For research use

Loopamp™
Verotoxin Typing Kit

[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 condition[1) 2)]; (2) 4 primers recognizing 6 distinct regions on the target achieve high specificity, (3) High amplification efficiency allows amplification within a shorter 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 the sequences related to Verotoxin (VT) as the primers, and VT related gene in the specimen is typed.

By using the specifically designed Loopamp Real-time Turbidimeters, detection does not need electrophoresis and all steps from amplification to detection are done within one reaction tube. Simple and rapid typing of VT related gene can be achieved[6)7)8)].

[Contents of the kit]

		24 tests
(1) Extraction Solution for Foods	(EX F)*	1.8mL × 1 tube
(2) Reaction Mix.VT1	(RM V1)*	0.5mL × 1 tube
(3) Reaction Mix.VT2	(RM V2)*	0.5mL × 1 tube
(4) *Bst* DNA Polymerase	(Bst DNA Polymerase)*	60μL × 1 tube
(5) Control DNA VT1	(Cont V1)*	0.1mL × 1 tube
(6) Control DNA VT2	(Cont V2)*	0.1mL × 1 tube

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

[Intended use]

Typing of Verotoxin I (VT1) and II (VT2) produced by Verotoxin producing *Escherichia coli*.

[Principle]

This kit uses LAMP method as assay principle. First, conduct enrichment culture of the food specimen and then after Alkalis heat extraction, the solution can be used as sample. Mix the sample solution with Reaction Mix.VT1 (RM VT1) or Reaction Mix.VT2 (RM VT2) and *Bst* DNA polymerase, and then incubate them. When Verotoxin I (VT1) and II (VT2) gene sequence that can be recognized by the primers exists, its DNA will be amplified with the activity of *Bst* DNA polymerase. . Gene amplification is detected using Loopamp Real-time Turbidimeter by monitoring turbidity of white precipitates of magnesium pyrophosphate, which is a by-product of amplification reaction[2)].Based on the amplification curve, the type of VT related gene in the specimen is judged. For details of the LAMP method, refer to the Eiken GENOME SITE (URL; http://loopamp.eiken.co.jp/e/)

[How to use]

1. Materials required but not provided

- Distilled Water
- Sterilized tubes for master mix preparation (0.5mL or 1.5mL)
- Micropipette (0.5~10μL,10～100μL,100～1,000μL)
- Pipette tips with filter
- Sterilized tubes for pre-treatment of specimen (0.5mL)
- Heat block (Use at 95°C)
- Loopamp Reaction Tube
- Aluminum rack for cooling tubes
- Crushed ice and ice box
- Loopamp Realtime Turbidimeter
- Centrifuge for microtubes
- Centrifuge for 8-strip tube
- Vortex mixer

2. Sample solution preparation

Suspension (3×10^6 CFU/mL) of bacteria suspected to be STEC by food microbe testing

Microbe culture or the colony formed on the agar plate →
Suspend in DW (McFarland No.1[8)]) → Dilute 100 times.
→ Dilution (3×10^6 CFU/mL)

1) Pretreatment of testing specimen.
   (1) Prepare necessary quantity of sterilized tube for pretreatment of specimen, and pour 50μL each Extraction Solution for Foods (EX F) in the tubes.
   (2) Add 50μL of testing specimen (pre-enrichment culture) into each tube.
   (3) Close the cap of the tubes, invert them several times to mix thoroughly and spin down with the centrifuge. Heat the tubes at 95°C for 5 minutes. Then centrifuge the tubes for 1 minute and place them on ice (Sample Solution). The Sample Solution can be kept for 4 hours at 0~4°C.

3. Reagents preparation

1) Take out the reagents stored at -20°C, and thaw then at room temperature. Once the reagents are thawed, keep them on ice.
2) Preparation of master mix. (Operation on ice)
   (1) Dispense the appropriate amount (including positive and negative control tests) into the separately sterilized tube under the proportion of Reaction mix.VT1 (RM V1) 20μL and *Bst* DNA polymerase 1μL per test (Master Mix. VT 1). Prepare Master Mix VT2 in a same manner
   (2) After dispensing mix the solution by gently tap the tubes a few times (hereinafter referred to as tapping) or invert the tube or vortex them 3 times for 1 second. 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.

