NESTED PCR KIT FOR ENTEROCYTOZOON HEPATOPENAEI (EHP) DETECTION

-Intended for molecular detection of EHP in shrimp

CATALOG NUMBER: DIG-EHP-N25

For Research Use Only

Contents

Kit Contents

Components	Volume
Master mix	950 µL×1 vial
EHP Primer mix 1 [ready to use]	60 µL × 1 vial
EHP Primer mix 2 [ready to use]	60 µL × 1 vial
Internal control primer mix [ready to use]	60 µL × 1 vial
EHP Positive control	30 µL × 1 vial
Internal positive control	30 µL × 1 vial
Nuclease free water	1 mL × 1 vial

Storage

The contents of the kit should be stored at –20 °C and are stable until the expiration date. Repeated thawing and freezing (>2 x) should be avoided, as this may reduce assay sensitivity. If the reagents are to be used only intermittently, they should be frozen in aliquots.

Precaution before use

- Before use, make sure the reagents are evenly mixed by gently inverting the tube several times without creating bubbles. Uneven reagent mixing will result in inadequate reactivity. Do not mix by vortexing.
- Place reagents on ice immediately after it has thawed.
- Use fresh disposable tips to minimize potential cross-contamination between samples when preparing reaction mixtures or dispensing aliquots.
- Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.

Equipment and materials to be supplied by User

- PCR tubes
- Pipets and pipet tips (aerosol resistant)
- Thermal cycler
- Mini Spin Centrifuge
- Gel electrophoresis setup
- Gel documentation system

Introduction

The aquaculture industry is one of the fastest-growing food production sectors and constitutes an important economic activity in developing countries. However, climate change and the emergence of pathogens have negatively impacted the aquaculture industry over the past 20 years. Infectious diseases caused by viruses, bacteria, fungi, and microsporidia pose a serious threat to shrimp aquaculture. Among them, viral diseases are by far the most difficult to manage.

Product Description

The Nested PCR kit for Enterocytozoon hepatopenaei (EHP) is intended for the molecular detection of EHP in shrimp. Hepatopancreatic microsporidiosis (HPM) is a disease caused by infection with *Enterocytozoon hepatopenaei* (EHP), a microsporidian pathogen. EHP belongs to the family *Enterocytozoonidae*, phylum Microsporidia, which comprises highly reduced and specialized, spore-forming, unicellular parasites of animals, including humans, insects and crustaceans. Externally visible clinical signs are often used for presumptive diagnosis of suspected infections in shrimp. In the case of EHP, however, externally visible signs are often absent, apart from retarded growth over time. This assay involves nested polymerase chain reaction (PCR) which allows the use of two sets of primers. The first set of primer amplifies the target sequence. The second primer set lies internal to the first amplicon.

Protocol

1. The necessary reagents required for PCR are allowed to thaw on ice or cooling box.
2. The PCR tubes are labelled and the amount are calculated based on the total reaction volume.
3. For each PCR reaction a positive and negative control are included.
4. The reaction for internal control is run separately following the reaction and the program set up as mentioned below.
5. For Nested PCR, the components for **Step 1 PCR reaction setup (Table 1A)** are added and the tube is spun gently to collect all the components to the bottom of the tube.
6. The program setup-1 **(Table 1B)** is allowed to run for first round of PCR amplification.
7. Once the reaction is complete, the PCR product from first round of amplification is used as the template for the second round of amplification.
8. The components for **Step 2 PCR reaction setup (Table 2A)** are added and the tube is spun gently to collect all the components to the bottom of the tube.
9. The program setup-2 **(Table 2B)** is allowed to run for second round of PCR amplification
10. Gel electrophoresis is carried out to observe the amplified product using gel documentation system.

