Instructions for Use

LyteStar™
TB/NTM PCR Kit 3.1

LyteStar™
TB/NTM PCR Kit 3.1

For detection and differentiation of the *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM) DNA from human specimens

[TB/NTM extraction reagents included]

For use with

Rotor-Gene Q5/6 Plex Platform (Qiagen)
CFX96™ (BioRad)
CFX Opus 96 (BioRad)
Magnetic Induction Cycler (Mic; BioMolecular Systems)
ABI Prism® 7500 SDS (Applied Biosystems)
QuantStudio™ 5 (Applied Biosystems)

IVD For *in vitro* diagnostic use

REF Product No.: 883103

96 reactions

Please refer to Storage and Shelf Life in this IFU

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Content

1. Intended Use

The LyteStar™ TB/NTM PCR Kit 3.1 is intended for the specific detection and differentiation of the *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM) DNA in human sputum, bronchial washings, cerebrospinal fluid (CSF), urine, tissue, and EDTA-whole blood specimens. The LyteStar™ TB/NTM PCR Kit 3.1 is a multiplex kit comprising a dual-target assay targeting the MTBC insertion sequence (*IS6110*) and mannose binding protein (*MPB64*) genes and targeting the NTM 16S rDNA (*rrs*) and RNA polymerase β subunit (*rpoB*) genes.

The LyteStar™ TB/NTM PCR Kit 3.1 is for professional use only
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2. Kit Components

Catalog no.	883103
(A) Extraction Reagents	
Extraction Buffer	4 x 1.5 ml
Pre-Treatment Solution 1 (10X)	2 x 40 ml
Pre-Treatment Solution 2 (10X)	2 x 40 ml
(B) PCR Reagents	
Master A	2 x 480 µl
Master B	2 x 240 µl
Internal Control (IC)	800 µl
Positive Control (PC)	200 µl
PCR grade water	500 µl

3. Storage and Shelf Life

- The LyteStar™ TB/NTM PCR Kit 3.1 has a shelf life of 12 months from the manufacturing date.
- The Pretreatment Solutions 1 & 2 are shipped at ambient temperature.
- The Extraction Buffer and all PCR Reagents are shipped on dry ice.
- Store Pretreatment Solutions 1 & 2 (10X) at room temperature (18 - 28°C). After opening, Pretreatment Solutions 1 & 2 are stable for three months or until the kit expiration date. After dilution, Pretreatment Solution 1 (1X) is

stable at room temperature for only 12 hours and Pretreatment Solution 2 (1X) for one month.

- Store Extraction Buffer at -15°C to -25°C. After opening, Extraction Buffer is stable for three months or until the kit expiration date
- Store all PCR reagents at -15°C to -25°C upon arrival.
- Repeated thawing and freezing should be avoided, as this might affect the performance of the assay. Master B should be frozen in aliquots, if they are to be used intermittently.
- Mix Master A thoroughly by vortex mixing, and centrifuge briefly before use.
- Protect Master B from light.
- All frozen reagents should be completely thawed to room temperature before use. Immediately return unused portions to the freezer for storage.

4. Quality Control

In compliance with AstronDX Technologies' EN ISO 13485 certified Quality Management System, each lot of the LyteStar™ TB/NTM PCR Kit 3.1 is tested against pre-determined specifications to ensure consistent product quality.

5. Product Use Limitations and Precautions

- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR procedures.
- Specimens should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures.
- Wear protective disposable powder-free gloves, a laboratory coat and eye protection when handling specimens.
- Avoid microbial and nuclease (DNAse/RNAse) contamination of the specimen and the components of the kit.
- Always use DNAse/RNAse-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (i) specimen preparation, (ii) reaction set-up and (iii) amplification/detection activities.
- Workflow in the laboratory should proceed in unidirectional manner.

- Always wear disposable gloves in each area and change them before entering different areas.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not use components of the kit that have passed their expiration date.
- Discard sample and assay waste according to your local safety regulations.
- Wash hands thoroughly after handling specimens and test reagents.
- Do not use kits from different lots together.
- Do not use an expired kit.
- In case of damage to the packaging and leaking vials, do not use the kit (possible contamination or deterioration that can cause false interpretation)

6. Product Warranty

AstronDX Technologies guarantees the performance of the LyteStar™ TB/NTM PCR Kit 3.1 for applications as described in the manual. The user must determine the suitability of the product for the particular intended use. Should the product fail to perform satisfactorily in the described applications, please contact AstronDX Technologies Technical Support (**16. Technical Support**) for troubleshooting.

AstronDX Technologies reserves the right to change, alter, or modify any product to enhance its performance and design.

7. Product Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles/face masks. For more information, please consult the appropriate material safety data sheets (MSDSs).

If buffer bottles are damaged or leaking, wear gloves and protective goggles when discarding the bottles in order to avoid any injuries or exposure to hazardous chemical.

