	Contains sufficient for <N> reactions (Pack Size)
	Manufacturer
	Temperature limitation (Storage Condition)
	Use by MMM-YYYY (Expiry Date)
	Biological risk (handle carefully)
	Important Note

Kit Contents

Cap Color	Contents	Description	50 Rxns (EP-MTB-50)	100 Rxns (EP-MTB-100)
Yellow	RAS PCR Mix	DNA Amplification Reagent	1 x 500 µL	2 x 500 µL
Green	MTB PM1	MTB Primer mix 1	1 x 50 µL	2 x 50 µL
Green	MTB PM2	MTB Primer mix 2	1 x 100 µL	2 x 100 µL
Lavender	MTB PC	MTB Positive Control	1 x 60 µL	2 x 60 µL
White	MBGW	Molecular Biology Grade water	1 x 1.0 ML	2 x 1.0 ML

Materials required but not supplied

The materials which are required but not supplied are listed below:

1. DNA Extraction kit
2. Biosafety Cabinet
3. PCR Hood
4. Calibrated variable micropipettes
5. Sterile pipette filter tips (aerosol free)
6. Vortex mixer
7. Dry Bath
8. Benchtop centrifuge with rotor for 1.5 mL reaction tubes
9. Thermocycler
10. Strip Tubes and Caps (0.2 mL) or PCR Tubes (0.2 mL) or 96 well plate
11. Cooling block (96 x 0.2 mL tubes)
12. 1.5 mL centrifuge tubes
13. 1.5 mL centrifuge tube stand
14. Cooling block (2 mL tubes)
15. Sterile powder free gloves
16. Facemask
17. Head cap
18. Laboratory Coat

Quality Systems

In accordance with ISO-certified Quality Management System (9001:2008 and 13485:2003) of RAS Lifesciences, each batch and components of Amplisure® MTB PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Sample Type/Collection/Storage/Transport

Sample Type

Body fluids (CSF, Pleural Fluid, Ascitic Fluid and Synovial fluid) Sputum, Pus, Menstrual fluid, Urine and Tissue.
Heparinized Samples must not be used as they inhibit the PCR reaction

Sample Collection, Storage and Transport

Please follow the guidelines mentioned below for collection, storage and transport of different types of samples. The yield and quality of extracted DNA would vary based on the quality of sample received and if the following conditions are not followed.

Sl. No.	Sample Type	Collection Requirement	Transport	Storage/Processing
1.	Body Fluids	1 ml of Body fluids in a sterile container	In a leak proof box containing frozen cool packs (4^0C)	Store the sample at 4^0C /Sample to be processed for DNA extraction within 24 hours
2.	Tissue	10-20 mg of tissue in a sterile container containing 1X PBS	In a leak proof box containing frozen cool packs (4^0C)	Store the sample at 4^0C /Sample to be processed for DNA extraction within 24 hours
3.	Sputum, Pus, Menstrual fluid and Urine	1 ml of Sample in a sterile container	In a leak proof box containing frozen cool packs (4^0C)	Store the sample at 4^0C /Sample to be processed for DNA extraction within 24 hours

Assay Procedure

DNA EXTRACTION

Amplisure® MTB PCR Kit has been validated using RAS DNA Extraction Kit (Cat. No. R-DXT).

Follow the manufacturer's instructions mentioned in the manual for DNA extraction. Different pack sizes of the above mentioned kits can be used. However the customer can validate their own DNA extraction systems.

USE OF INTERNAL CONTROL (IC)

In addition, the Amplisure® MTB PCR Kit contains a second amplification system to identify possible PCR inhibition by using an endogenous Internal control (IC). This allows the user to check for possible PCR inhibition.

NESTED PCR PROTOCOL

Qualitative PCR procedure with Amplisure® MTB PCR Kit involves the setting up of nested PCR which involves two PCR cycling steps.

1. The components for first cycle (described in Table below) are taken from the Amplisure® MTB PCR Kit and a master mix is prepared as described below.
2. Label the tubes properly.
3. For proper interpretation of results a positive control and a negative control is necessary to be included in the run.
4. After addition of all the first cycle components the tubes are placed in the thermocycler with the following Thermal profile:

Reaction Composition for First Cycle

COMPONENTS	VOLUME (µL) (FOR FINAL VOLUME OF 25 (µL)
RAS PCR MIX	5.0
MTB PM1	1.0
Extracted DNA/MTBPC/MBGW	5.0
MBGW	14.0
TOTAL	25.0

Cycling conditions for first cycle

No. of cycles	Temperature (°C)	Time
1	95	5 min.
30	94	1 min
	72	40 sec
1	72	5 min
1	4	hold

1. After the completion of first cycle, the second cycle components from the Amplisure® MTB Qualitative PCR Kit are taken and a master mix is prepared.
2. The reaction master mix is aliquoted into PCR tubes and the kit components are replaced back into the kit and placed in -20 ^{0}C so as to avoid contamination.
3. Add the template from the 1st round PCR tubes.
4. Label the tubes properly.
5. After addition of all the second cycle components the tubes are placed in the thermo cycler with the following Thermal profile:

Use the DNA template obtained from the first cycle as a template in the second cycle. The first cycle amplified product acts as a template for the second cycle reaction hence care should be taken that the first cycle products are opened only after the second cycle reaction master mix is made.

