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Protocol

# Rapid and sensitive detection of infectious hypodermal and hematopoietic necrosis virus by loop-mediated isothermal amplification combined with a lateral flow dipstick

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A B S T R A C T

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is an important shrimp pathogen that causes mortality in *Penaeus stylirostris* and stunting (called runt deformity syndrome or RDS) in *Penaeus vannamei*. Loop-mediated isothermal amplification (LAMP) allows rapid amplification of nucleic acids under isothermal conditions. It can be combined with a chromatographic lateral flow dipstick (LFD) for highly specific, rapid and simple visual detection of IHHNV-specific amplicons. Using this protocol, a 30-min amplification followed by 5 min hybridization with an FITC-labeled DNA probe and 5 min LFD resulted in visualization of DNA amplicons trapped at the LFD test line. Thus, 10 min for rapid DNA extraction followed by LAMP combined with LFD detection resulted in a total assay time of approximately 50 min. Detection sensitivity was comparable to other methods used commonly for nested PCR detection of IHHNV but had the additional advantages of reduced assay time, confirmation of amplicon identity by hybridization and elimination of electrophoresis with carcinogenic ethidium bromide.


## 1. Introduction

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is an important shrimp pathogen that causes mortality in *Penaeus stylirostris* and stunting (called runt deformity syndrome or RDS) in *Penaeus vannamei* (Bell and Lightner, 1984; Castille et al., 1993). LAMP combined with agarose gel electrophoresis (LAMP-AGE) and SYBR Green I staining has been reported for the detection of IHHNV (Sun et al., 2006). The requirement for electrophoresis and UV detection reduced the suitability of that method for field applications. In this report, combination of a modified LAMP reaction (i.e., addition of a pair of loop primers) with lateral flow dipstick (LFD) to improve assay specificity, sensitivity and speed was developed for the detection of IHHNV. For this purpose, generic LFD strips (Milenia GenLine HybriDetect) were employed. These strips detect the biotin-labeled LAMP product that has been hybridized with an FITC-labeled DNA probe complexed with a gold-labeled anti-FITC antibody. The resulting triple complex is trapped at the test line by an antibody specific to biotin. Non-hybridized FITC probe is bound by the gold-labeled anti-FITC to form a double complex without biotin and moves through the test line to be trapped at the control line. The LFD does not require special instrumentation since the user simply dips the LFD into an appropriately buffered LAMP reaction solution. Using the combined LAMP and LFD system (LAMP–LFD), the total assay interval was approximately 40 min, excluding the DNA extraction time. In this report, it is also demonstrated that DNA extracted using commercial kits or by simple boiling of samples yielded comparable assay results.

## 2. Materials and methods

### 2.1. Samples infected with IHHNV

IHHNV-infected *P. vannamei* were collected from shrimp farms in Samutsakorn and Surat Thani provinces, Thailand. The pleopods were tested for the presence of IHHNV using TakaRa PCR Amplification kit designed by Takara Bio Inc., Dalian, China.

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**Table 1**
Primers and probes used for IHHNV LAMP detection.

| Primer name | Genome | Sequences (5′–3′) |
|---|---|---|
| IHHNV-F3 | 1957–1975 | AGTCAGCAAGAACAGTACA |
| IHHNV-B3 | 2185–2164 | AACATGTATTTTATCCACTGCA |
| IHHNV-FIP | 2039-2020/TTTT/1979-2000 | TTGTCCTCTGGTACGGGTCCTTTTACTTGTCAATAAACTT GACGAT |
| IHHNV-BIP-Biotin | 2079–2103/TTTT/2149-2125 | Biotin-AACAAGAAAAAGTCAAACCAAAGCCTTTTGGTTTTCTTTTGGATATATTCTGCA |
| IHHNV-LF | 2018–2002 | TAGTTGTTTATATTCTT |
| IHHNV-LB | 2105–2122 | ACTGTC ACTAATTAC AAA |
| FITC-probe | 2042–2061 | FITC-TAAAGACAAACTCAGAGGAA |

### 2.2. DNA template preparation

Pleopods of IHHNV infected *P. vannamei* and SPF *P. vannamei* were used for total DNA extraction using a Genomic DNA Purification Kit (Fermentas) according to the manufacturer's protocol. The DNA pellet was dissolved in 200 μl of DNase-RNase-free water. The DNA concentration and quality were measured by spectrophotometric analysis at 260 and 280 nm.

A simple and rapid boiling method for DNA extraction was also performed as described by Jaroenram et al. (2009). Briefly, a pleopod of IHHNV infected *P. vannamei* was homogenized in a mixture of 200 μl of 0.025 N sodiumhydroxide (NaOH) and 0.0125% sodium dodecyl sulphate (SDS). After boiling for 5 min, the sample was placed immediately on ice. After a brief interval to allow most solids to settle, 200 μl of supernatant solution were used directly for subsequent steps without centrifugation.

