For research use

Loopamp ™

Cryptosporidium Detection Kit

[Characteristics]

LAMP (Loop-mediated Isothermal Amplification) method is a novel 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) 6)]. Amplification of nucleic acids with this kit is conducted by the LAMP method using *Cryprosporidium* 18S rRNA sequence as the primers. From whether the amplification occurred or not, the existence of *Cryptosporidium* 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.

[Contents of the kit]

	48 tests
(1) 2 × Reaction Mix. (RM) [*1]	0.6 ml × 1 tube
(2) Primer Mix. Cry (PM Cry)[*1]	0.12 ml ×1 tube
(3) *Bst* DNA Polymerase (Bst DNA Polymerase)[*1]	60 µl × 1 tube
(4) Distilled Water (DW)[*1]	1.0 ml × 1 tube
(5) Positive Control Cry (PC Cry)[*1]	0.1 ml × 1 tube

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

[Intended Use]

Detection of *Cryptosporidium* spp. in environmental specimens.

[Assay principle]

This kit uses LAMP method as assay principle. First, concentrate environmental water sample, then extract DNA and use it as sample solution. Mix the sample solution with 2 x Reaction Mix (RM), Primer Mix. Cry (PM Cry), Distilled Water (DW) and *Bst* DNA Polymerase and incubate it. When *Cryptosporidium* 18S 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)] caused by the amplification by-product magnesium pyrophosphate (white precipitate) and then determines whether there is *Cryptosporidium* spp. For further details of the LAMP principle, refer to Eiken GENOME SITE (URL: http://loopamp.eiken.co.jp/e/).
This kit is developed for the purpose of qualitative analysis, not for quantitative analysis.

[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
- Heat block (Capable of heating up to 95 ºC)
- Aluminum rack for cooling tubes
- Crushed ice and ice box
- Centrifuge for microtube
- Centrifuge for 8-strip tube
- Vortex mixer
- Ultrasonic Cleaner
- Loopamp Reaction Tube
- Loopamp Realtime Turbidimeter
- Proteinase K solution[*2]

*2: Regarding preparation of this solution, refer to “Loopamp *Cryptosporidium*/*Giardia* Detection Kit Operation Manual”.

2. Preparing sample solution

1) Prepare the beads-oocyst and/or -cyst complex from environmental water sample by immunomagnetic beads separation method[7)]. (Before dissociation of beads-oocysts/-cysts complex)
2) DNA Extraction[8)]
Regarding DNA extraction, refer to “Loopamp *Cryptosporidium /Giardia* Detection Kit Operation Manual.
3) Before the measurement, heat the sample solution at 95ºC for 5 minutes, and then immediately place them on the ice for more than 5 minutes.

3. 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)
Dispense the appropriate amount according to the number of tests to be conducted into the separately prepared sterilized tube under the following proportion per test:

	<for 1 test>	<for 10 tests>
2 × Reaction Mix. (RM)	12.5µl	125µl
Primer Mix. Cry (PM Cry)	2.5µl	25µl
Bst DNA Polymerase	1.0µl	10µl
Distilled Water (DW)	4.0µl	40µl
Total	20µl	200µl

The prepared master mix should be used as soon as possible.

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.
(3) For control reactions, use 5µl of Positive Control Cry (PC Cry) as positive control and 5µl of Distilled Water for as negative control.
Be careful not to cause air-bubbles when mixing.
By comparing the solution volume in all tubes, check visually if proper amount of sample solution/master mix has been dispensed into the reaction tube.
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 63ºC, Hot Bonnet 73ºC
[Measurement Time]: 60 min
[Inactivation]: 80ºC, 5 min
(2) Confirm that the temperature has reached 63ºC
(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 turbidity rose and the negative control's 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.
(6) The rising timing or turbidity of samples might be different from the positive control.
(7) After enzyme inactivation (80ºC, 5 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