Reaction Composition for second cycle

COMPONENTS	VOLUME(µL) (FOR FINAL VOLUME OF 25(µL)
RAS PCR MIX	5.0
MTB PM2	2.0
DNA TEMPLATE FROM FIRST CYCLE	1.0
MBGW	17.0
TOTAL	25.0

Cycling conditions for second Cycle

No. of cycles	Temperature (°C)	Time
1	95	5 min.
30	94	40 sec
	62	1 min
	72	40 sec
1	72	5 min
1	4	Hold

Gel Electrophoresis

The PCR products of 2nd cycle are resolved on 2% Agarose gel in 1X TAE or 1X TBE buffer along with 50 bp DNA ladder for size determination and result interpretation.

Interpretation

RESULT	INTERPRETATION	Conclusion
MTB complex specific amplification, 157bp : Present Internal Control specific amplification, 350bp : Present	*Sample is positive for MTB complex*	Proceed for further Analysis
MTB complex specific amplification, 157bp : Present Internal Control specific amplification, 350bp : Absent	*Sample is positive for MTB complex*	
MTB complex specific amplification, 157bp : Absent Internal Control specific amplification, 350bp : Present	*Sample is negative for MTB complex*	
MTB complex specific amplification, 157bp : Absent Internal Control specific amplification, 350bp : Absent	*Sample may have inhibitors*	Dilute sample 1:20 and Repeat the reaction

Troubleshoot

Observation	Probable Cause(s)	Solution(s)
No amplification product seen	Some reaction component missing	Repeat reaction setup
	Target sequence not present in template DNA	Try other sources of template DNA (positive control)
	Inhibitory substance in reaction	Decrease sample volume/ Dilute the sample /Repeat the DNA extraction procedure
	Inconsistent block temperature	Test calibration of heating block of thermocycler
	Reaction components or solutions contaminated	Use a fresh aliquot of reagents if available
Multiple or nonspecific products	Premature *Taq* DNA Polymerase replication	Set up reactions on ice with chilled components. Add samples to pre-heated (95°C) thermocycler
	Insufficient mixing of reaction mix	Reaction components must be thoroughly mixed.

Assay Characteristics

Analytical Sensitivity

To determine the analytical sensitivity for Amplisure® MTB PCR Kit, plasmid DNA control and positive genomic DNA samples ranging from 15 copies/reaction to 1 copy/reaction were tested using the Amplisure® MTB PCR Kit as described in the table below.

The different copy number templates were generated by serial dilution of the positive genomic DNA samples and plasmid DNA control. The testing was performed in triplicates for 5 consecutive days.

SL. No.	Sample Concentration (Copies)	Day 1 Result Triplicates	Day 2 Result Triplicates	Day 3 Result Triplicates	Day 4 Result Triplicates	Day 5 Result Triplicates
1.	15	+ + +	+ + +	+ + +	+ + +	+ + +
2.	10	+ + +	+ + +	+ + +	+ + +	+ + +
3.	5	+ + +	+ + +	+ + +	+ + +	+ + +
4.	3	+ + +	+ + +	+ + +	+ - -	- - -
5.	1	- - -	- - -	- - -	- - -	- - -

The analytical sensitivity of Amplisure® MTB PCR Kit is 5 copies per reaction.

Analytical Specificity

The analytical specificity of the Amplisure® MTB PCR kit for MTB complex was assured by primer design and stringent PCR conditions. Blast search was done to avoid any homology on primers with other organisms.

MTB Complex Panel	Result
Mycobacterium tuberculosis	+
Mycobacterium microti	+
Mycobacterium africanum	+
Mycobacterium bovis	+
Mycobacterium canettii	+

The specificity of primers was checked for the following possible cross reactive pathogens utilizing International positive controls/ confirmed positive samples and human DNA.

Organism/Sample Tested	Result
Mycobacterium smegmatis	-
Mycobacterium fortuitum	-
Mycobacterium kansasii	-
Mycobacterium phlei	-
Mycobacterium vaccae	-
Mycobacterium wolinskyi	-
E. coli	-
Citrobacter sps	-
Nocardia sps	-
Cryptococcous sps	-
Salmonella sps	-
Vibrio Cholera	-
Listeria sps	-
Campylobacter sps	-
Staphylococcus aureus	-

Precision

The precision data of the Amplisure® MTB PCR Kit has been generated with MTB positive clinical specimens and tested for 10 days in triplicates.

Day of Testing	Sample 1 Test result (10 copies)	Sample 2 Test result (5 Copies)	Sample 3 Test result (2.5 Copies)
1	+ + +	+ + +	+ + +
2	+ + +	+ + +	+ + +
3	+ + +	+ + +	+ + +
4	+ + +	+ + +	+ + +
5	+ + +	+ + +	+ + +
6	+ + +	+ + +	+ + +
7	+ + +	+ + +	+ + +
8	+ + +	+ + +	+ + +
9	+ + +	+ + +	+ + -
10	+ + +	+ + +	- - -

Abbreviations

Abbreviation	*Expansion*
MTB complex	Mycobacterium tuberculosis complex
DNA	Deoxy Ribonucleic Acid
IC	Internal Control
DNases	Deoxy Ribonucleases
PCR	Polymerase Chain Reaction
BSL2	Bio Safety Level 2
BSL3	Bio Safety Level 3
mL	Milli Liters
µL	Micro Liters
K_2EDTA	Potassium Ethylene Diamine Tetra Acetate
X g	Relative Centrifugal Force
MBGW	Molecular Biology Grade Water
NTC	No Template Control
BLAST	Basic Local Alignment Search Tool
Sps	Species
Rxn	Reaction
TAE	Tris-Acetate EDTA
TBE	Tris-Borate EDTA

References

1. Kirschner P *et al*. Diagnosis of mycobacterium infections by nucleic acid amplification: 18-month prospective study. J Clin Microbiol 1996; 34(2): 304-12.

Ordering Information

EP-MTB-50 : 50 rxns
EP-MTB-100 : 100 rxns