For research use

Loopamp™
Primer Set for Koi herpesvirus

[Characteristics]

LAMP (Loop-mediated Isothermal Amplification) method is a gene amplification method captured the following main characteristics: (1) Only one enzyme is required and the amplification reaction proceeds under isothermal condition[1)2)]. (2) Four 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) 6)].

This reagent consists of the primer set for the detection of the genomic DNA of koi herpesvirus; KHV [7)], which is specifically designed for Loopamp DNA Amplification Kit[8)].

[Contents of the kit]

			48 tests
(1)	Extraction Solution for F	(EX F) *1	1.8 mL × 3 tubes
(2)	1M Tris-HCl: pH 7.0	(Tris) *1	1.0 mL × 1 tube
(3)	Primer Mix. KHV	(PM KHV) *1	0.12 mL × 1 tube
(4)	Positive Control KHV	(PC KHV) *1	0.1 mL × 1 tube

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

[How to use]

1. Materials required but not provided

- ○ 1.5mL Homogenization Pestle for 1.5mL microtube
- ○ Sterilized tubes for sample preparation (1.5mL)
- ○ Sterilized tubes for master mix preparation (0.5mL or 1.5mL)
- ○ Micropipette (0.5~10μL, 10~100μL, 100~1000μL)
- ○ Pipette tips with filter
- ○ Aluminum rack for cooling tubes
- ○ Crushed ice and ice box
- ○ Heat block (Capable of heating up to 95 ºC)
- ○ Centrifuge for microtube
- ○ Centrifuge for 8-strip microtube
- ○ Vortex mixer
- ○ Loopamp Reaction Tube
- ○ Loopamp Realtime Turbidimeters
- ○ TE buffer for simplified extraction method (10mM Tris-HCl,1mM EDTA:pH8.0)
- ○ Commercially available DNA isolation reagent (DNeasy Tissue Kit: made by QIAGEN etc.)
- ○ Loopamp DNA Amplification Kit

2. Preparation of the specimen

1) For use commercially available DNA isolation reagent (for example, DNeasy Tissue Kit, QIAGEN.)

(1) When carry out the extraction of DNA from the specimen using a commercially available DNA isolation reagent, refer to the instruction manual in the kit.

(2) After extraction, incubate at 95 ºC for 5min then keep it on ice.

2) For use simplified extraction method for LAMP

(1) Put 5~10mg of specimens taken from infected fishes in the sterilized tubes for sample preparation, and add 100μL of TE buffer, then homogenize the specimen with Homogenization Pestle.

(2) Add 100μL of Extraction Solution for F (EX F), and close the cap, then mix it by vortex mixer (1 second × 3 times).

(3) Centrifuge for a few second (hereinafter referred to as "spin down"), then incubate at 95 ºC for 5min.

(4) Add 10μL of 1M Tris-HCl: pH7.0 (Tris), then mix by vortex mixer.

(5) After mixing, centrifuge at over 2,000×g (room temperature) for 30 seconds, then keep it on ice. Use the supernatant as sample solution.

* If the simplified extraction method is used, its sensitivity might decrease in comparison with commercially available DNA isolation reagent.

<u>* When handling the sample, adopt appropriate measures to avoid spreading of KHV to environment and prevent contaminations of the DNA to the sample.</u>

3. Reagents preparation

1) Take out the reagents from its storage at -20°C, thaw them at room temperature. Right after they are thawed out, keep them on ice.

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

Referring to the table below, dispense the necessary amount of the reagents into the sterilized tube for master mix. preparation. Primer Mix. KHV (PM KHV) is contained in this reagent, and 2×Reaction Mix. (RM), *Bst* DNA Polymerase, and Distilled Water(DW) in Loopamp DNA Amplification Kit.

〈Reagents〉	〈Amount: 1 test〉	〈Amount: 10 tests〉
2 × Reaction Mix. (RM)	12.5 μL	125 μL
Primer Mix. KHV (PM KHV)	2.5 μL	25 μL
Bst DNA Polymerase	1.0 μL	10 μL
Distilled Water (DW)	4.0 μL	40 μL
Total	20.0 μL	200 μL

3) Fluorescent visual detection

Substitute Loopamp Fluorescent Detection Reagent (FD) for 1μL of Distilled water in master mix. Add 3μL of Distilled Water (DW) and 1μL of Fluorescent Detection Reagent (FD) in the master mix and the total amount is 20μL per 1 test.

4. Operation procedures (Follow the instruction of Loopamp DNA Amplification Kit).

1) Mixing master mix. solution and the sample solution (<u>Operate on ice</u>).

