For research use

Loopamp ™

Legionella Detection Kit *E*

[Characteristics]

LAMP (Loop-mediated Isothermal Amplification) method is a gene amplification method capturing the following characteristics: (1) Only one enzyme is required and the amplification reaction proceeds under isothermal conditions [1) 2)]. (2) 4 primers recognizing 6 distinct regions on the target achieve high specificity. (3) High amplification efficiency allows amplification within a short time. (4) It produces tremendous amount of amplified products which makes simple detection possible [3) 4) 5)]. Amplification of nucleic acids with this kit is conducted by the LAMP method using *Legionella spp.* 16S rRNA sequence as the primers. The homology between the sequence for primers and that of non-*Legionella* spp. are very low, in contrast, high specificity in detection of the 11 species and 21 strains *Legionella* including the 11 serotypes *Legionella pneumophila* when using LAMP method for amplification.

From whether the amplification occurred or not, the existence of *Legionella* spp. can be determined. By using the specifically designed Loopamp Realtime Turbidimeters, detection does not need electrophoresis and all steps from amplification to detection are done within one reaction tube. Comparing with conventional culture methods[6)], LAMP detection of *Legionella* can be done much faster[7)8)].

[Contents of the kit]

		48 tests
(1) Extraction Solution for *Legionella*	(EX Leg) [*1]	1.8 ml × 2 tubes
(2) 1M Tris-HCl:pH7.0	(Tris)[*1]	1.0ml × 1 tube
(3) Reaction Mix. Leg	(RM Leg) [*1]	1.0mL× 1 tube
(4) *Bst* DNA Polymerase	(Bst DNA Polymerase)[*1]	60 µl × 1tube
(5) Positive Control Leg	(PC Leg)[*1]	0.1 ml × 1 tube

*1: The notation on each reagent tube is shown in ( ).

[Intended Use]

Detection of *Legionella* spp. in environmental specimens (hot spring water, bath tub water, cooling tower water etc.) and concentrate it 100times by filtrate concentrating or cooling centrifugation concentrating to detect *Legionella* spp[6)].

[Assay principle]

This kit uses LAMP method as assay principle. First, extract DNA from concentrated environmental water specimen (concentrated sample water) with alkalis heat extraction method and then neutralize the DNA solution, and use it as sample solution. Mix the sample solution with Reaction Mix. Leg and *Bst* DNA Polymerase and incubate it. When *Legionella* 16S rRNA sequence can be recognized by the primer, its DNA will be amplified with the activity of *Bst* DNA polymerase. The detection of the nucleic acid is done by detecting the turbidity change[3)4)] caused by the amplification by-product magnesium pyrophosphate (white precipitate) and then determines whether there is *Legionella* spp.

For further details of the LAMP principle, refer to Eiken GENOME SITE (URL: http://loopamp.eiken.co.jp/e/).

[How to use]

1. Materials required but not provided

- Sterilized tubes for master mix preparation (0.5mL or 1.5mL)
- Micropipette (0.5~10µL, 10~100µL, 100~1000µL)
- Pipette tips with filter
- Sterilized tubes (2.0mL)
- Loopamp Reaction Tube
- Aluminum rack for cooling tubes
- Heat block (Capable of heating up to 95 ºC)
- Crushed ice and ice box
- Loopamp Realtime Turbidimeter
- Refrigerated centrifuge for microtube
- Centrifuge for 8-strip tube
- Vortex mixer

2. Preparing the reagent

1) Take out the reagents stored at -20ºC, and thaw them at room temperature. Once the reagents are thawed, keep them on ice immediately.
2) Preparation of master mix (Operate on ice)
   (1) Dispense the appropriate amount according to the number of tests to be conducted into the separately prepared sterilized tube under the proportion of Reaction Mix. Leg (RM Leg) 20µL and *Bst* DNA Polymerase 1µL per test (including positive control and negative control tests).
   (2) After dispensing, mix the solution well by gently tap the tube a few times (hereinafter referred to as "tapping"), invert the tube, or vortex mixer at 1 second ×3 times. After mixing well, spin down the tube and the mixture can be used as the master mix for the reaction. Notice that too much mixing by the vortex mixer might inactive the polymerase, and assure that vortexing is conducted at 1 second×3 times. The prepared master mix should be used as soon as possible.

3. Preparing sample solution

DNA extraction solution can be prepared by using alkali heat extraction method[9)].

