Instructions for Use

380109-C

REF LMC521

Loopamp™ MTBC Detection Kit

INTENDED USE

Loopamp™ MTBC Detection Kit is a qualitative in vitro diagnostic test for the detection of *Mycobacterium tuberculosis* complex (MTBC) DNA extracted from sputum in patients with any symptom indicative of MTBC infection.

TEST PRINCIPLES

This product is based on the nucleic acid amplification method, LAMP (Loop-mediated Isothermal Amplification) developed by Eiken Chemical Co., Ltd.

The LAMP method has the following characteristics : (1) Only one enzyme is required and the amplification reaction proceeds under isothermal conditions;[1),(2)] (2) it has extremely high specificity because of the use of four primers recognizing six distinct regions on the target; (3) it has high amplification efficiency and enables amplification within a short time; and (4) it produces tremendous amount of amplified product which makes simple visual detection possible.[3), (4)]

The primers provided with this product have been designed in the *gyrB* and *IS* region of the MTBC genome DNA. This region has been confirmed by alignment analysis of selected base sequences of MTBC and nontuberculous mycobacteria to have a relatively well-preserved base sequence in MTBC.

DNA from untreated sputum or NALC-NaOH treated sputum is extracted using Loopamp™ PURE DNA Extraction Kit (available for sale separately). The DNA solution is then dispensed into a reaction tube. The strand displacement DNA polymerase, deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), calcein and MTBC-specific primers are stored in dried form in the cap of the reaction tube. This dried LAMP reagent (MTBC detection reagent (dMTB)) is reconstituted when adding the DNA solution. The reaction tube is then incubated at 67.0°C and the DNA is amplified through catalysis by the strand displacement DNA polymerase in accordance with LAMP reaction.

The detection of amplified products is based on turbidity measurement of a by-product, magnesium pyrophosphate (white precipitate).[3)] Also, visual judgment under UV irradiation may be used instead of turbidity measurement. During amplification the calcein contained in the dried LAMP reagent is released, generating fluorescence light that can be detected by eye.[4)] Before reaction, calcein contained in the reagent is in the quenched state due to bound manganese ions bound thereto; however, once the LAMP reaction is started, pyrophosphate ions are generated and bind out the manganese ions, and calcein becomes fluorescent.[4)]

CONTENTS OF THE KIT

Reagents are stable until the date on the label assuming the container remains unopened under a storage temperature of 1 – 30°C.

MTBC detection reagent ····· 2 X 48 tubes
The following ingredients in dried form are contained in each reaction tube.
Bst DNA polymerase[*1]
Deoxynucleotide triphosphates
Magnesium sulfate
Calcein
Manganese chloride
Primers[*2]
Positive control MTBC (PC MTB)[*3] ····· 1 X 0.4 mL
Negative control MTBC (NC MTB) ····· 6 X 0.5 mL
30 µL dropper ····· 1 X 12 droppers

*1: *Bst* DNA polymerase derived from *Bacillus stearothermophilus* is a strand displacement DNA polymerase that lacks 5'→3' exonuclease activity.

*2: Primers designed in the DNA gyrase subunit B (*gyr*B) and Insertion sequence IS6110 (IS) region of the MTBC genome DNA, purified from synthesized oligonucleotides by HPLC.

*3: PC MTB contains a product resulting from *in vitro* amplification of a template genome DNA of *Mycobacterium tuberculosis* H37Rv (GenBank No. NC_000962) origin.

The abbreviations of names of the following reagents and the Lot No. are printed on the containers as below, and also manufacturer (EKN) is printed.

Reagents	Labelling on the tube	Code on the cap
Positive control MTBC	PC MTB Lot No., EKN	PC MTB
Negative control MTBC	NC MTB Lot No., EKN	NC MTB