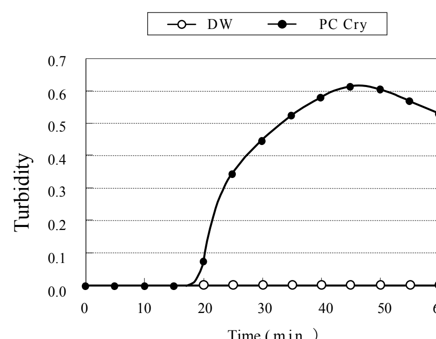
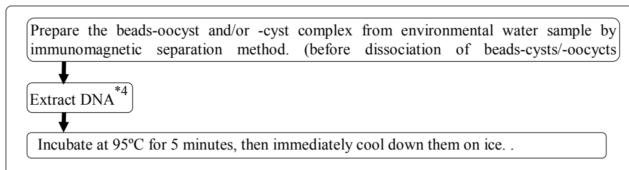


Fig.1 The amplification curve pattern of Positive Control Cry (PC Cry) [*3]
(Loopamp Realtime Turbidimeter LA-320C is used)

*3: The copy numbers do not necessarily correlate with rising timing.

■ **Protocol**

<Pre-treatment; DNA extraction solution>



<Preparation of master mix.> (Operate on ice)

Prepare the necessary volume of master mix. and mix well, (including positive, negative control)

Preparation of master mix. (per test)

2 × Reaction Mix. (RM)	12.5µl
Primer Mix. Cry (PM Cry)	2.5µl
Bst DNA Polymerase	1.0µl
Distilled Water	4.0µl
Total	20.0µl

Mix well *5 and spin down the master mix, then keep it on ice

<Procedure> (Operate on ice)

Dispense 20µl of master mix. into each reaction tube

Add 5µl of sample solution, or control (Total of 25µl as LAMP reaction)

Add 5µl of Positive Control Cry (PC Cry) for positive control and add 5µl of Distilled Water (DW) for negative control

Mix by tapping or pipetting then spin down

Be careful not to cause air bubbles

<LAMP reaction>

Set the samples in turbidimeter reaction block and start reaction

63ºC, 60 min

Inactivation (80ºC, 5 min)

(Loopamp Realtime Turbidimeter will perform inactivation automatically)

Turbidity detection, judgment

*4: Regarding DNA extraction, please refer to "Loopamp *Cryptosporidium /Giardia* Detection Kit Operation manual.

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

[Sensitivity]

Minimum detection limit: 60 copies/test*5

*6: The sensitivity is determined by using plasmid DNA which target gene sequence of *Cryptosporidium parvum* is inserted.

[Cautions for operation]

1. Sample handling

1) 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 Cry (PC Cry) contains high number of target DNA. In order to prevent Positive Control Cry 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 Cry in any other way not written in this instruction.
4) Keep positive and suspected positive samples away from the reagent when handling
5) Use the kit before the expiration date, which is labeled on the outer box (Exp. Date).
6) If there is any reagent left, do not mix or use it kits even if they are in the same lot.

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 Cry (PC Cry) 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.

[Cautions for handling]

1. This kit is for the purpose of environment detection of *Cryptosporidium* 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 *Cryptosporidium* spp. Combined interpretations with other test results are recommended.

3. Abide by the necessary counter biohazard regulations for sample taking and handling[9].

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 the 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. 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™ *Cryptosporidium* Detection Kit	48 tests	-20°C	1 year	LMP741

[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.: Biochem. Biophys. Res. Commun. **289**, No.**1**, 150-154 (2001)

4）Tomita N. et al..: Abstract for the 73rd Annual Meeting of the Biochemistry Society of Japan（2000）
5）Mori Y. et al.: Abstract for the 23rd Annual Meeting of the Molecular Biology Society of Japan（2000）
6）Tomita N. et al.: Abstract for the 26th Annual Meeting of the Molecular Biology Society of Japan（2003）
7）Japan Water Works Association: Cryptosporidium-Commentary and test procedure-（in Japanese, 2003）
8）Endo. T. et al; Study report (in Japanese, 2006).
9）National Institute of Infectious Disease: The guideline for the bio-safety and bio-hazard.(in Japanese, 2007).

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