1) Put 2mL of concentrated sample water (100 times concentrated sample water[6)]) in the separately prepared sterilized tube (2mL) and centrifuge at 4 ºC, 13,000×g for 10 minutes then remove the supernatant (about 1960µL) and leave around 40µL.
2) After spin down, add 50µL of Extraction Solution for *Legionella* (EX Leg) and mix it well using vortex mixer.
3) After spin down, incubate at 95 ºC for 15 minutes and cool it on ice immediately.
4) After spin down, add 8µL of 1M Tris-HCl: pH7.0 (Tris) and mix it well using vortex mixer.
5) Centrifuge under 4 ºC, 13,000×g for 10 minutes, then keep it on ice, use the supernatant as sample solution (equivalent 2000 times concentrated sample water).

4. Operation procedure

1) Mixing Master Mix with sample solution (Operate on ice)
   (1) Dispense 20µl of Master Mix into each Loopamp Reaction Tube.
   (2) Add 5µl of sample solution to the master mix, and the volume of the solution should be 25µl in total. Mix the solution well by pipetting or tapping the tube with the cap closed and then spin down. Be careful not to cause air-bubbles when mixing.
   (3) For control reactions, use 5µl of Positive Control Leg (PC Leg) as positive control and 5µl of Extraction Solution for *Legionella* (EX Leg) for as negative control.
2) The amplification reaction and realtime turbidity detection
   (1) Loopamp Realtime Turbidimeters are applicable to this kit. Turn on the device about 20 minutes before use. Set the parameters as follows:
   [Temperature]: Reaction Block 65ºC, Hot Bonnet 75ºC
   [Measurement Time]: 60 min
   [Inactivation]: 80ºC, 2 min
   (2) Confirm that the temperature has reached 65ºC
   (3) Set the prepared reaction tubes and immediately start reaction.
   (4) Check whether the positive and negative controls' turbidity rose from the display screen of the device. If the positive control's: Positive Control Leg (PC Leg) turbidity rose and the negative control's: Extraction Solution for *Legionella* (EX Leg) turbidity didn't, then LAMP reaction proceeded properly (Fig.1). If not, there might have been error in the process. Restart from reagent preparation and check again.
   (5) If the increase in turbidity is confirmed within 60 minutes, then it is judged as positive, if not, negative (Fig.2).
   (6) The rising timing or turbidity of samples might be different from the positive control.
   (7) After enzyme inactivation (80ºC, 2 min), done automatically by Loopamp Realtime Turbidimeter, is confirmed to have ended, remove the used reaction tubes and dispose them with caps closed.

■Amplification Curve Pattern

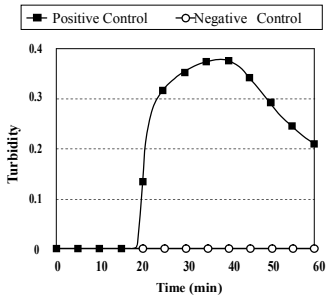


Fig.1 The amplification curve pattern of controls.

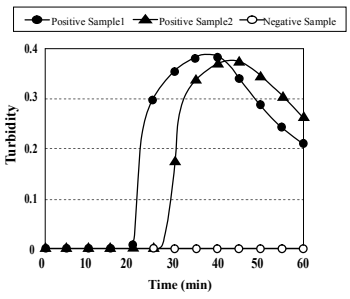


Fig.2 Samples amplification curve pattern.

This kit is not developed for the purpose of quantitative analysis, therefore, the copy numbers do not necessarily correlate with turbidity increment time.

[Cautions for operation]