WARNINGS AND PRECAUTIONS

(1) For in vitro diagnostic use only.
(2) This product is designed only for clinical diagnosis of MTBC from sputum samples of human origin. Do not use for other purposes.
(3) When using this product, always follow this package insert.
(4) Do not freeze the reagents.
(5) Do not use any expired reagent.
(6) Do not mix different lots.
(7) Do not replenish any reagent.
(8) Performance of Loopamp™ MTBC Detection Kit is dependent on operator proficiency and adherence to procedural directions. Testing should be performed by properly trained personnel.
(9) Remove the required number of reaction tubes from the packaging just before use and seal the aluminium pouch immediately.
(10) Do not remove the desiccant from the aluminium pouch. High level of humidity may deteriorate the dried LAMP reagent in the reaction tubes.
(11) Exposure to heat and light might deteriorate the dMTB. Remove only the required number of reaction tubes (number of samples + number of controls) and seal any unused tubes immediately.
(12) Read the instruction manual of equipment involved incubator before use.
(13) Sputum samples pose a potential risk for infection. Take all necessary preventive measures to avoid biohazard.[5)]
(14) PC MTB and NC MTB both contain a small amount of sodium azide as preservative. Sodium azide is classified as toxic. Avoid any contact with eyes, mouth, or skin.
(15) In case of accidental contact of any reagent with eyes, mouth, or skin, immediately rinse the affected site with plenty of water and, if necessary, seek medical advice.
(16) Do not dilute or add the PC MTB to the samples. Use the PC MTB only as described in this package insert in order to avoid DNA contamination.
(17) Store the PC MTB positive control and any positive sputum samples separately from the other kit reagents.
(18) The cap of each reaction tube contains dMTB in the dried form. Do not touch the inside of the cap.
(19) Before using the reaction tubes, check carefully to see if they have any cracks or scratches. Damaged tubes might give false results and lead to DNA contamination of the incubator and work area.
(20) Do not expose reaction tubes to UV light before the end of the LAMP reaction. Prolonged exposure to UV light might damage the tubes and lead to false results.
(21) When a UV lamp is used for visual fluorescence judgment, do not stare directly at UV light. Since UV light is harmful to the eyes, even watching for a short period would irritate eyes and cause symptoms similar to conjunctivitis. Use a glass screen or wear goggles or a protective eye mask whenever looking directly at the UV lamp.
(22) Refer to the manual of the incubator. When LF-160 or the real-time turbidimeter is used, be careful in removing the reaction tube from the incubator to avoid burns.

WASTE DISPOSAL

(1) Do not open the tubes after DNA amplification. Leave the cap closed and dispose of the used tubes as medical waste by incineration or after double bagging with sealable plastic bags.

(2) Never autoclave or re-use the reaction tubes, else amplified products will disperse and cause contamination.
(3) The main material for the reaction tubes, reagent tubes, and 30μL dropper is polypropylene (PP); for the reaction tube tray it is PET; for the aluminium pouch it is aluminium; for the kit case it is paper.
(4) Dispose of any used reagent, container, or lab ware in accordance with local regulations.

SPECIMEN COLLECTION

(1) Use the most purulent part of the sputum sample.
(2) Sputum samples should be used immediately after collection.
(3) Collect sputum in a separate room from the LAMP amplification room. Aerosols containing MTBC DNA can be generated during sputum collection and may cause contamination.

MATERIALS REQUIRED BUT NOT PROVIDED

Loopamp™ PURE DNA Extraction Kit (REF LMC802)

For Visual fluorescence detection

(For LF-160)

- Pipette-60 Set (REF MVL310)
- LF-160 (REF MVKM17)

(For other incubator using UV lamp)

- Incubator (temperature accuracy: ±0.5°C; with hot bonnet)
- Heating block
- UV lamp (wavelength: 240 to 260 nm, and 350 to 370 nm)
- Goggles and a protective eye mask

For Real-time turbidity detection

(For Real-time turbidimeter)

- Pipettes (10 to 100 μL, and 20 to 200 μL) and pipette tips with filter
- Centrifuge for micro-tubes
- Centrifuge for eight connected tubes
- Real-time turbidimeter (only for use in the LAMP method; wavelength: 600 to 700 nm; amplification temperature: 67.0°C)

PREPARATION OF REAGENTS

1) MTBC detection reagent

Remove the required number of tubes from the aluminium pouch and put them on the rack provided. (number of samples + number of controls).

Note: After removing the necessary tubes, seal any unused tubes in the original aluminium pouch immediately.

2) Negative control MTB (NC MTB)

Flick down (Spin down) the tube before use to collect the content in the bottom of the tube. Pipette 60 μL of NC MTB into the Heating Tube provided in Loopamp™ PURE DNA Extraction Kit. Follow the instruction for use of Loopamp™ PURE DNA Extraction Kit to process the NC.