(1)Dispense 20μL of the master mix. to each Loopamp Reaction Tube.

(2) Add 5μL of sample solution, Positive Control KHV (PC KHV) or Negative Control (Distilled Water (DW)) to the master mix., and the volume of the solution is 25μL in total. Mix the solution well by pipetting or tapping the tube with cap closed and then spin down.

* Be careful not to cause air-bubbles on mixing.

* Check visually if proper amount of sample solution has been dispensed into the reaction tube.

2) Amplification and detection

(1) Set the temperature at 65°C with Loopamp Realtime Turbidimeters or incubator with hot bonnet (temperature accuracy within ±0.5°C). Confirm that the temperature has reached at 65 °C.

(2) Incubate the reaction tube at 65 °C for 60min.

(3)After the amplification reaction, inactivate the polymerase with heat block (80°C for 5min or 95°C for 2min)and terminate the reaction.

* Loopamp realtime turbidimeters perform enzyme inactivation automatically.

* Make sure that the enzyme is inactivated when detection process is performed after the amplification reaction as the separate step (the visual fluorescence detection).

<Detection with Loopamp Realtime turbidimeter>

(1) Loopamp Realtime Turbidimeters are applicable to this kit. Turn on the device about 20 minutes before use. Set the parameters for detection of KHV.

(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 KHV (PC KHV)) turbidity rose and the negative control's (Distilled Water (DW)) 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) After enzyme inactivation (80ºC, 2 min), is confirmed to have ended, done automatically by LoopampRealtime Turbidimeter, remove the used reaction tubes and dispose them with caps closed.

* The rising timing or turbidity of samples might be different from the positive control.

■ **Amplification Curve Pattern**

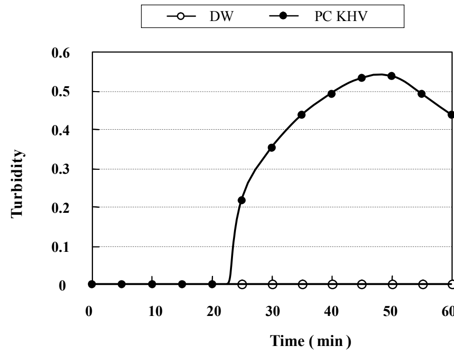

Fig.1 The real-time amplification of Positive Control KHV (PC KHV)*2

*2: There is no correlation between the initial template number and the turbidity increment time.

<Fluorescence visual detection>

(1) After the enzyme inactivation, irradiate the reaction tube with UV lamp (wavelength 240~260nm or 350~370nm) from the bottom and observe the fluorescent light from the side of the tube. When green fluorescent light similar to positive control is observed, the sample is judged as positive. When no fluorescence like negative control is observed, the sample is judged as negative.

(2) When observing the reaction tube, wear protective goggles that can efficiently block ultraviolet light.

(3) If necessary, save the detection result as picture format using digital camera.

* The copy number does not necessarily correlate with the fluorescent intensity.

* If the infected fish which were bred in a large amount of metallic contained ground water is tested, there is a possibility of interfering LAMP reaction.

* If the judgment with UV lamp does not make clear, use the following methods,
1) observe using commercially available black light from the side of the tube, 2) observe under the light by naked eye, 3) observe the detection result as picture format using digital camera.

* For examples of detection cases using this reagent, refer to the products information page in EIKEN Genome Site (URL; http://loopamp.eiken.co.jp/e/) .

[Caution for Handling]

1. This reagent is designed for research use only.
2. If the operator does not have the experience or knowledge in the field of the nucleic acid testing, there's a possibility of false judgment. Therefore, please make sure that the reagent is used under the supervision of the experienced and knowledgeable technician.
3. If any false judgment problem that is not related to capability of the reagent itself shall occur (operation error for instances), Eiken Chemical Co., Ltd. does not bear any responsibility.
4. Please use the reagent before the expiration date, which is labeled on the outer box (Exp. Date).
5. 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, Code No.]

Product Name	Unit	Storage	Expiration	Code No.
Loopamp™ Primer Set for Koi herpesvirus	48 tests	-20°C	1 year	PM0003

[References]

1) Notomi T. *et al.* : Nucleic Acids Research **28**, No.12, e 63 (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)
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6) Tomita N. *et al.* : Abstract for the 26th Annual Meeting of the Molecular Biology Society of Japan (2003)
7) Gilad O. *et al.* : Dis. Aquat. Organ. **11**, No.48, 101-108 (2002)
8) Yoshino M. *et al.* : Fish Pathology, **41**, No.1, 19-27 (2006)

Manufacturer

8028	AMK

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