The following risk and safety phrases apply to components of the LyteStar™ TB/NTM PCR Kit 3.1:

Pretreatment Solution 1

Contains 40% sodium hydroxide: corrosive. Risk and safety phrases:
*R35, S 26-3-37/39-45

*R-phrase(s):
R35 Causes severe burns

*S-phrase(s):
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S37/39 Wear suitable gloves and eye/face protection
S45 In case of accident or if you feel unwell, seek medical advice immediately (show label where possible)

24-hour emergency information
Emergency medical information can be obtained 24 hours a day in English and Malay language from:
National Poison Centre, Malaysia
Tel: +604-657 0099

For other countries, please visit link below to search for local poison center: http://apps.who.int/poisoncentres/

8. Introduction

Tuberculosis (TB) is a global issue and the most affecting disease worldwide. According to the 2022 WHO report, around 2 billion people, or one-quarter of the world's population, are latently TB infected with an estimation of 5-10% for TB reactivation [1]. The main cause for tuberculosis disease is the *Mycobacterium tuberculosis*, which is part of the *Mycobacterium tuberculosis* complex (MTBC), a group of significantly genetically similar bacteria. Although primarily a pulmonary pathogen, *M. tuberculosis* can also cause disease throughout the body (extrapulmonary TB). TB can present as a dynamic spectrum, from asymptomatic infection to a life-threatening disease. Patients with active TB disease experience general symptoms, such as fever, fatigue, lack of appetite and weight loss, and those with pulmonary disease can have persistent cough and hemoptysis (coughing up blood) in advanced disease [2].

Nontuberculous mycobacteria (NTM) are mycobacteria other than *M. tuberculosis* and *M. leprae*. NTM is also known as Mycobacteria other than tuberculosis (MOTT), atypical mycobacteria, or environmental mycobacteria. Although anyone can get an NTM infection, a small percentage of individuals are at an increased risk, including those with underlying lung disease or weakened immune systems. In these individuals, NTM may be established in the lungs as an infection. Currently, there are more than 200 species of NTM with many unique virulence characteristics [3]. For the diagnosis of NTM lung disease, patients are required to meet all clinical criteria such as pulmonary symptoms, nodular or cavitary opacities on chest radiograph or multifocal bronchiectasis with small nodules using chest high-resolution computed topography scan and microbiologic criteria with at least two separate positive cultures from sputum or at least one positive culture of bronchial wash, lavage, or biopsy [4]. Similar to TB, NTM infections can occur throughout the body. The most commonly described attributable human infections are pulmonary infections, lymphadenitis and skin and soft tissue infections.

Standard treatment for TB comprises of four first-line antimicrobials: isoniazid, rifampicin, pyrazinamide, and ethambutol, administered for a minimum of six months. However, resistance to these drugs can occur. Treatment of multidrug-resistant (MDR-TB) and extensively drug-resistant (XDR-TB) TB cases requires additional antibiotics for a prolonged duration [5, 6]. NTM diseases do not respond to anti-TB drugs. Treatment of NTM diseases follow specific guidelines, based on the nature of identification, and requires species identification [4].

Early diagnosis and successful treatment of TB is crucial to prevent further spread of the bacteria and development of resistant strains. The differentiation of NTM from MTBC is of important diagnostic value as the pathogenesis and treatment for these diseases are different. Several diagnostic techniques are commonly employed to rule out TB infection. Chest X-rays is a screening tool used to diagnose active pulmonary TB; however, it cannot detect latent TB infection. Sputum smear microscopy with Ziehl-Neelsen stain is a widely used tool in diagnosis but has low sensitivity and is unable to differentiate MTBC and other acid-fast bacilli such as NTM. Sputum culture method using Lowenstein-Jensen medium is highly specific and sensitive for MTBC and NTM but takes at least two weeks for the colonies to appear, which further delays the diagnosis and treatment [7].

Molecular techniques, such as polymerase chain reaction (PCR) overcome many limitations of traditional methods and are increasingly used also in TB diagnosis due to rapid and reliable results with high sensitivity and specificity. Thus, the LyteStar™ TB/NTM PCR Kit 3.1 was developed based on PCR technology to help in swift and accurate diagnosis of TB and NTM infection.

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[1] World Health Organization (WHO). (2022). Global Tuberculosis Report 2022. https:///www.who.int/publications/i/item/9789240061729

[2] Centers for Disease Control and Prevention (CDC) (2024). Clinical Symptoms of Tuberculosis. https://www.cdc.gov/tb/hcp/clinical-signs-and-symptoms/index.html

[3] Johansen MD, Herrmann JL, Kremer L. (2020) Non-tuberculous mycobacteria and the rise of Mycobacterium abscessus. *Nature Review Microbiology*. Jul;18(7):392-407. doi: 10.1038/s41579-020-0331-1.