1. Sample handling

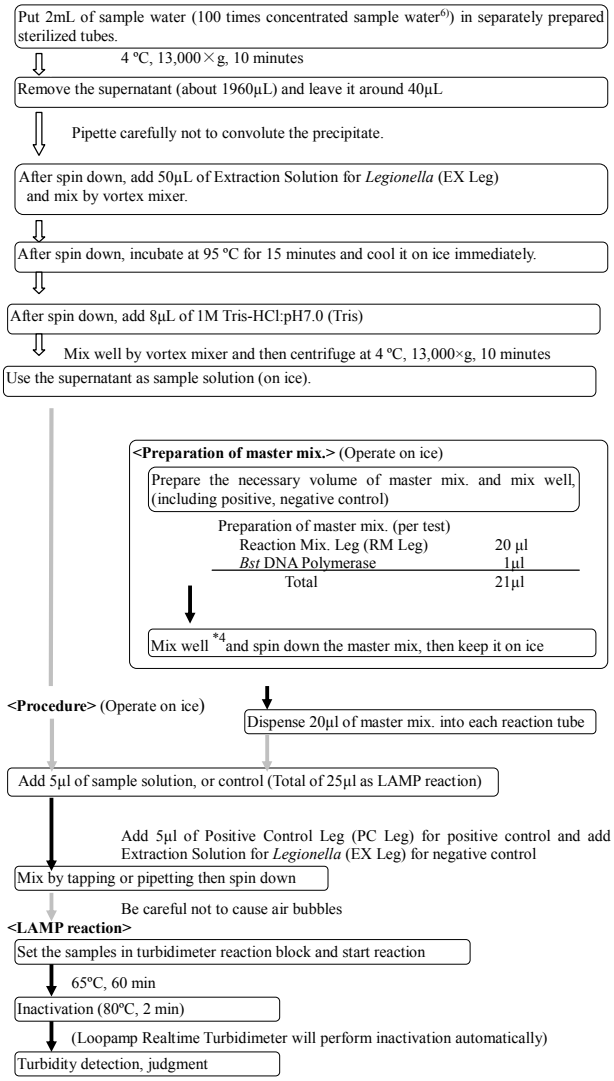
1) When removing the supernatant (about 1960µL) from 2mL of 100times concentrated water sample, pipette carefully as to convolute the precipitate.
2) The sample (DNA extraction solution) should be used immediately. However, if it is to be stored for long period of time, it should be stored under -80ºC and repeated freezing and thawing should be avoided.

2. Reagent handling

1) The reagent kit should be stored at -20ºC. To prevent the reagents from deterioration, only take out the necessary amount of reagents from the freezer before use. No decline was observed in the kit performance even after repeated freezing and thawing for 20 times in the quality control test. But, in order to maintain the reagent performance, avoid unnecessary freezing and thawing.
2) Thaw the reagent at room temperature, and keep them on ice for reagents preparation. Before use, spin down the tubes to drop down the solution staying on the tube wall or on the cap. Mix well the solution and spin down again before use. Notice that fierce mixing should be avoided as it can inactivate the *Bst* DNA Polymerase.
3) Positive Control Leg (PC Leg) contains high number of target DNA. In order to prevent Positive Control Leg from contaminating other samples or reagents, always spin down the tube, and open the cap as shortly as possible. Moreover, to avoid contamination, do not use Positive Control Leg in any other way not written in this instruction.
4) Keep positive and suspected positive samples away from the reagent when handling
5) If there is any reagent left, do not mix or use it kits even if they are in the same lot.

■ Protocol

<Pre-treatment; DNA extraction solution> (Operate on ice)



*4:Mix by tapping or invert them several times or vortex them for 1 second × 3 times.

3. Handling the reaction tube

1) Only use the specified Loopamp Reaction Tube for turbidity detection. Other reaction tubes might have different optical transparency and can cause misjudgment.
2) Take full care when handling reaction tubes, as they are vulnerable to scratches or damages.
3) Check carefully to see if the reaction tubes have any crack or scratch before use. Crack or scratch on the tube might not only cause false judgment but also contaminate the equipment. If the tubes are broken inside the reaction block of Loopamp Realtime Turbidimeter, the reaction mixture can spill inside the equipment and cause unrecoverable contamination and malfunction.
4) By comparing the solution volume in all tubes, check visually if proper amount of sample solution and master mix has been dispensed into the reaction tube.

4. Cautions for amplification reaction

Since bubbles in the solution will interfere the turbidity measurement and cause false judgment, try not to cause any bubble when mixing the master mix and the sample solution. If bubbles appear, spin down to remove them.

5. Cautions for detection and judgment

1) Use only Loopamp Realtime Turbidimeter and Loopamp Reaction Tube for the assay.
2) Start up Loopamp Realtime Turbidimeter at least about 20 minutes before use it.
3) For judgment, check whether the turbidity of Positive Control Leg (PC Leg) has risen to determine whether the reagent is performing properly (if the nucleic acid amplification reaction is performed properly, the turbidity will start to rise around 20 minutes after the reaction started). There are cases where the turbidity of the sample starts rising later than the positive control.