Note: A negative control should be included in every LAMP run.

3) Positive control MTB (PC MTB)

Flick down (Spin down) the tube before use in order to collect the content in the bottom of the tube.

Note: PC MTB should be measured every time.

MEASUREMENT PROCEDURE

DNA Extraction

To extract the DNA from a sputum sample, follow the instruction for Loopamp™ PURE DNA Extraction Kit (sold separately). Use the DNA solution obtained for LAMP amplification.

Reagent and Sample Mixing

(1) Turn on the incubator or the real-time turbidimeter.
(2) Dispense 30 μL of sample solution DNA into a reaction tube using Loopamp™ PURE DNA Extraction Kit, and close the cap.

Note: The volume between the two lines on the reaction tube corresponds to approx. 30μL.

(3) Dispense 30 μL of NC MTB into a reaction tube using Loopamp™ PURE DNA Extraction Kit and close the cap.

Note: The volume between the two lines on the reaction tube corresponds to approx. 30μL.

(4) Dispense 30 μL of PC MTB into a reaction tube using the dropper provided, and close the cap.
(5) Flick down (Spin down) all tubes to collect the solution to the bottom of the tubes.

Note: Make sure the liquid level is in the middle of the two lines on a reaction tube to ensure 30 μL of pipetting.

(6) Reconstitute the dried reagent in the cap by inverting the reaction tubes and collecting the DNA solution in the cap. Leave the tubes standing upside down for 2 minutes to reconstitute the dried reagent.
(7) Invert the reaction tubes five times to mix the content. Make sure that the dried reagent in the cap is fully dissolved.
(8) Flick down (Spin down) all tubes to collect the solution to the bottom of the tubes.

Amplification

For Visual fluorescence detection

(For LF-160)

(1) Check that the temperature on the incubator is 67°C.
(2) Load the reaction tubes into LF-160 incubator and press the green button to start the LAMP reaction (40 minutes at 67.0°C). See LF-160 instruction manual for details on how to operate the incubator.
(3) Confirm the completion of polymerase inactivation (automatically completed by LF-160). Take all reaction tubes from LF-160.

(For other incubator using UV lamp)

(1) Set the temperature of the incubator (with hot bonnet; temperature accuracy: ±0.5°C) at 67.0°C. Wait until the temperature displayed reaches the set value.
(2) Load the reaction tubes, and then start amplification reaction (for 40 minutes at 67.0°C).
(3) Forty minutes later, inactivate the polymerase using the heating block (for 5 minutes at 80°C, or for 2 minutes at 95°C) to terminate the reaction.

For Real-time turbidity detection (see Flow chart of procedure)

(1) Configure the real-time turbidimeter for detection with this product.
(2) Check if the temperature displayed reaches 67.0°C (Allow the turbidimeter to warm up for 20 minutes before use).
(3) Load the reaction tubes, and start measurement.
(4) Watch the display of the turbidimeter to check the positive and negative controls for any increase in turbidity. If the turbidity increases in PC MTB but doesn't in the negative control solution, amplification reaction is proceeding properly (Fig1). If any other situation occurs, however, amplification reaction may be proceeding in a wrong way. In such a case, restart testing from reagent preparation.
(5) Confirm the completion of polymerase inactivation (automatically completed by the turbidimeter). Take all reaction tubes from the turbidimeter and discard them without opening.

Amplification plots

(Analyzer: Loopamp Realtime Turbidimeter LA-320C)

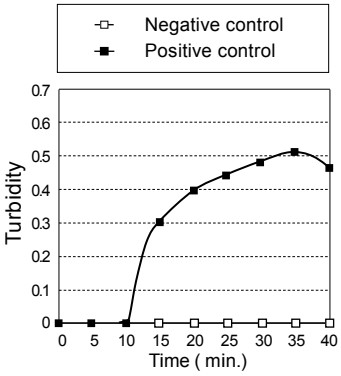

Fig1 : Amplification plots for controls

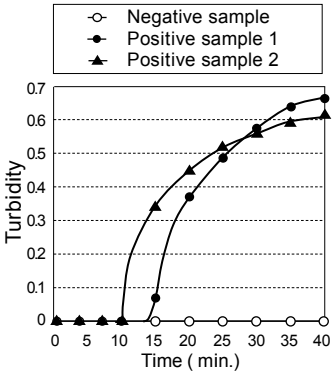

Fig2 : Amplification plots for samples

PROCEDURAL NOTES

(1) Clean benches with over 0.5% sodium hypochlorite before performing the test.
(2) The LAMP reaction is very sensitive, and contamination with small amounts of amplified product might lead to false positive results.
(3) Separate the sputum collection and the LAMP testing areas.
(4) Take all measures necessary to avoid contamination, in particular change gloves after transferring the sputum or if the gloves come into contact with the DNA solution.