[4] Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, Holland SM, Horsburgh R, Huitt G, Iademarco MF, Iseman M, Olivier K, Ruoss S, von Reyn CF, Wallace RJ, Jr, Winthrop K. (2007). An official ATS/IDSA statement: diagnosis, treatment, and prevention of non-tuberculous mycobacterial diseases *American Journal of Respiratory and Critical Care Medicine*, 175(4):367–416.

[5] Lewinsohn DM, Leonard MK, LoBue PA, Cohn DL, Daley CL, Desmond E, Keane J, Lewinsohn DA, Loeffler AM, Mazurek GH, O'Brien RJ, Pai M, Richeldi L, Salfinger M, Shinnick TM, Sterling TR, Warshauer DM, Woods GL. (2017). Official American Thoracic Society/Infectious Diseases Society of America/ Centers for Disease Control and Prevention Clinical Practice Guidelines: Diagnosis of Tuberculosis in Adults and Children. Clinical Infectious Diseases. 2017;64(2): e1–e33

[6] World Health Organization (WHO). (2022). WHO consolidated guidelines on tuberculosis. Module 4: treatment - drug-resistant tuberculosis treatment, 2022 update. https://www.who.int/publications/i/item/9789240007048

[7] Acharya B., Acharya A., Gautam S., Ghimire S.P., Mishra G., Parajuli N., Sapkota B. (2020). Advances in diagnosis of Tuberculosis: An update into molecular diagnosis of Mycobacterium tuberculosis. *Molecular Biology. Reports*, 47:4065–4075. doi: 10.1007/s11033-020-05413-7.

9. Product Description

The LyteStar™ TB/NTM PCR Kit 3.1 is an *in vitro* diagnostic test system, based on real-time PCR technology, for the qualitative detection and differentiation of *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM) specific DNA. The LyteStar™ TB/NTM PCR Kit 3.1 consists of a single tube assay targeting the insertion sequence (*IS6110*) and mannose binding protein (*MPB64*) genes for the detection of MTBC and the 16S rDNA (*rrs*) and RNA polymerase β

subunit (*rpoB*) genes for the detection of NTM. The LyteStar™ TB/NTM PCR Kit 3.1 includes a heterologous amplification system (Internal Control) to identify possible PCR inhibition and to confirm the integrity of the reagents of the kit. The Internal Control used in the LyteStar™ TB/NTM PCR Kit 3.1 is an artificial DNA sequence with no homology to any known genomes.

The LyteStar™ TB/NTM PCR Kit 3.1 utilizes real-time polymerase chain reaction (PCR) technology for the amplification of specific target sequences and target specific probes for the detection of the amplified DNA. The probes are labelled with fluorescent reporter and quencher dyes.

In the assay, probes specific for *IS6110* and *MPB64* genes of MTBC are labelled with the fluorophore FAM, probes specific for *rrs* and *rpoB* genes of NTM are labelled with the fluorophore Cy5™, and the probe specific for the Internal Control (IC) is labelled with the fluorophore HEX™. The *IS6110* and *MPB64* gene probes detect all members of the MTBC, while the *rrs* and *rpoB* gene probes detect clinically important NTM species, and allow differentiation between MTBC and NTM. Using probes linked to distinguishable dyes enables the parallel detection of MTBC and NTM specific DNA and the Internal Control in the corresponding detector channels of the real-time PCR instrument.

The oligonucleotides in the two assays were designed, modified, and based on sequences/targets published in the articles listed below:

Target	**Publication**
MTBC (*IS6110* gene)	NCBI Genbank (2024)
MTBC (*MPB64* gene)	Huang *et al.,* (2021)
NTM (*rrs* gene)	NCBI Genbank (2024)
NTM (*rpoB* gene)	Park *et al.,* (2023)
Internal Control	Deer *et al*., (2010)

The test consists of two processes in a single tube assay:

- PCR amplification of target and Internal Control cDNA
- Simultaneous detection of PCR amplicons by fluorescent dye labelled probes

The LyteStar™ TB/NTM PCR Kit 3.1 consists of:

- Three Extraction reagents (Extraction Buffer, Pre-Treatment Solution 1 & Pre-Treatment Solution 2)

- Two Master reagents (Master A and Master B)
- The template of the Internal Control (IC)
- The template of the Positive Control (PC)
- PCR grade water (for setting up of “No Template Control”, NTC)

Pre-Treatment Solution 1 and Pre-Treatment Solution 2 contains components for the pretreatment of specimens prior to extraction.

The Extraction Buffer contains components for the extraction and purification of mycobacterial DNA from sputum, bronchial washings, cerebrospinal fluid, urine, tissue and EDTA whole blood.

Master A and Master B reagents contain all components (buffer, enzymes, primers, and probes) to allow PCR mediated amplification and target detection of *IS6110* and *MPB64* genes of *Mycobacterium tuberculosis* complex (MTBC) specific DNA, *rrs* and *rpoB* genes for specific detection and differentiation of nontuberculous mycobacteria (NTM) specific DNA and Internal Control in one reaction setup.