4. Operation procedure

1) Mixing of master mix. and sample solution (Operate on ice)
   (1) Dispense 20μL of Master Mix.VT1 or Master Mix.VT2 into each Loopamp Reaction Tube.
   (2) Add 5μL of sample solution to the master mix. and the volume of the solution should be 25μLin 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 bubble when mixing.
   (3) For control reactions, use 5μL of Control DNA VT1 (or VT2) as positive control and 5μL of Extraction Solution for Foods as negative control instead of sample solution.
2) Amplification reaction and real-time detection
   (1) Loopamp Real-time turbidimeter is applicable to this kit. Set the parameter as follows:
   [Temperature]; Reaction Block: 65°C, Hot bonnet: 75°C
   [Measurement Time]; 60 min
   [Inactivation]; 80 °C, 2 min
   (2) Confirm that the temperature has reached 65°C.
   (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 turbidity of control rose and turbidity of negative control 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) Next, the judgment of each sample is conducted. 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; Control DNA VT1 or VT2 (Cont V1 or V2).
   (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 patterns

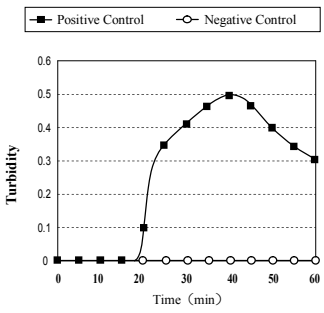


Fig.1 The amplification curve pattern of controls

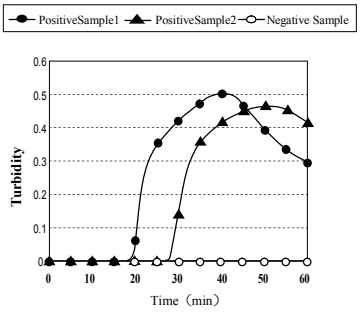


Fig.2 Samples amplification curve pattern

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

[Cautions for operation]

1. Sample handling

1) When collecting pre-enrichment culture, pipette carefully as to not convolute the precipitate.
2) Basically, 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) This reagent kit should be stored at -20°C. 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 reagents performance, avoid unnecessary freezing and thawing.
2) Thaw the reagents at room temperature and keep them on ice for reagents preparation. Before use, spin down the tubes to drop down the reagents staying on the tube wall or on the cap, mix well the reagents and spin down again. Notice that fierce mixing should be avoided as it can inactivate the *Bst* DNA polymerase.
3) Extraction Solution for Foods (EX F) gradually deteriorates when exposed to air. Opening and closing the cap of Extraction Solution for Foods should be limited as minimum as possible so that the time for exposing the solution to air can be limited as minimum as possible. Please add Extraction Solution for Foods (EX F) as soon as possible for pre-treatment. When storing Extraction Solution for Foods, keep its cap tightly closed, and do not aliquot the solution.
4) Control DNA VT1 (Cont V1) and Control DNA VT2 (Cont V2) contains high number of target DNA. In order to prevent Control DNA VT1 and Control DNA VT2 (Cont V2) from contaminating other samples or reagents, always spin down

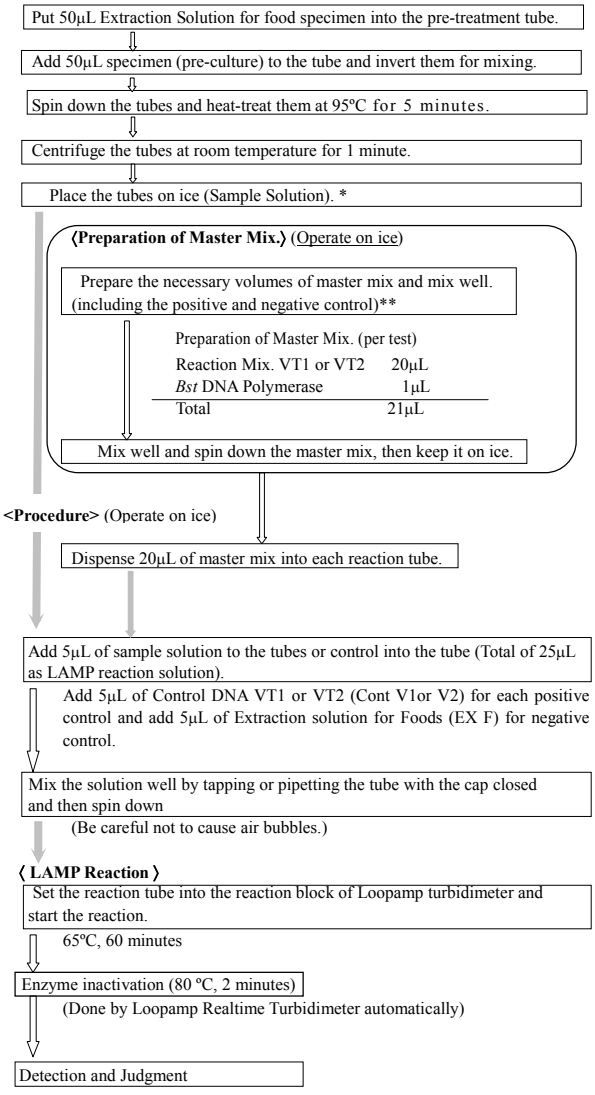
before opening the tube and open the cap of the tube as shortly as possible. Also add into the reaction tubes under the following order from negative control (Extraction Solution for Foods (EX F)), sample solution (extracted DNA), and leave the adding of Control DNA VT1(Cont V1) and Control DNA VT2 (Cont V2) to the last and make sure that all other tube caps are closed when adding it. Moreover, to avoid contamination, do not use Control DNA VT1 (Cont V1) and Control DNA VT2 (Cont V2) in any other way not written in this instruction (such as diluting the positive control or adding it to samples).

