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# Real-time PCR measures IHHNV in shrimp

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## Tests confirmed that a line of *P. stylirostris* showed no signs of infection 32 days after ingesting IHHNV-infected shrimp tissue

Much research effort has been directed toward the detection and quantification of viral infections in cultured shrimp. Molecular techniques have proven very effective, and determination of the number of viral genomes in infected individuals has become one of the most important means of monitoring the progression of shrimp viral diseases.

Determination of viral loads in other organisms is based on methods that require cell cultures, but reliable methods for maintaining continuous shrimp cell cultures have not yet been achieved. Work at the University of Arizona in Tucson, Arizona, USA, however, has developed a real-time polymerase chain reaction (PCR) method that allows the quantification of viral genomes in individual shrimp without the need for cell cultures.



Real-time PCR instrument used in aquaculture pathology Lab at the University of Arizona, USA.

## Real-time PCR

The PCR method involves the addition of a TaqMan probe in a 5' nuclease assay. In this application, the TaqMan probe is labeled with a reporter dye at the 5' end and a quencher dye at the 3' end. The quencher suppresses the fluorescence emitted by the reporter through Forster resonance energy transfer.

During PCR, the Taq polymerase extends the new DNA from its upstream primer. Its 5' to 3' exonuclease activity cleaves the reporter dye from the TaqMan probe, which hybridizes to an internal portion of the template DNA. This results in the destruction of the quenching effect and, subsequently, an increase in fluorescence. The Taq polymerase synthesizes amplicons that hybridize to the TaqMan probe such that the amount of fluorescence is in proportion to the number of amplicons generated.

A major feature of real-time PCR is the measurement of DNA amplification at each cycle, as contrasted to traditional PCR, which measures the accumulated amplification at the completion of cycling. In addition, real-time PCR measures fluorescence intensity. This eliminates post-PCR processing, greatly increases throughput, and reduces the risk of contamination.

## IHNV PCR

During the development of a PCR assay for infectious hypodermal and hematopoietic necrosis virus, a pair of PCR primers and a TaqMan probe were selected from open reading frame 1 of the IHNV genome to amplify an 81-bp DNA fragment. The upstream (IHNV1608F) and downstream (IHNV1688R) primer sequences are: 5'-TAC TCC GGA CAC CCA ACC A-3' and 5'-GGC TCT GGC AGC AAA GGT AA-3, respectively. The sequence of TaqMan probe is: 5'-ACC AGA CAT AGA GCT ACA ATC CTC GCC TAT TTG-3'.

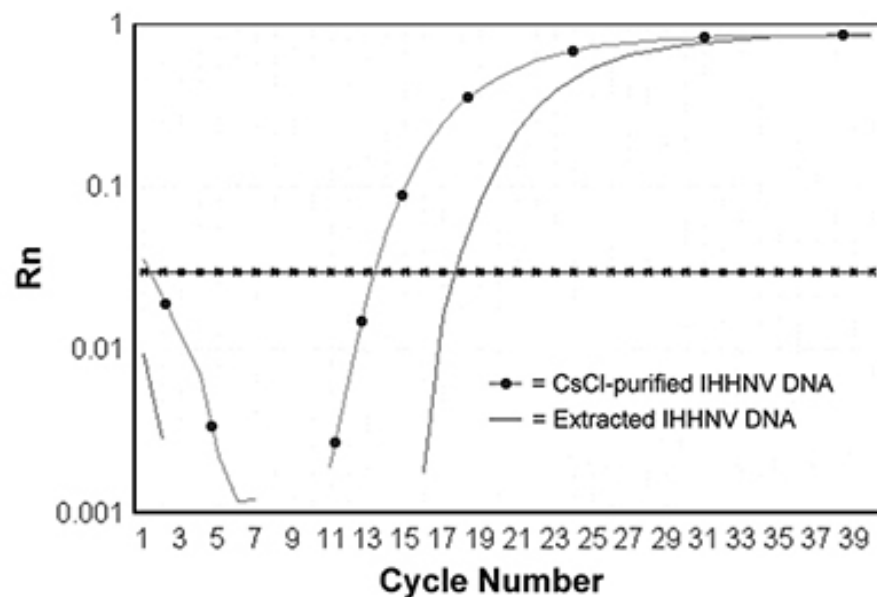


Fig. 1: Amplification plots for CsCl-purified IHNV and extracted IHNV DNA.