The Positive Control (PC) contains synthesized target genes of *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM).

The Internal Control used in the LyteStar™ TB/NTM PCR Kit 3.1 is DNA of an artificial sequence with no homology to any known genomes.

The LyteStar™ TB/NTM PCR Kit 3.1 was developed and validated to be used with the following real-time PCR instruments:

- Rotor-Gene Q5/6 Plex Platform (Qiagen)
- CFX96™ (BioRad)
- CFX Opus 96 (BioRad)
- Magnetic Induction Cycler (MIC; BioMolecular Systems)
- ABI Prism® 7500 SDS (Applied Biosystems)
- QuantStudio™ 5 (Applied Biosystems)

10. Material and Devices required but Not Provided

- Appropriate real-time PCR instrument
- 1.5 ml microcentrifuge tubes (with safe-lock or screw cap)

- Microcentrifuge (with speed ≥ 13,000 rpm)
- Pipettes, adjustable (range: 10 µl, 100 µl, 200 µl, 1000 µl)
- Pipette tips (with aerosol barriers)
- Disposable gloves (powder-free)
- Heating block for lysis of specimens during extraction
- Vortex mixer
- Sterile, nuclease-free water
- Appropriate 96-well reaction plates or reaction tubes with corresponding (optical) closing material.

11. Specimen Storage

- Suitable specimens include sputum, bronchial washings, cerebrospinal fluid (CSF), urine, tissue, and EDTA-whole blood specimens.
- Follow specimen transport and storage conditions outlined in the following guidelines:

  - World Health Organization (2000). Guidelines for the collection of clinical specimens during field investigation of outbreaks. https://apps.who.int/iris/handle/10656/66348
  - Ministry of Health (MOH) (2023). Guidelines for the safe transport of clinical specimens and infectious substances in Malaysia 2023. https://imr.nih.gov.my/images/intranet/UPS/2024/GUIDELINES-FOR-THE-SAFE-TRANSPORT-OF-CLINICAL-SPECIMENS.pdf
  - Ministry of Health (MOH) (2021). Clinical practice guidelines Management of tuberculosis (4th edition). https://www.moh.gov.my/moh/resources/Penerbitan/CPG/Respiratory/CPG-_Management_of_Tuberculosis_(4th_Edition).pdf
  - Association of Public Health Laboratory (APHL). Specimen collection, handling, transport and processing. https://www.aphl.org/programs/infectious_disease/tuberculosis/TBCore/TB_Specimen_Collection_thru_Processing_TrainerNotes.pdf
  - Centers for Disease Control and Prevention (CDC) (2024). Clinical and diagnosis guidelines for tuberculosis. https://www.cdc.gov/tb/hcp/testing-diagnosis/clinical-and-laboratory-diagnosis.html

12. Instructions for Use

12.1. Preparation of Reagents and Buffers

Prepare the following before each use:

1. Extraction Buffer - Thaw completely at room temperature.

2. Pretreatment Solution 1 (10X) - Dilute with sterile, nuclease free water to 1X concentration just before use. 6 ml Pretreatment Solution 1 (1X) is needed per sample. E.g. dilute 600 µl Pretreatment Solution 1 (10X) with 5.4 ml water and store up to 12 hours at 18 - 28°C.

3. Pretreatment Solution 2 (10X) - Dilute with sterile, nuclease free water to 1X concentration before use. 6 ml of Pretreatment Solution 2 (1X) is needed per sample. E.g. dilute 600 µl Pretreatment Solution 2 (10X) with 5.4 ml water. The solution is stable for one month at 18 - 28°C.

12.2. Pretreatment of Specimen

Things to do before starting:

- Switch on heating block and set to 65°C or 100°C, respectively, for pretreating paraffin embedded specimen or for performing mycobacteria DNA extraction.

Note: All centrifugation steps are carried out at room temerature (18 - 28°C)

12.2.1. Sputum and bronchial washings

1. Add equal volume of Pretreatment Solution 1 (1X) to sample (preferably >1ml) in a 1.5 ml / 2.0 ml microcentrifuge tube or conical tube. Vortex vigorously. Incubate at room temperature (18 – 28°C) for 5 min and vortex vigorously for 10s at one-minute intervals.

2. Centrifuge at 13,000 rpm for 3 min and discard supernatant.

3. If the specimen is visibly not viscous after Step 2, skip to Step 7.

4. Add 1 ml of Pretreatment Solution 1 and mix well to resuspend pellet. Transfer suspension to 1.5 ml microcentrifuge tube.