(5) When handling this product, avoid microbial contamination and nuclease contamination. Even a small amount of DNase transmitted from sweat or saliva to the reaction tube might decompose DNA and cause false result.
(6) Do not use sputum samples containing a large amount of blood since this may affect measurements.
(7) The DNA solution should ideally be used immediately after preparation. If it is impossible, the DNA solution can be stored at room temperature and used within 72 hours.
(8) When dispensing the solution into the reaction tube, avoid contact between the Injection Cap on Loopamp™ PURE DNA Extraction Kit and the inner wall of reaction tubes. Hold the tube rack upright and fill the tube until the level of DNA solution is between the two lines (30 µL).
(9) **(For LF-160 or other incubator using UV lamp)**
If bubbles are present, flick down the tubes to get rid of them.
(For Real-time turbidimeter)
Since bubbles in reaction solution will interfere with turbidity measurement and cause false judgment, try not to cause any bubble when mixing reagent and sample solution. If bubbles are present, spin down to get rid of the bubbles.
(10) dMTB should be fully dissolved. Any undissolved portion may influence performance, such as causing a decrease in sensitivity.
(11) The PC MTB contains high copy number control DNA. Avoid any contamination of other samples with the PC MTB. Dispense the samples and the NC MTB and close all reaction tubes before dispensing the PC MTB.
(12) Flick down (Spin down) the PC MTB tube before opening it, in order to collect the content at the bottom of the tube. Close the tube immediately after dispensing the PC MTB.
(13) When LF-160 incubator or the real-time turbidimeter is used, polymerase inactivation is automatically performed by LF-160 or the real-time turbidimeter.
(14) Never open the reaction tubes once the LAMP reaction has started or after completion. Be particularly careful when unloading the reaction tubes from the incubator to avoid opening the tubes accidentally.
(15) Do not reuse any amplified product in the tubes for electrophoresis or other applications.
(16) For the real-time turbidimeter or other incubator, when visual fluorescence judgment is chosen, inactivate the polymerase (for 5 minutes at 80°C, or for 2 minutes at 95°C) before judgment, or false judgment will be caused.
(17) For the real-time turbidimeter or other incubator, when a UV lamp is used, do not stare directly at UV light. Use a glass board or wear goggles or a protective eye mask whenever looking at the lamp.

INTERPRETATION OF RESULTS

For Visual fluorescence detection

(For LF-160)

Set the each reaction tube into Fluorescence Visual Check Unit, irradiate and observe the tube from the side.

(For other incubator using UV lamp)

Irradiate the bottom of each reaction tube from the side through goggles or any other protective equipment.

For a valid run, the following results must be obtained:

- Positive Control: Green fluorescent light is emitted.
- Negative Control: No fluorescent light is emitted.

If any control is invalid, all samples in the run should be reported as invalid and the test should be repeated.

After confirming that the run is valid, evaluate samples as follows:

- Positive Sample: Green fluorescent light is emitted.
- Negative Sample: No fluorescent light is emitted.

For Real-time turbidity detection

After confirming that the turbidity increases in PC MTB but doesn't in the negative control solution, evaluate samples in accordance with the following criteria (Fig 1 and 2).

- Positive: Some increase is observed in turbidity.
- Negative: No increase is observed in turbidity.

Notes:

(1) The minimum detectable sensitivity of this product is 0.38 genomes equivalent per test. Even with a negative test, patients with any persisting symptom indicative of infection by MTBC should undergo re-examination.
(2) Although the primers have been designed to target a region containing a relatively small number of variations, MTBC may possibly acquire further variations in this region and become less sensitive to this product. Therefore, a negative test does not always rule out infection by MTBC.
(3) Test results may be affected by specimen collection and transport, specimen preparation, inhibitors, and other laboratory procedural errors. A negative test does not exclude the presence of MTBC from the specimen. In making a clinical diagnosis, take into account the patient's clinical condition and all other available laboratory results.
(4) This product is a kit for qualitative detection; it is not designed for quantitative measurement. The intensity of fluorescent light observed or the rise time of turbidity measured by the real-time turbidimeter does not correlate with the number of template DNA.