### 2.3. Recombinant plasmid construction

IHHNV target sequences were amplified by PCR using primers designed from the target region 1957–2185 of the GenBank reference sequence AF218266. These were F3 (5′ AGTCAGCAAGAACAGTACA 3′) (reference positions 1957–1975) and B3 (5′ AACATGTATTTTATCCACTGCA 3′) (reference positions 2185–2164) yielding a PCR amplicon of 229 bp. The reaction mixture contained 0.3 μM each of F3 and B3, 0.2 mM of dNTP mix (Promega, Madison, WI, USA), 1.5 mM of $MgCl_2$, 1×PCRbuffer, 2 U of Taq DNA polymerase (Invitrogen, USA), 100 ng $\mu l^{-1}$ of IHHNV DNA from *P. vannamei* template in a final volume adjusted to 25 μl with sterile distilled water. The PCR thermal cycling protocol was 2 min at 90 °C (to activate the Hot start polymerase) followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 5 min ending in 4 °C hold. After ligating the PCR product into plasmid pGEM®-T Easy Vector (System II, Promega, Madison, WI, USA) according to the manufacturer's protocol, the recombinant plasmid was transformed into JM109 competent cells followed by blue-white colony selection. White colonies were picked for colony PCR and then amplified for 16 h in 2 ml of LB broth medium containing 100 μg $ml^{-1}$ of ampicillin. The plasmid DNA was purified using GeneJET™ Plasmid Miniprep Kits (Fermentas), followed by UV spectrophotometric analysis at 260 and 280 nm. The plasmid was 10-fold serial diluted to prepare stocks containing $1 \times 10^{10}$–1 copies $\mu l^{-1}$.

### 2.4. LAMP primer design

LAMP primers were designed according to the published sequence of IHHNV structural gene (GenBank accession no. AF218266) using the Primer Explorer version 3 (http://primerexplorer.jp/lamp3.0.0/index.html). Details of the final primers are shown in Fig. 1 and Table 1 and targeted positions 1957–2185 of the reference sequence. The normal primers and biotin-labeled BIP primer were synthesized by Bio Basic Inc., Ontario, Canada.

### 2.5. Optimization of LAMP reaction conditions

The LAMP reactions were performed using a heating block set at 60, 63 and 65 °C for 1 h, followed by 90 °C for 5 min to terminate the reaction. The reaction mixture contained 2 μM each of inner primer (5′ biotin-labeled BIP and FIP) and loop primers (LF and LB), 0.2 μM each outer primer (F3 and B3), 1.4 mM of dNTP mix (Promega, Madison, WI, USA), 0.06 M betaine (USB Corporation, USA), 6 mM $MgSo_4$, 8 U of *Bst* DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA), 1× of the supplied buffer and variable amounts of template DNA in a final volume of 25 μl. DNA

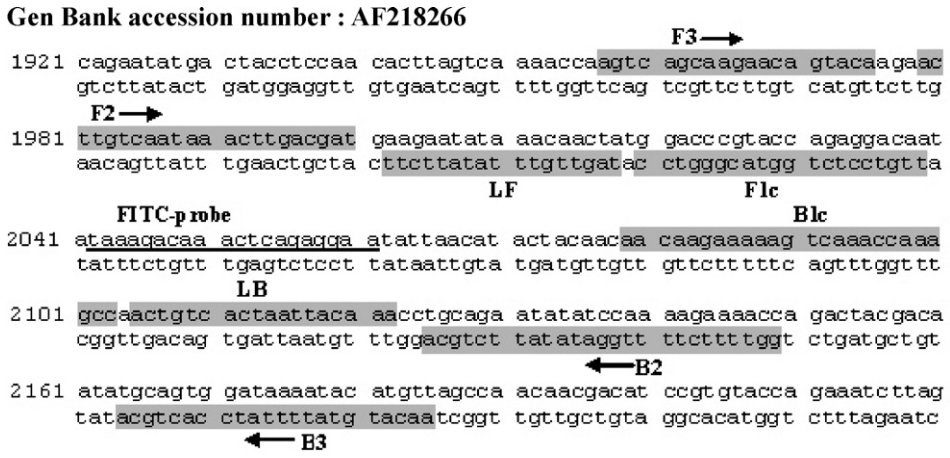


**Fig. 1.** Nucleotide sequence of IHHNV genome (GenBank accession no. AF218266). The sequences used to design primers F3, B3, FIP (F1c/TTTT/F2), BIP (B1c/TTTT/B2), LF and LB are shown by shaded boxes and arrows. The FITC-labeled probe sequence is shown in underlined typeface.