5. Incubate at room temperature (18 – 28°C) for 5 min and vortex vigorously.

6. Centrifuge at 13,000 rpm for 3 min and discard supernatant.

7. Use pellet for DNA extraction (section 12.3).

12.2.2. CSF, urine, and body fluids

1. Centrifuge specimen (in a 1.5 ml microcentrifuge tube) at 13,000 rpm for 3 min and discard supernatant.

2. Add 1 ml of Pretreatment Solution 1 (1X) and mix well to resuspend pellet.

3. Incubate at room temperature (18 - 28°C) for 5 min and vortex vigorously.

4. Centrifuge at 13,000 rpm for 3 min and discard supernatant.

5. Use pellet for DNA extraction (section 12.3).

12.2.3. EDTA whole blood

1. Transfer 1 ml whole blood into a 1.5 ml microcentrifuge tube and keep at -20°C for 4 hours or -70°C for 2 hours.

2. Thaw frozen blood completely at room temperature (18 - 28°C).

3. Centrifuge at 13,000 rpm for 3 min and discard supernatant.

4. Add 1 ml of nuclease-free water and vortex vigorously for 30 s.

5. Centrifuge at 13,000 rpm for 3 min and discard supernatant.

6. Repeat Step 4-5 until all visible stain of red blood cells is removed.

7. Use pellet for DNA extraction (section 12.3).

12.2.4. Tissue

i. General tissue

1. Cut tissue samples into small pieces and homogenize.

2. Transfer homogenized tissue into a 1.5 ml tube and use for DNA extraction (section 12.3).

ii. Paraffin embedded tissue

1. Thinly slice paraffin embedded tissue and dissolve at 65°C for 20 min in a 1.5 ml microcentrifuge tube. Remove liquefied paraffin by using a pipette.
2. Add 1 ml Pretreatment Solution 2 to sample and heat at 100°C for 10 min.
3. Centrifuge at 13,000 rpm for 1 min and discard supernatant by using a pipette.
4. Repeat Step 2-3 to remove paraffin completely.
5. Use pellet for DNA extraction (section 12.3)

12.3. Boiling Lysis Extraction of Mycobacterial DNA

1. Add 1ml Pretreatment Solution 2 to pellet obtained from section 12.2.
2. Vortex vigorously for 10 s.
3. Centrifuge for 13,000 rpm for 3 min and discard supernatant.
4. Mix Extraction Buffer well by pipetting and dispense 50 µl into pellet obtained in Step 3. Mix Extraction Buffer again by pipetting before dispensing into the pellet of the next sample.
5. For large pellets, add 100 µl Extraction Buffer.
6. Vortex to resuspend pellet and briefly centrifuge all tubes and heat mixture at 100°C for 20 min.
7. Centrifuge at 13,000 rpm for 1 min and vortex for 10 s.
8. Centrifuge at 13,000 rpm for 3 min.
9. Aspirate 5 µl supernatant and use for downstream application, e.g. PCR.

Note: If retesting the same sample, repeat Step 8 before aspirating supernatant for PCR.

Extracted DNA is the starting material for the LyteStar™ TB/NTM PCR Kit 3.1. The quality of the extracted DNA has a profound impact on the performance of the whole test system. It has to be ensured that the nucleic acid extraction system used is compatible with real-time PCR technology.

The following nucleic acid extraction kits / systems are suitable for use with the LyteStar™ TB/NTM PCR Kit 3.1:

- LyteStar™ TB/NTM PCR Kit 3.1 - Extraction Reagents (AstronDX Technologies)
- SpinStar™ Total DNA Kit (AstronDX Technologies)

For detailed instructions regarding the extraction procedure using the SpinStar™ Total DNA Kit please contact our Technical Support (**16. Technical Support**).

Alternative nucleic acid extraction systems and kits might also be appropriate. The suitability of the nucleic acid extraction procedure for use with LyteStar™ TB/NTM PCR Kit 3.1 has to be validated by the user.

If using a spin column-based sample preparation procedure including washing buffers containing ethanol, an additional centrifugation step for 10 min at approximately 17000 x g (~ 13000 rpm), using a new collection tube, prior to the elution of the nucleic acid is highly recommended.

NOTE

Ethanol is a strong inhibitor in real-time PCR. If your sample preparation system is using wash buffers containing ethanol, you need to make sure to eliminate any traces of ethanol prior to elution of the nucleic acid.

12.4. Master Mix Setup

1. All reagents and samples should be thawed completely, mixed (by gentle vortex mixing) and centrifuged briefly before use. Prepare a marginal excess (additional 0.5 reaction) of the required Master Mix volume.

2. The LyteStar™ TB/NTM PCR Kit 3.1 contains a heterologous Internal Control (IC). The Internal Control is used ONLY as a PCR inhibition control when using the Extraction Reagents provided with the LyteStar™ TB/NTM PCR Kit 3.1.

NOTE

For the boiling lysis extraction protocol using the Extraction Reagents provided in the LyteStar™ TB/NTM PCR Kit 3.1, the Internal Control is used as a PCR inhibition control ONLY and is added to the Master Mix.

(i) The Internal Control can be used as both (i) a PCR inhibition control and (ii) a control of the sample preparation procedure if a spin column-based nucleic acid extraction protocol is used.