Both primers and TaqMan probe were shown to be specific for IHNV, and can be used to amplify IHNV from either purified virions or extracted DNA from IHNV-infected shrimp (Fig. 1).

## Quantitative studies

To determine the copy number of the IHNV genome, a plasmid containing the target IHNV sequence (designated pIHNV-P4) was constructed and used as a quantitative standard. A plasmid is a circular, double-stranded unit of DNA that replicates within a cell independently of the chromosomal DNA. Plasmids are most often found in bacteria, and used in recombinant DNA research to transfer genes between cells.

The concentration of pIHHNV-P4 was determined through spectrophotometric analysis, with varying amounts of plasmid used in the IHHNV PCR assay. This method has a detection limit of five copies and linear range up to a billion copies.

## Proven results

The assay was used to quantify IHHNV in infected shrimp collected from Mexico, Guam, the Philippines, and Belize, and from laboratory challenge studies. The quantitative analysis showed that wild-caught, large juveniles of *Penaeus stylirostris* collected in 1996 from the Gulf of California in Mexico were naturally infected with IHHNV and contained up to 10 billion copies of IHHNV per  $\mu\text{g}$  of DNA.

Similar quantities of IHHNV were detected in hatchery-raised, small juveniles of *P. stylirostris* collected from Guam in 1995, and in farm-raised postlarval (*P. monodon*) from the Philippines in 1996. Laboratory-infected *P. stylirostris* contained approximately one billion copies of IHHNV 31 days after being fed IHHNV-infected shrimp tissue.

Tests also confirmed that samples of Super Shrimp<sup>®</sup>, a line of *P. stylirostris* selected for IHHNV resistance, showed no signs of infection 32 days after ingesting IHHNV-infected shrimp tissue. In *P. vannamei* with Runt-Deformity Syndrome (RDS) collected in Belize this year, 10 billion copies of IHHNV were detected (Table 1).

## Tang, Quantitative analysis of IHHNV, Table 1

Source	Species	Origin	Tissue	Copy No. $\mu\text{g}^{-1}$ DNA
Mexico	<i>P. stylirostris</i>	Wild-Caught	Gills	$1.1 \times 10^9$
			Pleopods	$4.2 \times 10^8$
Guam	<i>P. stylirostris</i>	Hatchery	Heads	$1.2 \times 10^9$
Philippines	<i>P. monodon</i>	Farm	Whole Postlarvae	$1.6 \times 10^9$
Mexico	<i>P. stylirostris</i>	Lab Infection Day 31, Post-Feeding	Pleopods	$2.2 \times 10^8$
Panama	<i>P. stylirostris</i> (Super Shrimp <sup>®</sup> )	Lab Infection Day 32, Post-Feeding	Heads	Not Detected
Belize	<i>P. vannamei</i>	Farm	Pleopods	$4.5 \times 10^9$

Table 1. Quantitative analysis of IHHNV by real-time PCR.

Belize Aquaculture, Ltd. has used real-time PCR to evaluate IHHNV in *P. vannamei* showing RDS. Ten shrimp each of three sizes (20, 8 and 4 grams) were selected from a pond affected with RDS. Pooled pleo-pods of each group were submitted for real-time PCR analysis.

Results showed the number of copies of IHHNV varied by shrimp size. The smallest shrimp most affected by RDS had the heaviest viral load ( $1 \times 10^9$  copies of IHHNV), the intermediate size had an intermediate load ( $6.3 \times 10^8$ ), and the largest shrimp had the lightest viral load ( $4.4 \times 10^7$ ).

Based on these results, real-time PCR analysis was used to help select shrimp from a broodstock pond stocked with large non-runted shrimp from an IHHNV-infected production pond. The majority of shrimp from that pond had 107 to 109 copies of IHHNV, but 10 percent of the broodstock tested had only 103 to 104 copies of IHHNV. These broodstock are now being bred to determine if offspring will also have lower levels of IHHNV, and therefore less tendency to develop runt deformity.

## Conclusion

Real-time PCR is a practical technique that can rapidly quantify a large number of samples with high specificity and sensitivity. The use of this method to detect and quantify IHHNV in penaeid shrimp has already revealed new information regarding levels of IHHNV infection and the progress of viral infections.

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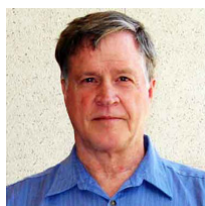
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