6. Handling reaction tubes after use

1) The caps of the used reaction tube should not be opened. Pay special attention not to accidentally open the cap when taking the tubes out of the turbidimeter. Contamination of amplified products on other samples may not only cause false judgment of the test result but also pollute testing area. In this case, a correct test result might not be obtained until the contamination is removed completely.
2) . Keep the cap of the used tube completely closed and dispose it according to the relevant regulations and instructions by incineration or after double bagging it with sealable vinyl bag. To prevent the amplified products from dispensing, do not conduct autoclave sterilization treatment for disposal.

7. Inhibitory substance

LAMP reaction will be inhibited if there is over 3µmol/L of Iron ion or 500µmol/L of Manganese ion in the 25µL of reaction solution. When use hot spring water which contains a large amount of metallic ion as sample water, be careful that it might be interfered LAMP reaction.

[Performance]

1. Specificity

The 11 spices 21 strain of *Legionella* spp. that contains 11serogroupe of *Legionella pneumophilla* can be specifically detected. The 19 species 19 strain of *non-Legionella* spp. that contains community acquired pneumonia pathogen cannot be reacted with this kit[7)8)].

2. Sensitivity

Minimum detection limit: 60 CFU (Colony Forming Unit)/test*5

*5: The sensitivity is determined by using preserved strains of *Legionella pneumophilla*. When use actual environment water, it cannot be compared directly with the performance and sensitivity of conventional culture method.

3. Correlation of culture method

49 water samples which showed the positive results with culture method are determined all positive by LAMP method, and these results do not find discrepancy. In the 51 water samples determined as negative with culture method showed 22 of them as positive results by LAMP method[11)].

[Cautions for handling]

1. This kit is for the purpose of environment detection of *Legionella* spp., not for medical or clinical diagnostic purposes on human or animal samples.
2. Although the test result is negative, this test is incapable of determining the complete absence of *Legionella* spp. Combined interpretations with other test results are recommended.
3. Abide by the necessary counter biohazard regulations for sample taking and handling[6)10)].
4. LAMP reaction is extremely sensitive and even the slightest amount of amplified products tainted into the reaction might cause the false result. Therefore, avoid this type of contamination by carrying out the sample and reagent preparation in different clean benches. Avoid electrophoresis or operations that need to handle amplified products.
5. Do not expose Loopamp Reaction Tube, master mix. preparation tubes to UV light. A change in color or degeneration caused by ultraviolet lamp sometimes results in misjudgment.
6. If there is DNase in the reaction solution, the reaction might cause the false result. Pay attention to handle the operation under proper environment with clean equipments and instruments.
7. If the operator does not have the knowledge or experience in genetic test, there is a possibility of false judgment. Therefore, be sure to use the kit under the supervision of the genetic testing experienced and knowledgeable technicians.
8. EIKEN CHEMICAL CO., LTD. does not bear responsibility for false judgment or any consequential damage from the misuse or mishandling.
9. Use the kit before the expiration date, which is labeled on the outer box (Exp. Date).
10. This kit should be stored at -20 ºC. Storing the kit at temperature lower than -20 ºC or repeated freezing and thawing might cause cracks on the tube of 1M Tris-HCl:pH7.0(Tris).
11. The reagent tube is made of polypropylene and the main material for kit case is paper. The institution disposing the reagents, reagent tubes, container, and lab-ware and case should bear the responsibility and abide by the clinical waste disposal regulations, water pollution prevention law, and any local regulation related.

[Unit, Storage, Expiration and code No.]

Product	Unit	Storage	Expiration	Code No.
Loopamp™ *Legionella* Detection Kit *E*	48 tests	-20°C	1 year	LMP661

[Reference]

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.: Abstract for the 23rd Annual Meeting of the Molecular Biology Society of Japan(2000)
4) Mori Y. et al.: Biochem. Biophys. Res. Commun. **289**, No.**1**, 150-154(2001)
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6) The supervised by the Ministry of health, labour and welfare, Living environment: The guideline for the prevent of Legionella disease (in Japanese, 2000).
7) Annaka T. et al.: J. Japanese society for Clin. Microbiol. **13**, No.1, 19-25 (2003).
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9) Beige, J. et al.: J. Clin. Microbiol. 33 : 90-95 (1995)
10) 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)
11) Inoue H, et al.: SAAAJ, **32**, No.10, 481-487 (2004)

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