(ii) **For the boiling lysis extraction protocol**, the IC is used as a PCR inhibition control, but not as a control for the sample preparation procedure, the Master Mix is set up according to the following pipetting scheme:

Number of Reactions	1	12
Master A	10 µl	120 µl
Master B	5 µl	60 µl
IC	0.5 µl	6 µl
Volume Master Mix	**15.5 µl**	**186 µl**

(iii) **For the spin column-based extraction protocol**, the IC may be used as a control for the sample preparation procedure and as a PCR inhibition control, therefore, the IC has to be added during the nucleic acid extraction procedure.

The IC **must not** be added directly to the specimen. The IC should always be added to the specimen/lysis buffer mixture.

The volume of the IC which has to be added depends always and only on the elution volume. It represents **10% of the elution volume**. For instance, if the nucleic acid is going to be eluted in 60 µl of elution buffer or water, 6 µl of IC must be added into the specimen/lysis buffer mixture.

If the IC was added during the sample preparation procedure, the Master Mix is set up according to the following pipetting scheme:

Number of Reactions	1	12
Master A	10 µl	120 µl
Master B	5 µl	60 µl
Volume Master Mix	**15 µl**	**180 µl**

NOTE

Never add the Internal Control directly to the specimen.

12.5. Reaction Setup

1. Pipette 15 µl Master Mix into each required well of an appropriate optical 96-well reaction plate or an appropriate optical reaction tube.

2. Add 5 µl of the sample (eluate from the nucleic acid extraction) or 5 µl of the controls (Positive Control; or water as No Template Control, NTC).

3. Make sure at least one Positive Control and one NTC are used per run.

4. Thoroughly mix the samples and controls with the Master Mix by pipetting up and down.

5. Close the 96-well reaction plate with an appropriate optical adhesive film and the reaction tubes with appropriate caps.

6. Centrifuge the 96-well reaction plate at 1,000 xg (~3,000 rpm) for 30s.

Reaction Setup	
Master Mix	15 µl
Sample or Control	5 µl
Total Volume	**20 µl**

13. Programming the Real-Time PCR Instrument

13.1 Settings

• Define the following settings:

Settings	
Reaction Volume	20 µl
Ramp Rate	Default
Passive Reference*	ROX

*Only for ABI7500 and QuantStudio 5

13.2 Fluorescent Detectors (Dyes)

• Define the following fluorescent detectors:

Detection	Detector Name	Reporter	Quencher
MTBC specific DNA (*IS6110* and *MPB64* genes)	MTBC	FAM	IBFQ
NTM specific DNA (*rrs* and *rpoB* genes)	NTM	Cy5	IBRQ
Internal Control	IC	HEX*	IBFQ

*Set VIC channel when using ABI7500 and QuantStudio 5

13.3 Temperature Profile and Dye Acquisition

• Define the temperature profile and dye acquisition:

	Stage	Cycle Repeats	Acquisition	Temperature	Time
UNG Reaction	Hold	1	-	35 °C	5:00 min
Initial Activation	Hold	1	-	95 °C	5:00 min
Denaturation	Cycling	40	-	95 °C	5 sec
Annealing			√	60 °C	20 sec
Extension			-	70 °C	30 sec
Final Extension	Hold	1	-	70 °C	3:00 min

√ Signal acquisition: activate FAM, Cy5, and HEX channels in all runs

14. Data Analysis

For basic information regarding data analysis on specific real-time PCR instruments, please refer to the manual of the respective instrument. For detailed instructions regarding data analysis of the LyteStar™ TB/NTM PCR Kit 3.1 on different real-time PCR instruments please contact our Technical Support (**16.Technical Support**).

14.1. Validity of Diagnostic Test Runs

14.1.1 Valid Diagnostic Test Runs (Qualitative)

For a **valid** diagnostic test run (qualitative), the following control conditions must be met:

Control ID	FAM / Cy5 Detection Channel	HEX Detection Channel
Positive Control	POSITIVE	POSITIVE
Negative Control	NEGATIVE	POSITIVE

14.1.2 Target CT values of PC and IC

	Positive Control *(MTBC)*	Positive Control *(NTM)*	Internal Control
Target CT value	< 35 cycles	< 35 cycles	≤ 40 cycles*

*Required for unknown samples that do not amplify in FAM and Cy5 channels

Note: The above CT target values are exclusively given for **monitoring the integrity of the product and validated assay conditions** and should be achieved **ONLY for the provided Positive Control (PC) and Internal Control (IC)** when used as per the instructions given under section 12.5. Reaction Setup. **The target CT values for PC MUST NOT be misinterpreted as the diagnostic cut-off values for clinical samples.**

14.1.3 Invalid Diagnostic Test Runs (Qualitative)

A **qualitative** diagnostic test run is **invalid**, (i) if the run has not been completed or (ii) if any of the control conditions for a **valid** diagnostic test run are not met.