INTERFERING SUBSTANCES

Our in-house studies have revealed that measurement was not affected by the presence of free bilirubin (91.0 mg/dL), conjugated bilirubin (101.0 mg/dL), chyle (formazine turbidity: 7,300), and hemolytic hemoglobin (2,475 mg/dL).

With regard to drugs, our in-house studies have revealed that measurement was not affected by the presence of isoniazid (100 µg/mL), ethambutol (20 µg/mL), rifampicin (100 µg/mL), pyrazinamide (500 µg/mL), kanamycin (20 µg/mL), and streptomycin (500 µg/mL).

PERFORMANCE CHARACTERISTICS

1. Sensitivity and Accuracy

In testing the following samples:

- negative sample (concentration: 0 genome equivalent/test)
- positive sample 1 (1.875 genomes equivalent/test)
- positive sample 2 (125 genomes equivalent/test);

The negative sample shall test negative, and the positive samples 1 and 2 shall test positive.

2. Within-run Reproducibility

In testing five negative and positive samples simultaneously, the negative sample shall test negative throughout, and the positive sample shall test positive throughout.

3. Limit of Detection

0.38 genomes equivalent/test

4. Cross-reactivity

As for nontuberculous mycobacteria and respiratory disease bacteria, the measurement system tested negative for all bacterial species as detailed in the table below; no cross-reaction occurred.

Nontuberculous mycobacteria	
Mycobacterium asiaticum	Negative
Mycobacterium kansasii	Negative
Mycobacterium marinum	Negative
Mycobacterium simiae	Negative
Mycobacterium scrofulaceum	Negative
Mycobacterium szulgai	Negative
Mycobacterium gordonae	Negative
Mycobacterium xenopi	Negative
Mycobacterium avium	Negative
Mycobacterium intracellulare	Negative
Mycobacterium gastri	Negative
Mycobacterium haemophilum	Negative
Mycobacterium malmoense	Negative
Mycobacterium chelonae	Negative
Mycobacterium fortuitum	Negative
Mycobacterium flavescens	Negative
Respiratory disease bacteria	
Streptococcus pneumoniae	Negative
Staphylococcus aureus	Negative
Haemophilus influenzae	Negative
Moraxella catarrhalis	Negative
Klebsiella pneumoniae	Negative
Escherichia coli	Negative
Legionella pneumophila	Negative
Pseudomonas aeruginosa	Negative
Mycoplasma pneumoniae (I)	Negative
Mycoplasma pneumoniae (II)	Negative

5. Reactivity against MTBC

Reactivity against *Mycobacterium tuberculosis* and *Mycobacterium bovis* was confirmed.

6. Information about a Calibrator

The performance test for this product used plasmid DNA containing the *gyr*B and IS region of the genome DNA of *Mycobacterium tuberculosis* H37Rv (GenBank No. NC_000962) as a calibrator.

7. Clinical performance

Tuberculosis is still one of the world's largest infections. In the world, the total estimated number of new cases of tuberculosis is about 9.4 million, and that of sputum smear-positive is about 4.3 million (2008; data published by WHO). [6] In Japan, 24,760 patients were newly registered as affected with tuberculosis in 2008, and 2,216 of them have died (*Kekkaku No Tokei* 2009 Statistics of TB 2009). [7]

This product was examined in two medical institutions designated for Class-2 infectious diseases (designated medical institutions that had beds exclusive for patients with tuberculosis) for performance in diagnosing patients suspected of tuberculosis. Along with PCR and TRC, both of which had already been approved in Japan, this kit was used to test samples of sputum collected for two days (320 samples from 160 subjects), and results were compared between kits. Note that testing based on this product included evaluations for DNA extract solution obtained from untreated sputum (hereinafter, referred to as LAMP for untreated sputum) and those for DNA extract solution obtained from sputum pretreated by NALC-NaOH and some other processes (hereinafter, referred to as LAMP for pretreated sputum).