Internal control-Detection mix and Amplification protocol

Internal control- PCR Reaction Setup

Component	Volume/ Reaction
TAQ 2X Master Mix	12.5 µl
Internal control Primer Mix	2.0 µl
Template DNA	1.0 µl
Water	9.5 µl
Total volume	25 µl

Internal control-PCR Program Setup

Cycles	Duration of cycle	Temperature
35	3 minutes	95 °C
	30 seconds	95 °C
	30 seconds	55 °C
	45 seconds	72 °C
	3 minutes	72 °C

Nested PCR-Detection mix and Amplification protocol

Table 1A-Step 1: PCR Reaction Setup

Component	Volume/ Reaction
TAQ 2X Master Mix	12.5 µl
EHP Primer Mix 1	2.0 µl
Template DNA	1.0 µl
Water	9.5 µl
Total volume	25 µl

Table 1B-PCR Program Setup for Step-1

Cycles	Duration of cycle	Temperature
30	5 minutes	95 °C
	30 seconds	95 °C
	30 seconds	58 °C
	45 seconds	68 °C
	5 minutes	68 °C

Table 2A-Step 2: PCR Reaction Setup

Component	Volume/ Reaction
TAQ 2X Master Mix	12.5 µl
EHP Primer Mix 2	2.0 µl
PCR product	1.0 µl
Water	9.5 µl
Total volume	25 µl

Table 2B-PCR Program Setup for Step-2

Cycles	Duration of cycle	Temperature
30	5 minutes	95 °C
	30 seconds	95 °C
	30 seconds	64 °C
	20 seconds	68 °C
	5 minutes	68 °C

Specificity

The primers have been designed for specific and *in vitro* detection of EHP. This kit has high priming efficiency of ≥ 95 % and can detect less than 10 copies of target template.

Expected PCR product

- Internal control band is observed at 848 bp.
- When the sample is positive for EHP containing 20,000 copies of target DNA, the resulting band is observed at 514 bp in step 1 whereas the sample having less than 200 copies of target DNA, a target band at 148 bp is observed in step 2.
- No band is observed in the sample negative for EHP PCR.

Data interpretation

	Outcome 1	**Outcome 2**	**Outcome 3**	**Outcome 4**
Test sample	Positive	Negative	Positive	Negative
Positive control	Positive	Positive	Positive	Negative
Negative control	Negative	Negative	Positive	Negative
Internal control	Positive	Positive	Positive	Negative
Interpretation	Infection detected	Infection not detected	PCR Contamination	Experiment failed

Troubleshooting guide

- No PCR product
    - Incorrect programming of PCR cyclic conditions-Double check the settings on PCR machines.
    - Missing reaction component-Repeat the reaction setup.
    - Insufficient number of cycles-Re-run the reaction with more cycles.
- Primers degraded
    - Depending on the primer sequence and length certain primer sets are vulnerable to the effect of frequent freeze thawing.

    - Inappropriate storage of primers such as at room temperature may affect the integrity-Aliquot the working primer working solutions
- Template DNA has PCR inhibitors
    - Certain chemicals, ethanol and EDTA can inhibit the PCR reaction-Dilute the template DNA to reduce the PCR inhibitors in the reaction and internal control is used to check the correctness of PCR.
    - Autoclave the empty reaction tubes prior to use to eliminate biological inhibitors.
- Bands in negative control
    - Use autoclaved and filtered water dedicated for pre-PCR setup use.
    - Avoid cross over contamination and make sure to use filter tips for setting up the reaction.
    - Aliquot PCR reagents before setting up the reaction.
- Multiple/Non-specific products-Template or reaction mixture components are contaminated
    - Re-extract template and try new reaction mixture
    - Reduce environmental cross contamination to eliminate false positives/ negatives in the reaction.
- Contamination with exogenous DNA
    - Setup dedicated work space for pre-PCR setup and post-PCR work.
    - Use separate pipettes and sterile filter tips
    - Wear clean gloves and a dedicated pre-PCR lab coat before entering the work area and change them regularly.

TECHNICAL ASSISTANCE

For technical assistance and enquiries

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