In case of an **invalid** diagnostic test run, **repeat testing by using the remaining purified nucleic acids** or start from extracting the original samples again.

14.2 Interpretation of Results

FAM *MTBC*	Cy5 *NTM*	HEX *Internal Control*	Result Interpretation
+	+	+*	MTBC and NTM specific DNA detected *Positive for MTBC and NTM*
+	-	+*	MTBC specific DNA detected *Positive for MTBC*
-	+	+*	NTM specific DNA detected. *Positive for NTM*
-	-	+	Both MTBC and NTM specific DNA not detected. The sample does not contain detectable amounts of MTBC and NTM specific DNA.
-	-	-	PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

Note: For MTBC *IS6110* and *MPB64* genes (FAM channel) and NTM *rrs* and *rpoB* genes (Cy5 channel) "+" refers to amplification curve detected at CT ≤ 40 cycles. "-" refers to no amplification / no CT obtained.

* Detection of the Internal Control in the HEX channel is not required for positive results in the FAM and Cy5 detection channels. A high MTBC and/or NTM load in the sample can lead to reduced or absent Internal Control signals.

14.2.1 Threshold Settings for Cycler Software

Cycler	Threshold		
	FAM MTBC Channel	Cy5 NTM Channel	HEX IC Channel
Rotor-Gene Q	0.05 norm. fluoro.	0.05 norm. fluoro.	0.05 norm. fluoro.
CFX96™	100 RFU	100 RFU	100 RFU
CFX Opus 96	100 RFU	100 RFU	100 RFU
Mic qPCR	Auto	Auto	Auto
ABI7500	15,000 ΔRn	15,000 ΔRn	15,000 ΔRn
QuantStudio 5	50,000 ΔRn	50,000 ΔRn	50,000 ΔRn

14.2.2 CT Cut-Off Values for Clinical Samples

	FAM MTBC Channel	Cy5 NTM Channel
CT Cut-Off Value	< 40 cycles	< 37 cycles

15. Performance Evaluation

The analytical performance evaluation of the LyteStar™ TB/NTM PCR Kit 3.1 was accomplished using quantified MTBC and NTM specific DNA.

15.1 Analytical Sensitivity

The analytical sensitivity (limit of detection: LoD) of the LyteStar™ TB/NTM PCR Kit 3.1 is defined as the concentration of MTBC and NTM DNA molecules that can be detected with a positivity rate of ≥ 95%. The analytical sensitivity was determined by analyzing MTBC and NTM genomic DNA of known concentration.

A dilution series of AmpliRun® Mycobacterium tuberculosis and AmpliRun® Mycobacterium avium (a representative of NTM species) DNA control was prepared by using 1× TE buffer as diluent. Dilutions of MTBC and NTM DNA were tested with LyteStar™ TB/NTM PCR Kit 3.1. Results were analyzed by Probit analysis (Table 1 and Table 2).

The analytical sensitivity of the LyteStar™ TB/NTM PCR Kit 3.1 in combination with the Rotor-Gene Q 5-plex platform (Qiagen) was determined at 0.36 copies/µl for *Mycobacterium tuberculosis* complex (MTBC) *IS6110* and *MPB64* genes target and 9.52 copies/µl for nontuberculous mycobacteria (NTM) *rrs* and *rpoB* genes target ($p \leq 0.05$).

Table 1. PCR results used for the calculation of the analytical sensitivity of *Mycobacterium tuberculosis* complex (MTBC) *IS6110* and *MPB64* genes target for the LyteStar™ TB/NTM PCR Kit 3.1 in combination with the Rotor-Gene Q 5-plex platform (Qiagen).

Concentration (copies/µl)	No. of Samples Tested	No. of Samples Positive	Hit rate (%)
6.33	12	12	100
2.00	12	12	100
0.63	12	12	100
0.20	12	10	83.33
0.063	12	4	33.33
0.020	12	1	8.33
0.0063	12	0	0
0.0020	12	0	0

Table 2. PCR results used for the calculation of the analytical sensitivity of nontuberculous mycobacteria (NTM) *rrs* and *rpoB* genes target for the LyteStar™ TB/NTM PCR Kit 3.1 in combination with the Rotor-Gene Q 5-plex platform (Qiagen).

Concentration (copies/µl)	No. of Samples Tested	No. of Samples Positive	Hit rate (%)
101.12	12	12	100
32.00	12	12	100
16.00	12	12	100
10.13	12	11	91.67
5.06	12	9	75.00
3.20	12	2	16.67
1.01	12	0	0
0.32	12	0	0
0.10	12	0	0
0.03	12	0	0
0.01	12	0	0

15.2 Analytical Specificity

There have been approximately 200 NTM species identified to-date. The clinically important disease-causing agents among NTM differ geographically. The most common species that are frequently isolated from patients with NTM are *Mycobacterium avium* complex (MAC), *Mycobacterium abscessus* complex and *Mycobacterium kansasii*.