As shown in the tables below, the overall concordance rate was favorable as follows: LAMP for untreated sputum vs. PCR: 91.5% (291/318 subjects); LAMP for pretreated sputum vs. PCR: 92.1% (293/318); LAMP for untreated sputum vs. TRC: 94.3% (230/244); LAMP for pretreated sputum vs. TRC: 93.0% (227/244). In addition, the evaluations made by real-time turbidity detection and those made by visual fluorescence detection were in complete agreement except for one sample. (This sample was bloody sputum and tested false negative in visual fluorescence detection because of disturbance by blood). So, we regard the two types of evaluations as identical with each other, and the tables show test results obtained by real-time turbidity detection. The results of the visual fluorescence detection by LF-160 were the same results by the real-time turbidimeter.

LAMP		PCR		Overall concordance rate
		Positive	Negative	
Untreated sputum	Positive	196	7	91.5% (291/318)
	Negative	20	95	
Pretreated sputum	Positive	194	3	92.1% (293/318)
	Negative	22	99	

LAMP		TRC		Overall concordance rate
		Positive	Negative	
Untreated sputum	Positive	163	5	94.3% (230/244)
	Negative	9	67	
Pretreated sputum	Positive	157	2	93.0% (227/244)
	Negative	15	70	

ORDERING INFORMATION

Product Code	Product Name	Contents
LMC521	***Loopamp™ MTBC Detection Kit***	***96 tests***
LMC802	Loopamp™ PURE DNA Extraction Kit	90 tests
MVL310	Pipette-60 Set	1 pipette; 4 X 96 tips
MVKM17	LF-160	1 Main unit 1 Fluorescence Visual Check Unit

REFERENCES

(1) Notomi T., et al.: Nucleic Acids Research 28, No. 12, e63 (2000)
(2) Nagamine K., et al.: Clin. Chem. 47, No. 9, 1742–1743 (2001)
(3) Mori Y., et al.: Biochem. Biophys. Res. Commun. 289, No. 1, 150–154 (2001)
(4) Tomita N., et al.: Nat. Protoc. 3, No. 5, 877–882 (2008)
(5) The guideline for the bio-safety and bio-hazard (by the Japanese Society for Bacteriology): Japanese Journal of Bacteriology 54, No. 3, 667–715 (1999)
(6) WHO global TB database:
http://www.who.int/tb/country/en/
http://www.who.int/tb/publications/global_report/2009/update/a-1_summary.pdf
http://www.who.int/tb/publications/global_report/2009/update/a-3_summary.pdf
(7) Kekkaku No Tokei 2009 (Statistics of TB 2009): Japan Anti-Tuberculosis Association

Flow chart

Operation procedure for Real-time turbidity detection

Preparation of sample solution and negative control solution

Pipette 60 µL of suitable sputum containing a purulent portion. Pipette 60 µL of NC MTB.

With Loopamp™ PURE DNA Extraction Kit, extract DNA from the collected sputum and NC MTB. The obtained solutions are used as sample solution and negative control solution, respectively.

Reagent preparation

Take a required number of reaction tubes of dMTB.

Mixing of reagent and sample solution

(For samples, negative and positive controls)

Transfer 30 µL of sample or control solution into each reaction tube.

Use the negative control solution as negative control.
Use PC MTB as positive control.
(The positive control should be prepared last.)

Invert the reaction tubes to collect the solution on the cap.

Invert the reaction tubes five times to mix the contents, and then spin them down.

(Avoid any bubbles.)

Amplification

Load the reaction tubes onto the reaction block of the turbidimeter.

As directed in the instruction for use of the turbidimeter, start reaction and measure and evaluate the turbidity (for 40 minutes at 67.0°C).

Confirm the completion of polymerase inactivation (for 5 minutes at 80°C, or for 2 minutes at 95°C). Take all reaction tubes from the turbidimeter and discard them without opening. Be careful not to damage the tubes.

IVD

CE

EC REP

Advena Ltd.
Tower Business Centre, 2nd Flr., Tower Street, Swatar, BKR 4013 Malta

EIKEN CHEMICAL CO., LTD.
4-19-9, Taito, Taito-ku, Tokyo 110-8408, Japan

Date of Revision: April 1, 2019