5) Keep positive control and suspected positive samples away from the reagents when handling.

6) If there is any reagent left, do not use it with other kits even if they are in the same lot.

■Protocol

〈Pretreatment: DNA extraction solution preparation〉 (Operate on ice)



* : Sample Solution remains active for up to 4 hours at 0-4ºC.

** : Invert tubes for mixing or vortex the tubes 3 times for 1 second.

3. Handling 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 the 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. Caution 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 using it.
3) For judgment, check whether the turbidity of Control DNA VT1 or VT2 (Cont V1 or V2) 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 tubes should not be opened. Pay special attention not to accidentally open the cap when talking 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 may not be obtained until the contamination is completely removed.

2) Keep the cap of the used tube completely closed and disposed 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 dispending, do not conduct autoclave sterilization treatment for disposal.

[Performance]

Minimum detection limitation: 60 CFU (Colony Forming Unit) / test

[Caution for handling]

1. LAMP reaction is very sensitive and even the slightest amount of amplified product tainted into the reaction might cause 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.
2. This kit is not available to the test for the food specimen consists of the liver, because the LAMP reaction is susceptible to the liver ingredients.
3. The culture medium is handled according to each manual. Try the appropriate measure for the biohazard when the specimen is processed[9].
4. Do not expose the Loopamp Reaction Tube, master mix preparation tubes to UV light. A change in color or deterioration caused by ultraviolet lamp sometimes results in misjudgment.
5. This kit is for the purpose of foods and environment inspection, not for medical or clinical diagnostic purposes on human or animal samples.
6. If the operator does not have the experience or knowledge in the field of nucleic acid testing, there is a possibility of false judgment. Therefore, make sure that the kit is used under the supervision of the experienced and knowledgeable technicians.
7. This kit can detect VTEC through amplifying its gene, which is different from the conventional culture method that can only detect viable bacteria of VTEC. Use this kit as a part of self-imposed test.
8. The result of this kit might be different from that of the culture method.
9. Eiken Chemical Co., Ltd. does not bear any responsibility for false judgment or any consequential damage derived from the false judgment caused by non-capability problems such as operation error.
10. Use the kit before the expiration date, which is labeled on the outer box (Exp. Date).
11. The reagent tube is made of polypropylene and the main material for kit case is paper. The institution disposing the reagent tube and case should bear the responsibility and abide by the clinical waste disposal regulations, water pollution prevention law, and any other regulation related.

[Unit, Storage, Expiration, Code No.]

Product name	Unit	Storage	Expiration	Code No.
Loopamp™ Verotoxin Typing Kit	24 tests	-20 °C	1 year	LMP 641

[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)
5) Tomita N. et al.: Abstract for the 26th Annual Meeting of the Molecular Biology Society of Japan (2003)
6) Nemoto J. et al.: the 23rd Japan Food microbiology Conference. Abstract p 26 (2002)
7) Microbiology, Food sanitary testing guide, edited with Japan Food Sanitary association, compiled under milk and meat sanitary sector, Life-sanitary department, Minister of public welfare, Tokyo (1990), Addendum Ⅰ(1993), Addendum Ⅱ(1996)
8) Manual of Clinical Microbiology, fourth edition, p1095, American Society for Microbiology, Washington, D.C.(1985)
9) Japan bacteriology, **54**, No.3, 667-715 (1999)

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