The analytical specificity of the LyteStar™ TB/NTM PCR Kit 3.1 is ensured by the thorough selection of the oligonucleotides (primers and probes). The oligonucleotides were checked by sequence comparison analysis against publicly available sequences to ensure that the applied primer/probes in LyteStar™ TB/NTM PCR Kit 3.1 specifically detect all members of the *Mycobacterium tuberculosis* complex (MTBC) and clinically important nontuberculous mycobacteria (NTM) species. For detailed results of the *in silico* analysis please contact our Technical Support (**16. Technical Support**).

In addition to the *in silico* analysis, the specificity of the LyteStar™ TB/NTM PCR Kit 3.1 was evaluated by testing genomic DNA extracted from other pathogens likely to be present in the same sample material as MTBC and NTM, or that cause similar symptoms to these bacteria (Table 3).

Table 3. Microorganisms tested to demonstrate the analytical specificity of the LyteStar™ TB/NTM PCR Kit 3.1.

LyteStar™ TB/NTM PCR Kit 3.1			
Organisms	***MTBC (FAM channel)***	***NTM (Cy5 channel)***	***Internal Control (HEX channel)***
Mycobacterium tuberculosis	positive	negative	valid
Mycobacterium tuberculosis (Rifampicin resistant)	positive	negative	valid
Mycobacterium tuberculosis (Isoniazid resistant)	positive	negative	valid
Mycobacterium bovis	positive	negative	valid
Mycobacterium abscessus	negative	positive	valid
Mycobacterium avium	negative	positive	valid
Mycobacterium gordonae	negative	positive	valid
Mycobacterium intracellulare	negative	positive	valid
Mycobacterium kansasii	negative	positive	valid
Mycobacterium ulcerans	negative	positive	valid
Bordetella parapertussis	negative	negative	valid
Borrelia burgdorferi strain B31	negative	negative	valid
Haemophilus influenzae	negative	negative	valid
Klebsiella pneumoniae	negative	negative	valid
Legionella pneumophila	negative	negative	valid
Chlamydophila pneumonia	negative	negative	valid
Orientia tsutsugamushi	negative	negative	valid
Rickettsia conorii	negative	negative	valid
Human herpesvirus 5 HCMV	negative	negative	valid
Epstein-Barr virus	negative	negative	valid
Parvovirus B19 (PAB19)	negative	negative	valid
Bocavirus	negative	negative	valid

The LyteStar™ TB/NTM PCR Kit 3.1 did not cross-react with any pathogen or genotype/subtype other than its own target.

15.3 Diagnostic Sensitivity and Specificity

The clinical performance of the LyteStar™ TB/NTM PCR Kit 3.1, in regards to diagnostic sensitivity and specificity, was evaluated through a retrospective study at a private laboratory. The diagnostic sensitivity and specificity of the LyteStar™ TB/NTM PCR Kit 3.1 was compared to the clinical site's reference method.

In comparison to the reference test, the LyteStar™ TB/NTM PCR Kit 3.1 achieved a diagnostic sensitivity of 100% for the detection of both the *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM), and a diagnostic specificity of 100% for the detection of both the *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM).

		Reference Method			
		MTBC (n=19)	NTM (n=17)	MTBC (n=18)	NTM (n=18)
		Positive		Negative	
LyteStar™ TB/NTM PCR Kit 3.1	Detected	19	17	0	0
	Not Detected	0	0	18	18
	Total	**19**	**17**	**18**	**18**
Overall Concordance		100 % 19 / 19 Sensitivity	100 % 17 / 17 Sensitivity	100 % 18 / 18 Specificity	100 % 18 / 18 Specificity

To calculate the diagnostic sensitivity of LyteStar™ TB/NTM PCR Kit 3.1:

$$\text{Diagnostic Sensitivity [\%]} = \frac{\text{“correct positive”}}{\text{(“correct positive” + “false positive”)}} \times 100$$

To calculate the diagnostic specificity of LyteStar™ TB/NTM PCR Kit 3.1:

$$\text{Diagnostic Specificity [\%]} = \frac{\text{“correct negative”}}{\text{(“correct negative” + “false negative”)}} \times 100$$

16. Technical Support

For customer support, please contact our Technical Support:

e-mail: **techsupport@astrondx.com**
phone: **+603 7931 6760**

17. Appendix

Explanation of Symbols

Symbol	Explanation
	*In vitro* diagnostic medical device
	Product Number
	Batch Code
	Manufacturer
	Date of Manufacture
	Contains sufficient for “n” tests/rxns
	Temperature limitation
	Version
	Use-By Date
	Instruction for Use
	Caution
	Skull and crossbones
	Corrosion
	Keep away from sunlight

18. Ordering Information

Products	Packing (reactions)	Order No.
LyteStar™ TB/NTM PCR Kit 3.1	96	883103
SpinStar™ Total DNA Kit 2.0	100	821803

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